Evaluation of Oxidative-Antioxidative Balance in Serum of Patients With Non Acute Hepatitis Virus Type B

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Abstract. Oxygen free radicals play an important role in the pathogenesis of tissue damage in many pathological conditions, including liver diseases. Aim of the present study focused on the investigation the possible relationship between serum malondialdehyde level, an index of lipid peroxidation, and ceruloplasmin levels, as protective agents against lipid peroxidation, in hepatitis B virus. A group of 26 hepatitis virus type B patients enrolled in the study, while; control group consisted of 20 healthy subjects. In the present study, total proteins, malondialdehyde (µmol/L), ceruloplasmin oxidase activity (U/L) and ceruloplasmin concentration (g/L) were measured in sera samples of patients with non acute hepatitis B virus as well as in the healthy controls. Non significant variation (p>0.05) of total serum protein and malondialdehyde levels, while, highly significant variations were found for ceruloplasmin oxidase activity (p<0.000) and ceruloplasmin concentration (p<0.001) in patients with hepatitis B virus when compared with those of healthy individuals. The results revealed a significant elevation (p<0.000) of copper level in patients with hepatitis B virus when they were compared with healthy controls group, on the other hand, non significant variations (0.784) were observed when iron levels in patients group were compared with healthy individuals group. The levels of malondialdehyde and ceruloplasmin oxidase activity in sera of patents as well as in healthy controls group failed to illustrate a significant statistically correlation. We can conclude that a raise in the ceruloplasmin oxidase activity is not reflex to the ambulance in the oxidation – antioxidation status, but it is produced as one of the defense system’s proteins against the initial viral infection.

Key words: lipid peroxidation, malondialdehyde, ceruloplasmin

1. Introduction

Hepatitis B is a viral illness causing inflammation of the liver, resulting from infection with a DNA-type virus (Hepadnaviridae). This virus consists of an inner core surrounded by an outer capsule. The inner core contains the core antigen (HBcAg) and the antigen (HBeAg) also known as the "e" antigen. The outer capsule contains the hepatitis B surface antigen (HBsAg). Hepatitis B virus can be acute or chronic. It is a major public health concern worldwide which can lead to acute and chronic liver diseases including cirrhosis and hepatocellular carcinoma [1]. However, worldwide, about 400 million people have the virus, with most of these people living in Asia. Clearly, this is a significant public health and medical problem, about 5-10% of adult patients and 80-90% of children carriers became chronic carriers of the virus (basing to the World Health Organization at 2000) [2]. Moreover; 1.2 million people die from hepatitis virus type B and its related diseases every year [3].

Oxidative stress is a term denoting an imbalance between the production of oxidants and the respective defense systems of an organism [4, 5]. Lipid peroxidation occurs at low levels in all cells and tissues. In health, oxidation by free radicals and neutralization by antioxidants remain in balance, when the reactive oxygen species (ROS) are in abundance, oxidative stress occurs [6]. Oxygen free radicals play an important role in the pathogenesis of tissue damage in many pathological conditions, including liver diseases [7].

Ceruloplasmin is a ferroxidase enzyme that in humans is encoded by the CP gene [8-10]. Ceruloplasmin binds copper; appears to be more important as a copper storage pool than as a transport protein; integrates iron and copper homeostasis [11]. In addition to that, it have a protective effect as an antioxidant agent through its ability to prevent oxidative damage, using copper (II) centers[7, 12].
Various trace elements are responsible for many biochemical, immunological, and physiological activities. Essential micronutrients are involved in many metabolic pathways in the liver, such as enzymatic functions, protein synthesis, oxidative damage and anti-oxidant defense, immunological competence, interferon therapy response regulations and alterations of the virus genomes, copper and iron are example for the most trace elements importance [13].

Copper is essential trace metal which is a component of a wide range of intracellular metalloenzymes, including cytochrome oxide, superoxide dismutase, tyrosine, dopamine hydroxylase and lysyl oxidase, more of 75% the copper is associated with specific copper-binding protein, ceruloplasmin [14]. Aim of the present study focused on the investigation the possible relationship between serum malondialdehyde level, an index of lipid peroxidation, and ceruloplasmin levels, as protective agents against lipid peroxidation, in hepatitis B virus.

2. Materials and Methods

2.1 Individuals of the Study

The study group comprised 26 patients with newly diagnosed hepatitis B virus, between the age of 21-75 years who were admitted consecutively to the Liver and Digestive Tract Center of Al-Sader Medical City in Najaf, Iraq, between June and November 2010. All patients were enrolled in the study before receiving the course of drugs. The patients group consisted of 15 females and 11 males and their range age was 54 years. The control group comprised 20 healthy volunteers that included 11 females and 9 males aged between 19-70 (range of 51), the ratio of male to female was shown in figure 1.

![Gender Distribution of Patients with Hepatitis B Virus, and Healthy Individuals.](image)

All sera were collected in the morning after fasting 10 hour feature of the subjects in the present study are shown in the table 1. The healthy volunteers were selected on the basis of no alcoholic and smoking habits, without history of viral hepatitis, routing clinical check up during the entire period of research. Patients and control subjects residing in the same geographical area, and they were in the same socioeconomic status and similar diet habits. Patients with chronic hepatitis B diagnosed based on clinical, biochemical, histological and virological evidence in the same medical city. Blood samples were taken from subjects in accordance with standard procedure, 6-10 ml of blood was collected from vein and protected in evacuated tubes without anticoagulation agents.

2.2 Determination of Total Serum Proteins Levels

A total serum protein was estimated using Biuret method [15]. Biuret reagent supplied by the Manufacturing Company in a container contains 100 ml, that consist of sodium hydroxide (100 mM), sodium-potassium tartrate (16 mM), potassium iodide (15 mM), and cupric sulfate (6 mM). The bovine serum albumin was used as a standard protein. The procedure included mixing of 50 µl serum or standard with 2.5 ml Biuret reagent, then the mixture was incubated at room temperature for 30 minutes, and the absorbance was measured at 540 nm.

2.3 Measurement of Serum Malondialdehyde Level

Malondialdehyde level is measured by the thiobarbituric acid-reacting substances (TBARS) assay [16]. Abridgment, 150 µl of the serum sample was mixed with 1 ml of trichloro acetic acid (TCA) (17.5 %) and 1ml of thiobarbituric acid (0.6 %). Using the vortex, the final mixture was mix, the reaction mixture was then heated at 100ºC for 15 minutes in the water bath. After the mixture was cooled with tap water, it was
extracted with 1 ml TCA (70 %), the mixture was stand for 20 minutes at 25°C, and centrifuged at 3000 xg for 15 minutes. The organic phase was measured by use of a spectrophotometer with a wavelength of 534 nm.

### 2.4 Determination of Ceruloplasmin Oxidase activity

The activity of ceruloplasmin oxidase was determined in serum using the modified Rice method [17]. The procedure included two glass tubes, test (A) and blank (B). 1ml of substrate buffer was added to each tube, then incubated at 37ºC for 5 min. A 100μl of serum sample was added to tube A then incubated at 37ºC for 15 min. A volume of 3 ml of cold working inhibition solution was added to all of A and B tubes; at last 100μl of deionized water was added to tube B. The absorbance was measured at λ=540 nm.

#### Reagents

- **Preparation of Substrate Buffer:** Two gram of p-phenylenediamine was dissolved in the smallest volume of absolute ethanol, then filtered through double filter paper (Whitman number 1). Gently and gradually, concentrated hydrochloric acid was added. The pink precipitate was filtered and washed with methanol, then the product salt (p-phenylenediamine-2HCl) was dried at 70ºC. To purification of p-phenylenediamine-2HCl, the salt was dissolved in a minimum volume of hot water (60ºC), charcoal was added and left for 5 min, and then the mixture was filtered while hot. The purified salt was cold and precipitated from the filtrate by the addition of cold acetone until the turbidity was appeared (for the perfect results, all these step must be done in the ice bath). The mixture was refrigerated for several hours, filtered off the crystals, then it was dried in the dark in a vacuum desiccators over anhydrous calcium. To prepare substrate buffer, 0.1g of crystal p-phenylenediamine-2HCl was dissolved in 100ml of acetate buffer (0.4M, pH 5.2, containing 0.4 μM EDTA).

- **Working inhibition solution:** This solution was prepared by diluting 3 ml of stock inhibition solution (0.1 M of sodium azide and 0.5 M of sodium chloride) to 100 ml with deionized water, stored at 4ºC, and used cold [18].

\[
\text{Ceruloplasmin Oxidase Activity} = \text{The Absorbance of A–B tubes} \times 349.04
\]

Ceruloplasmin oxidase concentration was determined by measuring the absorbance of A and B tubes at wavelength = 605 nm.

\[
\text{Ceruloplasmin Oxidase Concentration} = \text{The Absorbance of A–B tubes} \times 87.5.
\]

### 2.5 Determination of Serum Copper and Iron Levels

The levels of serum copper and iron were determined by flame atomic absorption spectrophotometry (GBC-933plus).

### 2.6 Statistical Analysis

The findings were expressed as the mean ± standard deviation (S.D.). The data were analyzed with Student’s **independent t test**. All statistical analyses were performed with the program Statistical Package for the Social Science (SPSS for Windows, Version 14.0). Pearson’s correlation was applied to determined the relations among the laboratory parameters of the present study, significance was determined regression. A p-value of <0.05 was accepted as statistically significant.

### 3. Results

In the present study, total proteins, malondialdehyde (μmol/L), ceruloplasmin oxidase activity (U/L) and ceruloplasmin concentration (g/L) were measured in sera samples of patients with non acute hepatitis B virus as well as in the healthy controls. Table 1, shows that no significant variation (p > 0.05) of total serum protein levels in patients with hepatitis B virus when compared with those of healthy individuals. With same manner, the statistical evaluation failed to exhibit significant variation (p=0.180) for serum malondialdehyde when patients of hepatitis B virus were compared with those of healthy controls. On the other hand, when the comparison was carried out for hepatitis B virus patients and control group, highly significant variations were found for ceruloplasmin oxidase activity and ceruloplasmin concentration (p<0.000 and p<0.001 for ceruloplasmin oxidase activity and ceruloplasmin concentration; respectively).
Table 1: Levels (g/L) of TSP, (µmol/L) of MAD, (U/L) of Ceruloplasmin Oxidase Activity, and (g/L) of Ceruloplasmin Concentration in patients of Hepatitis B Virus and control subjects (mean ± S.D.)

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Age (Years)</th>
<th>TSP Level (g/L)</th>
<th>MAD (µmol/L)</th>
<th>Cp. Activity (U/L)</th>
<th>Cp. Level (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>49.000±16.122</td>
<td>66.120±10.500</td>
<td>5.753±1.390</td>
<td>109.130±37.544</td>
<td>19.981±6.612</td>
</tr>
<tr>
<td>(n=26)</td>
<td>21-75</td>
<td>42.000-82.000</td>
<td>3.440-8.650</td>
<td>55.491-199.279</td>
<td>10.063-33.513</td>
</tr>
<tr>
<td>Controls</td>
<td>41.850±15.246</td>
<td>74.050±6.492</td>
<td>5.305±0.987</td>
<td>43.573±11.822</td>
<td>9.492±3.285</td>
</tr>
<tr>
<td>(n=20)</td>
<td>19-70</td>
<td>59.000-83.000</td>
<td>3.440-7.240</td>
<td>15.705-63.169</td>
<td>2.363-14.000</td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td><strong>0.715</strong></td>
<td><strong>0.064</strong></td>
<td><strong>0.180</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.001</strong></td>
</tr>
</tbody>
</table>

The present study involved a wide age range of patients, according to that the correlation of case’s age to the serum total proteins, malondialdehyde, ceruloplasmin oxidase activity and ceruloplasmin concentration were studied. Significant negative correlation (r = - 0.646 at p < 0.005) was observed between patients age and total serum proteins, while less of this result was noted (moderate negative correlation, r = - 0.513 at p < 0.05) when the relation was between healthy individuals age and total serum proteins, as shown in figure 2 (A and B).

**Fig. 2: Correlation of Age to the Total Serum Proteins in A: Patients of Hepatitis B Virus, and B: Healthy Individuals**

Figure 3 A, illustrates a significant positive correlation (r = 0.632 at p < 0.005) of hepatitis patient’s age to the serum malondialdehyde level, in contrast to this result, figure 3 B, shows that no statistically correlation (r = 0.336 at p < 0.05) between control’s age and serum malondialdehyde level.

**Fig. 3: Correlation of Age to the Serum Malondialdehyde in A: Patients of Hepatitis B Virus, and B: Healthy Individuals**

Hepatitis B virus patients demonstrated significant positive correlation of their age to the serum ceruloplasmin oxidase activity (r = 0.716 at p < 0.005), however those of healthy individuals failed to do so (figure 4 A and B).
Highly significant positive correlation was also observed for patients age to serum ceruloplasmin concentration \( (r = 0.764 \text{ at } p < 0.0005) \) as shown in figure 5 A, no such finding was noticed when the correlation to the serum ceruloplasmin concentration of healthy individuals was studied (figure 5 B).

According to the fact that copper level basing on the activity and concentration of ceruloplasmin enzyme [19], in the same time the level of copper metal control and affect the level of iron metal [20, 21], for that, the levels of these metals were measured using flame atomic absorption technique. The results revealed a significant elevation \( (p < 0.000) \) of copper level in patients with hepatitis B virus when they were compared with healthy controls group (table 2). On the other hand, non significant variations \( (0.784) \) were observed when iron levels in patients group were compared with healthy individuals group, as shown in table 2.

Table 2: Serum Copper and Iron Levels (\( \mu g/ml \)) in Patient of Hepatitis B Virus and Healthy Controls (mean ± S.D.)

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Cu Level (( \mu g/ml ))</th>
<th>Fe Level (( \mu g/ml ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>Min-Max Range</td>
</tr>
<tr>
<td>Patients</td>
<td>1.788±0.668</td>
<td>0.887-3.232</td>
</tr>
<tr>
<td>(n=26)</td>
<td>0.678±0.291</td>
<td>0.047-1.022</td>
</tr>
<tr>
<td>Controls</td>
<td>0.678±0.291</td>
<td>0.047-1.022</td>
</tr>
<tr>
<td>(n=20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.000</td>
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</tbody>
</table>

In order to find the possible relation between the oxidation and antioxidation processes through the infection with B virus of hepatitis, the correlation between malondialdehyde as a profile to the peroxidation
process, and ceruloplasmin enzyme (ceruloplasmin oxidase activity) as an agent of antioxidation was studied. As shown in figure 6 A and B, the levels of malodialedehyde and ceruloplasmin oxidase activity in sera of patients as well as in healthy controls group failed to illustrate a significant statistically correlation.

Fig. 6: Correlation of Serum Malondialdehyde to the Ceruloplasmin Oxidase Activity in A: Patients of Hepatitis B Virus, and B: Healthy Individuals

4. Discussion

The outcome of hepatitis B infection parameters (which evaluated in the present research) largely depends upon the patient’s age at infection and immune status as well as the level of hepatitis B replication. According to the previous studies, normally; total serum proteins level decrease with the age progression; as well as this alteration in the protein levels was recorded in numerous diseases [22-25]. The result of current study agreed with these findings.

Lipid peroxidation occurs when lipids are attacked by free radical species and hydrogen atoms are extracted from the methylene carbon side chain, initiating a cascade of free ROS that can cause oxidative damage to cell structures [26]. In previous study (personality work in our laboratory) [27], it showed a progressive rise of malondialdehyde level in serum samples for individuals (patients and healthy) with age. This relationship was highly significant (r=0.68 at p<0.005). In the present work, there was a significant relation between serum malondialdehyde level and the age progression; this result agreed with the previous finding. On the other hand; malonaldehyde level in sera of patients didn’t elevated than those in sera of healthy individuals; according to this observation the lipid peroxidation status didn’t occurred in the primary stage of infection with hepatitis virus type B. This result is agreed with several studies that measured malonaldehyde level as a reflex for lipid peroxidation in numerous diseases [28, 29], while it disagreed with other studies [30], and especially those with liver diseases [31, 32].

According to the fact that ceruloplasmin is one of the acute-phase reactant [12], it is elevated in the cases of acute and chronic inflammation, basing to that, hepatitis viral infection may cause an elevation of ceruloplasmin levels in patients’ sera. This result agreed with several studies which recorded an increase in the ceruloplasmin levels [33-35]. Ceruloplasmin exhibits a copper-dependent oxidase activity, which is associated with possible oxidation of ferrous iron into ferric iron, therefore assisting in its transport in the plasma in association with transferrin, which can only carry iron in the ferric state [36, 37].

Results of the current study illustrated that rise of copper level in serum of patients with hepatitis virus type B comparison to healthy controls. This raise refers to the alterations of copper metabolism during the acute phase of uncomplicated hepatitis (hepatitis virus type B), that meaning an elevation may be resulted from defense frugalties of acute phase proteins response ; which leads to increase de nova synthesis (principally by the liver). This response is stimulated by cytokines and raised of the hormones cortisol and glucagon. As the disease progresses from chronic hepatitis to liver cirrhosis, copper will exhibit high concentrations in serum, It may be explained by the release of copper from damaged necrotic hepatocytes [13, 14, 38].

Highlight on the results of copper and iron levels in the patients’ sera illustrates that the elevated copper concentration can be explained as a reflex to the increases in the ceruloplasmin oxide activity and ceruloplasmin concentration as defense protein against chronic hepatitis B infection. On the other hand, the literatures recorded that raises of the iron levels can be refer to the damage of liver cells [20, 39], while; in
the present study the levels of iron in patients’ sera were within normal value, the matter can support the hypothesis that in the early stage of infection with hepatitis virus type B the liver stay hale.

In order to prove the hypothesis of liver cells safety during the prim infection period with hepatitis virus type B, the relationship of ceruloplasmin oxidase activity to the malondialdehyde levels. The study outcome showed there is no significant relation between these parameters; for that, we can conclude that a raise in the ceruloplasmin oxide activity is not reflex to the ambulance in the oxidation – antioxidation status, but it is produced as one of the defense system’s proteins against the initial viral infection.

5. References
