THE STATUS OF OXIDATIVE STRESS AND ANTIOXIDANT DEFENSE IN PATIENTS WITH CHRONIC HEPATITIS D

FEYZULLAH ÜÇMAK*, İHSAN SOLMAZ**, NAZIM EKİN*, İBRAHİM KAPLAN***, ELİF TUĞBA TUNCİL*, EBUBEKR ŞENATEŞ****, KENDAL YALÇIN

*Department of Gastroenterology, Dicle University School of Medicine, Diyarbakir, Turkey - **Internal Medicine Clinic, Siverek State Hospital, Şanlıurfa, Turkey - ***Department of Clinical Biochemistry, Dicle University School of Medicine, Diyarbakir, Turkey - ****Department of Gastroenterology, Medeniyet University School of Medicine, İstanbul, Turkey

ABSTRACT

Introduction: Oxidative stress is increasingly recognized as an important factor in the progression of chronic liver disease of varying etiologies and antioxidants are utilized in the treatment of some of them. Chronic viral hepatitis D continues to be a significant health problem in certain regions of the world. Rates of response to currently proposed treatments are rather low. The aim of this study was to investigate oxidative stress in patients with chronic viral hepatitis D.

Materials and methods: A total of 91 patients with chronic hepatitis D virus infection were included in this study (mean age: 42.2±11.7). In addition, 40 healthy volunteers were included in the study to form the control group. Patients were divided into two main sub-groups as cirrhotic (n=30) and non-cirrhotic (n=61) groups. Blood samples were taken from both patients and control subjects and compared for total oxidant status (TOS), total anti-oxidant status (TAS) and oxidative stress index (OSI).

Results: TOS levels were significantly higher in the patients compared to the control subjects (p<0.001). Moreover, TOS levels were higher in the cirrhotic patients compared to the non-cirrhotic patients (p=0.006). TAS levels were significantly lower in the patients compared to the control subjects (p=0.003). OSI levels were significantly higher in the patients compared to the control subjects (p<0.001). Moreover, OSI levels were higher in the cirrhotic patients compared to the non-cirrhotic patients (p<0.05).

Conclusion: These results are supportive of the role of oxidative stress in the pathogenesis of chronic viral hepatitis D. Antioxidant therapies might be considered in patients with chronic viral hepatitis D considering the presence of oxidative stress in these patients.

Keywords: Chronic hepatitis D, oxidative stress, antioxidants.

DOI: 10.19193/0393-6384_2016_6_175

Received May 30, 2016; Accepted September 02, 2016

Introduction

Hepatitis D is the most serious form of viral hepatitis in humans. It is a defective RNA virus and requires hepatitis B virus surface antigen for the life cycle. It poses a higher risk for cirrhosis, decompensation and hepatocellular carcinoma compared to chronic hepatitis B (CHB) monoinfection because of the faster progression of fibrosis in this disease. Globally, more than 15 million people are infected by Hepatitis Delta virus (HDV)(1,2). Although HDV infection has been reported worldwide, the prevalence of HDV is not uniform(3,4). Its prevalence is highly endemic in some areas such as Mediterranean basin, Middle East, Central Africa and Central Asia(1,3).

Severe and rapid progression makes HDV a high pathogenic virus. Despite various drug trials,
the only therapeutic option for chronic hepatitis D (CHD) remains to be pegylated interferon (p-INF)\(^6\). CHD is associated with a more threatening form of liver disease exacerbating the pre-existing liver damage leading to more rapid progression to cirrhosis in 70% to 80% of the cases\(^7\). It may lead to cirrhosis within 2 years in 10%-15% of patients\(^8\).

All the cells, as part of a metabolic process, constantly produce free radicals and reactive oxygen species. These free radicals and reactive oxygen species are neutralized by a complex antioxidant system. Any imbalance between this antioxidant system and free radicals and reactive oxygen species (ROS) causes oxidative stress, which might end up with cellular damage. Several in vitro and in vivo studies demonstrated the relationship between oxidative stress and associated damage and various forms of chronic liver damage and hepatic fibrosis\(^9\). In addition, previous studies showed that oxidative stress caused DNA and RNA damage in chronic viral hepatitis\(^9\). Reactive oxygen species contribute significantly to the progression of fibrosis by impacting on type I collagen gene regulation and cytokine release\(^9\).

Currently, p-INF are the most widely used treatments for CHD. However, response to these therapies are not satisfactory\(^6\). Some data suggested a beneficial effect of anti-oxidant treatments in chronic hepatitis. Using these agents alone or in conjunction with IFN therapies may serve an additional treatment option in the management of CHD. Available data regarding oxidative stress and CHD is currently limited. The aim of this study was to investigate oxidative stress in patients with CHD.

**Materials and methods**

**Patients**

One hundred six patients who visited Dicle University, Faculty of Medicine, Gastroenterology clinic who were HDV- and HDV RNA-positive and had received p-IFN therapy for a minimum of 12 months were reviewed retrospectively in this study.

Our hospital is a tertiary healthcare institution with a capacity of 1200 beds serving approximately 4 million people with the inclusion of surrounding provinces. Ninety-one of these patients with complete information who had signed the informed consent form were included in the study. Thirty-one of 91 patients were positive for both HDV and HDV RNA, and sixty patients were positive only for HDV. All of the included cases were managed as outpatients. The age range of the patients was 17-69 years. Patients with hepatitis C virus (HCV) anti-body or human immunodeficiency virus antibody positivity, acute exacerbation of chronic hepatitis B (CHB), history or evidence (serological and/or histological) of immunologically mediated liver disease, evidence of alcohol abuse (20 g/day for women and 30 g/day for men), use of vitamins and/or herbal products for their anti-oxidant effects within 6 month, presence of active bacterial infection, diagnosis of both liver or non-liver cancers and any organ transplantation were excluded from the study.

Thirty-four (37%) patients had liver biopsies, and thus histopathological data were available. Histopathological evaluations were performed to determine the Ishak scores.10 Nine-teen of the remaining 57 patients did not undergo liver biopsy due to compensated cirrhosis, and thirty-eight patients started PEG-IFN therapy without biopsy.

Liver cirrhosis was diagnosed by liver biopsy or comprehensively reviewing physical findings, laboratory parameters, endoscopic and/or ultrasonographic signs of portal hypertension and/or the finding of an irregular liver margin on ultrasonography (US).

As a control group we selected 40 age- and sex-matched healthy subjects.

The study was undertaken in compliance with the principles of the Helsinki declaration and was approved by the Local Ethics Committee of the Dicle University School of Medicine (Number; 26 December 2014-39, Diyarbakir, Turkey).

**Blood sample collection**

Blood samples were obtained following an overnight fasting state from healthy volunteers and patients with CHD after informed consent form was obtained. Samples were withdrawn from a cubital vein into a biochemistry tube without anti-coagulant for serum and immediately stored on ice at 4°C. The sera was then separated from the cells by centrifugation at 3000 rpm for 10 minutes. Sera were isolated and kept at -80°C until analysis.
Measurement of total oxidant status and total antioxidant status

Total oxidant status (TOS) and total antioxidant status (TAS) were determined using a novel method developed by Erel\(^{[11, 12]}\). TOS and TAS were determined on Abbott Architect C16000 autoanalyzer (Abbott, Illionis, USA) using calorimetric automated measurement methods developed by Erel\(^{[11, 12]}\) and employing commercially available kits (Relassay R, Turkey). In TAS measurement method of Erel, dark blue-green ABTS (2.2‘azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) is reduced back to its original colorless form. Change in absorbance at 660 nm is associated with total anti-oxidant level in serum. Anti-oxidative effect of the sample against the strong free radical reaction triggered by the hydroxyl radical is determined. Data are expressed in micromolar Trolox equivalents per liter. On the other hand, in TOS measurement method of Erel, oxidants present in the sample oxidize ferrous ion-chelator complex to ferric ion. In acidic medium, ferric ion makes a colored complex with chromogen. Color intensity, which can be measured spectrophotometrically, is associated with the amount of total oxidant molecules present in the sample. Data are expressed in micromolar hydrogen peroxide equivalents per liter.

Determination of the oxidative stress index

The ratio of TOS to TAS was accepted as the oxidative stress index (OSI). TAS concentrations were converted to \(\mu\)mol Trolox Eq/L, and OSI was calculated according to the following formula: OSI= 100 * TOS (\(\mu\)mol/ L \(H_2O_2\)) / TAS (\(\mu\)Mol/L Trolox).

Statistical analysis

The analysis of the data was performed by using the Statistical Package for the Social Sciences (SPSS) 15.0 (SPSS Inc., Chicago, IL, USA) statistical software. Inter-group comparisons were performed by using Student’s test when numeric data demonstrated a normal distribution and by using Mann Whitney U test when numeric data did not demonstrate a normal distribution. Groups with categorical variables were evaluated by using Pearson Chi-square test. A p value smaller than 0.05 was considered statistically significant.

Results

Demographic data

Among 91 patients with CHD infection enrolled in the study, 30 cases were diagnosed as cirrhosis. Fifty-one of patients with CHD infection (56%) were males while 40 were fe-males (44%); mean age was 42.2\(\pm\)11.7. Seventeen of patients with cirrhosis (57%) were males; mean age was 43.2\(\pm\)11.3. In the group of control, 18 were females (45%) and 22 were males (65%) while the mean age was determined as 39.8\(\pm\)6.0. No significant difference was found in comparison of control and patient groups in terms of age and gender.

Laboratory parameters

Laboratory parameters of patients and controls were shown in Table 1 and 2.

<table>
<thead>
<tr>
<th></th>
<th>Patients, n= 91 (mean±SD)</th>
<th>Control, n= 40 (mean±SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.70 ± 1.78</td>
<td>13.85 ± 1.16</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Leukocyte (K/uL)</td>
<td>5.67 ± 1.35</td>
<td>8.12 ± 2.20</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Thrombocyte (K/uL)</td>
<td>172 ± 71</td>
<td>285 ± 60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>58.9 ± 53.4</td>
<td>22.8 ± 8.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>48.0 ± 35.2</td>
<td>19.7 ± 3.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TAS ((\mu)Mol Trolox equiv/L)</td>
<td>0.93 ± 0.22</td>
<td>1.06 ± 0.19</td>
<td>0.003</td>
</tr>
<tr>
<td>TOS ((\mu)mol (H_2O_2) equiv/L)</td>
<td>366.13 ± 164.10</td>
<td>57.35 ± 41.86</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OSI (Arbitrary unit)</td>
<td>32.56 ± 16.08</td>
<td>3.88 ± 3.89</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 1: Comparison of laboratory parameters between the patients with chronic HDV and control subjects.

WBC: White Blood Cell, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, Total oxidant status: TOS, Total anti-oxidant status: TAS, Oxidative stress index: OSI.

There were statistically significant differences between the patient and control groups in hemoglobin, leukocyte, thrombocyte, alanine aminotransferase (ALT), aspartate aminotransferase (AST) levels (Table 1) and between cirrhotic and non-cirrhotic patients in leukocyte, thrombocyte, hemoglobin, albumin, AST, ALT and total bilirubin levels (Table 2).

Thirty-four percent of the patient population was HDV RNA positive. Twenty-six percent of the patient population was HBV DNA positive.

In the cirrhotic group, model of end-stage liver disease (MELD) scores ranged from 6 to 17 (mean 8.5\(\pm\)3.4), and a majority of the patients (87%) had a compensated liver disease.
The redox status contributes to the progression of inflammatory, metabolic and proliferative liver diseases. Investigation of oxidative stress might provide useful insight into the etiopathogenesis of liver diseases. It might also help identify the degree of liver damage and monitor the degree of liver damage and monitor the progression of chronic liver disease (CLD) and hepatocarcinogenesis.

**Table 2: Comparison of laboratory parameters between the patients with cirrhotic and non-cirrhotic patients.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cirrhotic, n=30 (mean±SD)</th>
<th>Non-cirrhotic, n=61 (mean±SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lökosit (K/μL)</td>
<td>4.52 ± 2.14</td>
<td>6.26 ± 1.41</td>
<td>0.001</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.2 ± 2.0</td>
<td>14.5 ± 1.4</td>
<td>0.007</td>
</tr>
<tr>
<td>Thrombocyte (K/μL)</td>
<td>89 ± 35</td>
<td>214 ± 43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>61.0 ± 40.9</td>
<td>41.7 ± 30.5</td>
<td>0.003</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>65.9 ± 50.4</td>
<td>56.0 ± 55.9</td>
<td>0.047</td>
</tr>
<tr>
<td>T protein (g/dL)</td>
<td>7.3 ± 0.7</td>
<td>7.7 ± 0.8</td>
<td>0.212</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.6 ± 0.5</td>
<td>3.9 ± 0.3</td>
<td>0.005</td>
</tr>
<tr>
<td>T bilirubin (mg/dL)</td>
<td>1.11 ± 0.64</td>
<td>0.85 ± 1.00</td>
<td>0.002</td>
</tr>
<tr>
<td>TAS (µMol Trolox equiv/L)</td>
<td>0.91 ± 0.23</td>
<td>0.95 ± 0.21</td>
<td>0.41</td>
</tr>
<tr>
<td>TOS (µmol H₂O₂ equiv/L)</td>
<td>399.76 ± 146.68</td>
<td>297.75 ± 178.45</td>
<td>0.006</td>
</tr>
<tr>
<td>OSI (Arbitrary unit)</td>
<td>34.89 ± 13.79</td>
<td>27.82 ± 19.35</td>
<td>0.025</td>
</tr>
</tbody>
</table>

**Parameters of oxidative stress**

The mean TAS, TOS and OSI levels were 0.93 ± 0.22, 366.13 ± 164.10 and 32.56±16.08, respectively in the patients with CHD infection and 1.06 ± 0.19, 37.35 ± 41.86 and 3.88 ± 3.89, respectively in the controls (Table 1). According to these results, there were statistically significant differences between the patients and the control subjects in TAS, TOS and OSI levels (p=0.003, p<0.001 and p<0.001, respectively). Comparison of laboratory parameters of cirrhotic and non-cirrhotic patients was shown in Table 2.

As to the comparison between the cirrhotic and the non-cirrhotic patients, the mean TAS, TOS and OSI levels were 0.91 ± 0.23, 399.76 ± 146.68 and 34.89 ± 13.79, respectively in the cirrhotic patients and 0.95 ± 0.21, 297.75 ± 178.45, and 27.82 ± 19.35, respectively in the non-cirrhotic patients (Table 2).

According to these results, there were significant differences between the groups in TOS and OSI levels (p=0.006 and p=0.025, respectively). The difference in TAS levels, on the other hand, was not statistically significant. (p=0.41).

Additionally, no significant correlation was shown between these markers and biochemical parameters, viral load (HDV RNA and HBV DNA), necro-inflammation and fibrosis of the liver in CHD patients.

**Discussion**

Oxidative stress is increasingly recognized as an important factor in the progression of chronic liver disease (CLD) and hepatocarcinogenesis. Generation of ROS and impairments in redox defense mechanisms with glutathione, catalase or superoxide dismutase cause an elevation in the oxidative stress levels. Mitochondria host the most abundant and the most significant sources of intracellular ROS. An imbalance that might occur in the mitochondrial respiratory chain is considered a major cause of ROS generation. In addition, the metabolically significant liver is considered a major reservoir of mitochondria that host the most abundant sources of ROS, which are known to play a key role in the initiation of necroinflammation. Under physiological conditions, antioxidant defenses and repair enzymes detoxify reactive and in-completely reduced forms of oxygen such as superoxide into water for maintenance of a reduced continuous level of toxic oxidants.

In case of acute liver damage and hepatic inflammation, neutrophils and Kupffer cells generate ROS, as the main toxic mediators to cause cellular death. The sources of oxidative stress in CLD (HBV, HCV) induced by the viral proteins. Previous studies demonstrated that viral protein large hepatitis D antigen (p27), which is significantly involved in the pathogenesis of HDV, affects various cellular functions, including the nuclear factor kappa B (NF-κB) pathway, through oxidative stress. The innate immune response results in chronic infection with hepatotropic viruses, inducing chronic inflammation, causing ROS generation and forming oxidative DNA lesions. Hepatotropic viruses and their proteins might cause endoplasmic reticulum stress and trigger lipid accumulation, leading to elevation in oxidative stress levels and formation of oxidative DNA lesions.

The rate of non-permanent oxidative DNA damage is normally counteracted by its rate of repair; however, chronic oxidative stress might cause permanent genetic damage that is involved in carcinogenesis.

The redox status contributes to the progression of inflammatory, metabolic and proliferative liver diseases. Investigation of oxidative stress might provide useful insight into the etiopathogenesis of liver diseases. It might also help identify the degree of liver damage and monitor the...
response to treatment. The significant role of oxidative stress in chronic viral hepatitis (types B and C), nonalcoholic steatohepatitis (NASH) was demonstrated by several studies. However, to the best of our knowledge, this is the first study in the literature that investigates the role of oxidative stress in CHD.

Duygu et al. reported that TOS and OSI levels were significantly higher in patients with CHB compared to inactive carriers and healthy controls. Bolukbas et al. investigated oxidative stress in patients with HBV infection (patients with chronic hepatitis, inactive carriers and cirrhotic patients) and compared the results to those of healthy control subjects. They found that antioxidant response was significantly lower in cirrhotic patients compared to healthy control subjects and inactive carriers.

In addition, they observed that total peroxide and OSI levels were significantly higher in cirrhotic patients and patients with cirrhotic hepatitis B compared to healthy control subjects and inactive carriers. Moreover, they found that serum ALT and total peroxide levels positively correlated with OSI levels only in patients with CHB. These results might be suggestive of the contribution of hepatitis B to oxidative stress. Namiduru et al. found that Malondialdehyde levels were significantly higher in patients with chronic hepatitis types B and C compared to control subjects. Antioxidant myeloperoxidase levels, on the other hand, were significant lower in patients with chronic hepatitis.

As stated earlier, this is the first study that investigates oxidative stress in patients with CHD. The results of this study were suggestive of the role of oxidative stress in CHD and, in this respect, were in agreement with the previous studies conducted among patients with chronic hepatitis types B and C which also indicated the role of oxidative stress in chronic hepatitis.

Bolukbas et al. reported that TOS and OSI levels were significantly higher in patients with hepatitis B virus-related cirrhosis compared to inactive carriers and control subjects. On the other hand, they found no significant difference between patients with hepatitis B virus-related cirrhosis and patients with CHB. In the present study, TOS and OSI levels were significantly higher in patients with hepatitis B virus-related cirrhosis compared to both patients with CHD and control subjects.

Of all the aforementioned authors, only Bolukbas et al. included cirrhotic patients in their study (hepatitis B virus-related cirrhosis), as was the case in the present study.

In the present study, oxidative stress levels were found to be higher in cirrhotic patients compared to CHD patients and control subjects. Hepatic fibrosis is a dynamic and orderly wound healing process that occurs in response to chronic hepatocellular damage. ROS are involved in generation of liver damage and initiation of hepatic fibrogenesis. Oxidative stress is capable of impairing proteins, lipids and DNA, triggering necrosis and apoptosis of hepatocytes and increasing the inflammatory response. ROS also induce the generation of profibrogenic mediators from circulating inflammatory cells and Kupffer cells and play a prominent part in the activation of hepatic stellate cells, eventually leading to initiation of fibrosis.

Several explanations can be made for these higher levels in cirrhosis compared to those in chronic hepatitis, in which inflammatory process is expected to be more remarkable. Firstly, a big part of the antioxidant capacity consists of hepatocellular parenchymal cells. Hepatocellular capacity is diminished in cirrhosis. Cirrhotic patients were shown to have reduced antioxidant capacity. In fibrotic process, the dynamic and active inflammation keeps oxidative stress present. In cirrhosis, reduced antioxidant capacity results in increased sensitivity and exposure of non-parenchymal cells (kupffer, stellate and endothelial cells) to oxidative stress.

Lastly, portal hypertension coexisting with cirrhosis has to be mentioned. Portal hypertension-induced impairments in intestinal epithelial barrier function, and accordingly transition of bacteria and bacterial products such as endotoxin into systemic circulation through intestinal lumen result in systemic inflammation and increased oxidative stress.

In their study, Zuwal et al. reported increased oxidative stress in cirrhotic patients as well as a significant correlation between MELD scores and oxidative stress. A previous study investigating oxidative stress in patients with various stages of CHB demonstrated that cirrhotic patients had the highest levels of oxidative parameters and lowest levels of anti-oxidative parameters. In this respect, the present study found similar results.
Today, medications recommended for treatment of CHD are p-IFN. The goal of treatment is to eradicate the disease or to suppress HDV RNA and HBV DNA for a long term. Rate of eradication achieved in today’s patients remains low. Some recent studies reported the potential usefulness of the long-term p-IFN therapy. In the present study, no significant difference was found between INF-treated patients and treatment-free patients in oxidative stress levels, which might be attributed to the finding that a majority of the patients remained HBV DNA and HDV RNA positive.

In a controlled study, antioxidant agents were added to interferon-alpha therapy in patients with chronic hepatitis C, and a significant reduction in viral load and 2.4 times higher probability of complete response was observed in the group that received additional vita-min E compared to the groups that did not receive vitamin E.

Dikici et al. showed that levels of oxidative stress markers were higher whereas levels of antioxidant markers were lower in patients with acute and CHB compared to control subjects. In addition, they found that this impairment returned to normal levels following interferon-alpha therapy in patients with CHB.

Chiou et al. showed that levels of oxidative stress markers were significantly higher in patients without a fast virologic response compared to those with a fast virologic response at the outset of the therapy and at the 24th week of the therapy. Moreover, TAS levels were significantly higher in patients with permanent virologic response compared to those without permanent virologic response at the fourth week after treatment.

Erel method employed in the present study is a novel measurement method that quantifies oxidant and antioxidant capacities simultaneously and reveals the total antioxidant re-sponse. This method has several advantages. First of all, it is reliable and sensitive. Secondly, it is a cheap and simple method that provides easy and fully automated measure-ments. Thirdly, it is not affected by serum contents such as bilirubin, lipids and anticoagulants.

The limitations of our study are as follows; The most important limitation of this study was the relatively small number of patients and a single-center design. Some may consider that the lack of patients with CHB may be a limitation of this study. However, it is known that HDV suppresses HBV in its all stages. Because of that we did not include patients with CHB without delta infection in this study. Furthermore, we did not observe any difference between HBVDNA positive and negative subgroups in terms of oxidative stress. Conduction of further comparative studies with the inclusion of HBV-monoinfected populations could provide additional insights into this area of research.

The present study showed the presence of oxidative stress in patients with CHD which was caused by elevated oxidant status and reduced antioxidant status. In the event that future studies support the results of this study, antioxidant therapies might be considered as an alternative to INF therapy in patients with CHD, who do not have a high probability of success with INF therapy, as well as patients with contraindications to INF therapy, patients with INF-related complications, and patients that are unresponsive to INF therapy. However, larger studies are needed to support the results of this study.

References

The status of oxidative stress and antioxidant defense in patients with chronic hepatitis D


Acknowledgements

We thank Mehmet Ali Kaplan for providing statistical assistance in the preparation of this manuscript.

Corresponding author

PEYZULLAH ÜCMAK, MD, Assistant Prof.
Department of Gastroenterology
Dicle University School of Medicine
21100, Diyarbakir (Turkey)