Alterations in Lipid Peroxidation and Antioxidants in Patients' with Different Stages of Hepatitis B Virus Infection in Egypt

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Abstract: Persistent hepatitis B virus (HBV) infection often leads to the development of chronic hepatitis, cirrhosis and hepatocellular carcinoma. The aim of this study was to estimate the oxidant/antioxidant status and liver function tests in different stages of HBV infection to investigate the role of oxidative stress in the pathogenesis of chronic liver diseases. The study was carried out on 150 control healthy persons (C) and 320 patients with HBV infection which included normal carriers (N), acute (A), chronic (CH), cirrhosis (C1) and hepatocellular carcinoma (HCC). The study focused on the determination of malondialdehyde (MDA), non enzymatic and enzymatic antioxidant parameters and liver function profile tests. The results showed that MDA and glutathione reductase (GSR) were increased, while reduced glutathione (GSH), total glutathione s-transferase (GST), total glutathione peroxidase (GPx), superoxide dismutase (SOD) were decreased in N, A, CH, C1 and HCC groups as compared to the C group. Also, the levels of total protein (TP) and albumin were decreased but alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities and total bilirubin (TB) level were increased in all studied groups as compared to the C group. This indicates that HBV induced oxidative stress, lipid peroxidation LPO, liver injury and cell death. We concluded that the determination of antioxidant system, oxidative stress and liver function tests may be considered as accurate biomarkers for determining the progression of HBV infection. Also treatment with antioxidant supplementation is very important in case of HBV infection.


Key Words: Hepatitis B virus, Liver function tests, Oxidative stress, Antioxidant enzymes.

1. Introduction

Oxidation is a metabolic process that leads to energy production necessary for essential cell activities. However, metabolism of oxygen in living cells also leads to the unavoidable production of reactive oxygen species (ROS) such as superoxide (O2⁻) radical, hydrogen peroxide (H2O2) and hydroxyl radical (OH⁻). The O2⁻ radical can react with nitric oxide to form the peroxynitrite radical (ONOO⁻) (1). There are two sources of free radicals, endogenous and exogenous sources. The endogenous FRs is derived from normal cellular metabolism and oxidative burst produced when phagocytic cells destroy invading microorganisms such as bacteria and viruses. Also the reactions of certain oxidases, cyclooxygenases, lipooxygenases, dehydrogenases, and peroxidases are the sources of ROS (1, 2). The exogenous sources of free radicals include tobacco smoke, air pollutants, organic solvents, anesthetics, pesticides, drugs, and radiation (3, 1). ROS have enormous ability to cause damage to biomolecules (lipids, protein or DNA), cells and tissues because of their highly reactive nature (4). ROS alterations in different signaling pathways may modulate gene expression, cell adhesion, cell metabolism, cell cycle and cell death (5). It has been reported that ROS are potential carcinogens because of their roles in mutagenesis, tumor promotion, and progression (5).

Polyunsaturated fatty acids (PUFAs) residues of phospholipids located in cell membranes and intracellular organelles are highly susceptible to reaction with free radicals. In addition, fatty acids that esterified in glycolipids, or cholesterol esters can undergo lipid peroxidation (LPO) by the help of free radicals (6). The LPO products such as aldehydes (malondialdehyde and hydroxynonenal), are capable of forming crosslinks with lipids, proteins, and nucleic acids, thereby causing damage to the macromolecules, which are essential components of biological tissues (1, 7).

A number of sophisticated antioxidant systems exist in aerobic organisms, and their action interactively and synergistically to balance the cellular production of ROS. They involve a variety of components, both endogenous and exogenous in origin (8). Endogenous antioxidant defenses include a
network of compartmentalized antioxidant enzymes that are usually distributed within the cytoplasm and among various organelles in cells. A variety of small non-enzymatic molecules present in the internal milieu are also capable of scavenging ROS. In eukaryotic organisms, several ubiquitous primary antioxidant enzymes are superoxide dismutase (SOD), catalase, and different forms of peroxidases. They work in a complex series of integrated reactions to convert ROS to more-stable molecules such as water and O2 as shown in Fig. 1 (9). These enzymes require micronutrients as cofactors such as selenium, iron, copper, zinc, and manganese for optimal catalytic activity and effective antioxidative defense mechanism. Besides the primary antioxidant enzymes, a large number of secondary enzymes present such as glutathione reductase, glucose-6-phosphate dehydrogenase. These enzymes help in maintaining a steady concentration of glutathione and NADPH necessary for optimal functioning of the primary antioxidant enzymes (10). Detoxicant enzymes, such as glutathione S-transferase (GST), metabolize toxic electrophiles and are considered also to be secondary antioxidant enzyme (11). Oxidative stress (OS) is a disturbance in the oxidant-antioxidant balance leading to potential cellular damage. Most cells can tolerate a mild degree of OS. The imbalance can result from a lack of antioxidant capacity caused by disturbances in production and distribution, or by an overabundance of ROS from other factors (3).

Extensive liver injury of various etiology is manifested as hepatocyte damage and associated with inflammatory and immune responses. It is the starting point for events which may result in fibrosis, often followed by cirrhosis (12). Liver damage commonly results by viral and protozoal infection, toxicity due to drugs, food additives and fungal toxins (13).

Hepatitis B is one of the world's most serious and widespread chronic diseases (14). The World Health Organization (WHO) estimates that hepatitis B infection leads to more than one million deaths every year (15). HBV infection can result in acute hepatitis, HBV carriage, chronic hepatitis, liver cirrhosis, and even primary hepatocellular carcinoma (HCC). Progression of hepatitis to cirrhosis and the response to therapy are variable among individuals. This variability is due to the primary cause of disease and inter-individual differences in target proteins and drug metabolism (16). Also the progress of HBV infection may be affected by host genetic susceptibility (17). It has been found that the total peroxide level is significantly higher in patients with CH. Also lipid peroxidation and oxidative DNA damage are enhanced in patients with HBV infection (18). In addition, the previous studies showed that ROS play a pathogenetic role in carcinogenesis (19). Therefore this study was carried out to investigate the changes in lipid peroxidation and the activities of the antioxidant enzymes in human liver at different stages of HBV. Also the study included the determination of liver function profile.

![Fig. 1: Chemical equations of antioxidant enzymes.](http://www.lifesciencesite.com)

Since SOD = superoxide dismutase, CAT = catalase, GPx = glutathione peroxidase, GSR = glutathione reductase, GST = glutathione S transferase, CYP3A4 = cytochrome P 3A4 and G6P = glucose 6 phosphate.

2-Materials and Methods

2.1. Chemicals and kits:

GST assay kit, OxiSelectTM MDA Quantitative assay kit, EnzyChromTM GSH/GSSG assay, SOD assay and GSR assay kits were obtained from Cell Biolab, BioAssay Systems, Cyman, USA, respectively. Total GPX Assay kit was obtained from Gentaur, Belgium. Total protein kit was obtained from Spinreact, Spain. Albumin kit was obtained from Borax diagnostic, England. AST kit was obtained from Prodia international, Germany. ALT kit was obtained from Crest biosystem, India and Bilirubin kit was obtained from Diamond, Egypt.

2.2. Patients and Control:

The present study was carried out on blood samples of 470 persons (310 males and 160 females) which included 150 healthy persons (control group, C) and 320 patients with HBV: The age of studied cases was (43±13) years. The patients were classified into five groups according to clinical history, serological tests and histological analysis as follow: 67 normal carriers (N), 53 acute hepatitis (A), 78 chronic hepatitis (CH), 62 cirrhotic liver (CL), and 60 hepatocellular carcinoma (HCC). Complete demographic data and full clinical history were recorded. Different blood samples were collected from different hospitals, medical laboratories and
hepatologist clinics in Egypt according to the rules of scientific research ethics.

**Serum preparation:** Five ml of blood from each person were collected, left at room temperature for 10 min and then centrifuged at 8000 rpm for 5 min at 4 °C. All serum samples were stored at -80 °C.

**2.3. Biochemical Assays:**

**2.3.1. Determination of molondialdehyde (MDA):** MDA, a marker of lipid peroxidation, was determined using kit according to the thiobarbituric acid (TBA) test (20). The pink product was measured spectrophotometrically at 530 nm. The specific unit is defined as μmol of MDA/L.

**2.3.2. Determination of reduced glutathione (GSH):** The GSH concentration in blood was determined according to the method of Hu et al., (21) using kit. 2-dinitrobenzoic acid (DNTB) was reacted with GSH giving 2-nitro-5-thiobenzoic acid, a yellow colored product with a maximum absorbance at 412 nm which determined spectrophotometrically. The specific unit is defined as mg/dL.

**2.3.3. Superoxide dismutase (SOD, EC 1.15.1.1) activity:** The superoxide dismutase was determined using kit by the indirect method using pyrogallol as described by Michiels et al (22). The product was read at 420 nm and the specific unit is defined as U/mL.

**2.3.4. Total Glutathione peroxidase (t-GPx, EC 1.11.1.9) activity:** T-GPx activity was determined spectrophotometrically using kit in which cumene hydroperoxide acts as a substrate (23). The product was read at 366 nm and the specific unit is defined as U/L.

**2.3.5. Glutathione reductase (GSR, EC 1.8.1.7) activity:**

GSR is a ubiquitous NADPH-dependent enzyme which catalyzes the reduction of oxidized glutathione (GSSG) to GSH and determined spectrophotometrically (24) using kit. The product was measured at 340 nm and the specific unit is defined as U/L.

**2.3.6. Total Glutathione S-transferase, t-GST (EC 2.5.1.18) activity:** GST assay kit measures total GST activity by measuring the conjugation of 1-chlor 2-4 dinitrobenzene (CDNB) with reduced glutathione forming a conjugate product. This conjugation is accompanied by an increase in absorbance at 340 nm (25). The specific activity is defined as U/L.

**2.3.7. Liver function tests:** The transaminases [aspartate transaminase (AST) and alanine transaminase (ALT)], total protein (TP), albumin and total bilirubin (TB) were determined using appropriate kits (26).

**3. Results**

**3.1. MDA concentration:** The results showed that serum MDA levels were increased significantly in N, A, CH, CI and HCC groups by about 39.4 %, 52.4 %, 100.9 %, 133.2 % and 168.5 %, respectively as compared to the C group (Fig. 2).

**3.2. Reduced (GSH) level:** The results showed that GSH levels were decreased significantly in N, A, CH, CI and HCC groups by about 38.4 %, 44.9 %, 52.8 %, 65 % and 72.4 %, respectively compared to the C group (Fig. 3 a).

**3.3. GSR activity:** The results showed that GSR activities were increased significantly in N, A, CH, CI and HCC groups by about 23.2 %, 40.7 %, 64.1 %, 90.6 % and 187.9 %, respectively as compared to the C group as compared to the C group (Fig. 3 b).

**3.4. SOD activity:** SOD activities were decreased significantly in N, A, CH, CI and HCC groups by about 45.6 %, 53.0 %, 57.7 %, 64.8 % and 76.8 %, respectively as compared to the C group (Fig. 3 c).

**3.5. T-GPx activity:** As shown from Fig. (3 d), the activities t-GPx were decreased significantly in N, A, CH, CI and HCC groups by about 57.1 %, 64.8 %, 75.5 %, 80.0 % and 84.5 %, respectively as compared to the C group.

**3.6. T-GST activity:** The results showed that the GST activities were decreased significantly in N, A, CH, CI and HCC groups by about 40.1 %, 52.9 %, 57 %, 54.3 % and 65.8 %, respectively as compared to the control group (Fig. 3 e).

**3.7. Liver function tests:** The levels of TP and albumin were decreased significantly in N, A, CH, CI and HCC groups as compared to the control group (Table 1). However AST and ALT activities and TB levels were increased in all groups compared to the control group (Table 1).

<table>
<thead>
<tr>
<th>Table 1: Liver function tests in different stages of HBV infection</th>
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<td><strong>Studied groups</strong></td>
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Values of $P < 0.05$ were considered statistically significant.
Fig. 2: Malonaldehyde (MDA) in different stages of HBV infection.
Since N=normal carriers, A=acute infection, CH=chronic infection, Cl= cirrhotic liver and HCC=hepatocellular carcinoma patients.

Fig. 3: The levels of antioxidant parameters in different stages of HBV infection.
(a): Serum reduced GSH levels, (b) Serum GSR activities, (c) Serum SOD activities; (c) Serum GPx activities and (e) Serum GST activities. Since C=control samples, N=normal carriers, A=acute infection, CH=chronic infection, Cl= cirrhotic liver and HCC=hepatocellular carcinoma patients.
4. Discussion

In the present study the relationship between HBV DNA titer and different stages of HBV infection was studied and the data showed that there was no significant relationship between them. Our results agree with the previous studies of Yuen et al. (27), however, other studies reported that patients with less liver damage had higher viral load (28, 29).

Oxidative stress has been shown to be linked to liver diseases such as hepatotoxicity and other liver pathological conditions (30). In the present study the levels of MDA were elevated in all studied groups and follow the order: HCC > C1 > CH > A > N as compared to the control. This indicates that HBV stimulates the generation of ROS and LPO. It had been reported that ROS act as second messenger molecules and key elements to activate the nuclear factor kapa beta (NFκβ) in the early events of inflammation. NFκβ activates the inducible nitric oxide synthase (iNOS) which produces high amount of NO (31). Yoshide & Oshima (32) reported that NO is considered likely to be an important endogenous factor of carcinogenesis, both by itself and also in association with other agents. Hence, it leads to phagocytosis, amplification of inflammation and tissue injury. In addition, it reacts with O₂− producing peroxynitrite (ONOO⁻), a powerful oxidant, which damage the biological molecules (33). The generation of O₂, NO and other ROS and reactive nitrogen species (RNS) accelerate peroxidation of native membrane lipids leading to loss of membrane integrity, membrane damage and subsequent release of the cytosolic contents (34). In addition, peroxidation of mitochondrial membrane leads to increase the membrane permeability and alteration of Ca²⁺ homeostasis that contribute to cell death due to alteration in the inner membrane potential (35, 36). Our results are in agreement with the previous studies which reported elevated lipid peroxidation levels in patients with chronic hepatitis B and C (37-39) and in patients with alcoholic liver disease among the chronic alcoholics (40). In addition the previous studies showed that patients with CH, liver cirrhosis (LC), and HCC due to HBV infection had more than 20 times higher urinary episolond A levels compared to uninfected individuals with no liver disease. Among the mechanisms involved in mediating the process of liver fibrosis, an important role is played by ROS. During the progression of liver injury, hepatic stellite cells (HSCs) become activated, which produce extracellular matrix such as collagen I. Collagen I gene regulation has revealed a complex process involving ROS as a key mediator. ROS-sensitive cytokines contribute to HSC activation during inflammation through paracrine signals released from immune cells. The activated HSCs become responsive to platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β. PDGF facilitates the progression of hepatic fibrosis in human chronic liver diseases (CLD). It increased the accumulation of H₂O₂ in HSCs (7).

Hepatic GSH plays a crucial role in both scavenging ROS and the detoxification of drugs (36). The possible involvement of GSH in the metabolism of such products is indicated by decrease in its level in all studied groups and follows the order: HCC < C1 < CH < A < N as compared with the control. This may be due to the increased OS. This caused insufficient synthesis of GSH to maintain its adequate intracellular levels and in turn led to further GSH depletion (3, 4). This alteration may influence the capability of the liver to provide protection against oxidative damage (41). Also, the decrease in GSH level may be due to its consumption by GST (3, 4). In addition, to GSH depletion in all groups, the activities of both GST and t-GPx enzymes were decreased significantly as compared to the control since they follow the sequence HCC < C1 < CH < A < N. GST and GPx enzymes are GSH dependent (42) so the decline in GSH level may lead to decrease in both enzyme activities. The depression in the levels of both GSH and the activities of GST & t-GPx may be an adaptive response to condition of increased OS. In addition, the decrease in the level of GSH and the activity of GST indicated that the liver had a lower ability to detoxify the ROS resulting in an increased availability of potentially carcinogenic molecules. These carcinogenic molecules targeted hepatocytes to the S-phase of mitosis, eventually leading to a higher number of initiated cells with the time (43).

GSR is an important flavo-enzyme involved with glucose-6-phosphate dehydrogenase (G6PDH) in the maintenance of a reduced intracellular environment. It plays a key role in the cellular defense against oxidative stress by preventing the accumulation of GSSG and thus maintaining the reduct state (44). To recycle GSSG, the cell utilizes NADPH-dependent GSR and the NADPH being supplied by G6PDH (45). The levels of hepatic GSH were severely depleted where GSR activity was enhanced in N, A, CH, C1 and HCC groups as compared to the control group. This may be related to the upregulation of G6PDH enzyme which led to increase the level of NADPH leading to elevation of GSR activity (45).

SOD is the first line of defense of the cellular antioxidant system against oxidative damage mediated by superoxide radicals and it is considered as the most effective antioxidant (36). The decline in the activity of SOD in all studied groups in the present study indicates the increased formation of O₂⁻ radicals in liver tissues. This result is in accordance with other
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