

Persistent Oxidative Stress in Patients with Chronic Active Hepatitis-C Infection After Antiviral Therapy Failure

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ABSTRACT

Background/Aims: Oxidative stress and hepatocellular pathological changes are common associations with chronic hepatitis C virus (CHC) disease. The aim of this study was to assess serum antioxidant-oxidant (Redox) balance in patients with CHC infection before and after intake of the traditional antiviral therapy (pegylated interferon α -2b and oral ribavirin). **Patients and Methods:** Blood samples from 50 biopsy-proven CHC patients, with no prior anti-viral treatment and persistently elevated serum transaminase levels for 6 months, as well as 15 age- and sex-matched healthy subjects were used for determination of the antioxidants: reduced glutathione (GSH), superoxide dismutase (SOD), α tocopherol and ascorbic acid as well as lipid peroxidation (LPO) index (malondialdehyde [MDA]). The measurements were repeated in the diseased group 25 weeks after pegylated interferon α -2b and ribavirin combination therapy. **Results:** Serum levels of bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were significantly higher in CHC patients than in the control group ($P < 0.05$). Pretreatment serum MDA values were significantly higher in patients with CHC infection than the control group ($P < 0.001$), while serum antioxidant levels were significantly lower ($P < 0.001$). Responders (10 patients) had lower pretreatment serum levels of MDA than non-responders (35 patients) ($P < 0.001$). Both groups were comparable for the antioxidant serum levels. There was significant negative correlation between serum MDA and serum SOD, GSH, α tocopherol, and ascorbic acid concentrations in CHC patients. On the other hand, there was no correlation between the studied parameters and serum bilirubin, albumin, ALT, and AST. **Conclusions:** Redox imbalance was detected in patients with CHC. Responders had significantly lower levels of MDA than non-responders. Serum MDA may be used as a pretreatment predictor of response to antiviral treatment in patients with CHC.

Key Words: Ascorbic acid, glutathione, lipid peroxidation, malondialdehyde, oxidative stress, superoxide dismutase, α tocopherol

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Hepatitis C virus (HCV) is a major cause of viral hepatitis. Although the molecular mechanisms of HCV pathogenesis remain unclear, oxidative stress is emerging as a key step and a major initiator in the development and the progression of liver damage, and the evaluation of oxidative stress may be useful for a better understanding of the pathogenesis of hepatitis.^[1]

Patients infected with HCV showed increased serum

lipid peroxidation (LPO) products due to breakdown of the polyunsaturated fatty acids in the cell membranes, endoplasmic reticulum, and mitochondria.^[2-4] LPO leads to oxidative destruction of polyunsaturated fatty acids constitutive of cellular membranes. Their destruction leads to the production of toxic and reactive aldehyde metabolites such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE). These highly cytotoxic metabolites, produced in relatively large amounts, can diffuse from their site of origin to attack distant targets and form covalent bonds with various molecules.^[5] The augmented oxidative stress may possess diverse effects on cell growth and later on induce apoptosis.^[6-8]

Furthermore, studies have indicated that HCV can directly induce oxidative stress intracellularly in hepatocytes. HCV core gene expression has been associated with increased ROS, decreased intracellular, and/or mitochondrial glutathione

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(GSH) content, and increased levels of oxidized thioredoxin and LPO products.^[6-7] The molecular mechanism of how the HCV core protein induces oxidative stress has been investigated. The core protein has now been shown to associate with the outer mitochondria membrane via its COOH-terminal region.^[6] Previously, the increased ROS generation with HCV core protein was shown to be inhibited with diphenyliodonium,^[9] on the basis of this finding, the core protein was suggested to stimulate ROS production by the electron transport chain of mitochondria.^[10]

In addition, chronic HCV infection is associated with decreased plasma levels of the antioxidant contents due to their inefficient intracellular synthesis.^[11,12] Therefore, host antioxidant defenses, such as GSH, catalase, superoxide dismutase (SOD), and hemo-oxygenase-1 are subnormal in Chronic hepatitis C (CHC) disease. These HCV-induced Redox reactions can occur through multiple liver injury mechanisms that include chronic inflammation, activated Kupffer cells, and iron overload. In health, cellular Redox environment is tightly regulated by antioxidants that directly catalyze the unneeded oxidants or reverse their structural chemistry.^[8] There is a reverse correlation between level of severity of HCV infection and blood antioxidant levels.^[12] Therefore, antioxidant supplement is highly supportive against this disease.^[1,12]

Although there is no vaccine available till now for HCV, the current therapy in early stages of CHC disease still needs further progress. The combination of interferon α -2b and a nucleoside analog, ribavirin therapy is the treatment of choice. However, sometimes these two drugs may produce severe side effects, and their combined usage generates sustained beneficial response in only some patients.^[13-16] In addition, liver transplantation is a major surgery with partial and time-limited benefit.^[1]

In this study, the extent of free radical-mediated damage on lipids (measured as MDA levels) and effect on antioxidant defense mechanism (measured as reduced glutathione (GSH), SOD, α -tocopherol, and ascorbic acid) was studied in patients with chronic active hepatitis C disease. Also, the relationship between oxidative stress and response to antiviral (parenteral interferon α -2b+ oral ribavirin) treatment in these patients was determined.

PATIENTS AND METHODS

Study population

Fifty patients (30 males and 20 females) with CHC, aged between 36 and 50 years, were included in the current study. Patients with autoimmune hepatitis or HBV coinfection were excluded from the study. None of the patients was treated with antiviral agents before inclusion into the study. None

of them had any evidence for decompensated cirrhosis or any other causes of chronic hepatitis. Informed consent was obtained from each patient. Medical histories were taken and detailed physical examinations were performed in both patients and healthy control group subjects (9 males and 6 females), and individual body mass index (BMI) (kg/m^2) was calculated before including them in the study.

CHC disease was diagnosed according to the National Institute of Health Consensus on Hepatitis C Virus recommendations.^[17] Primarily, serum anti-HCV antibody was tested by enzyme-linked immunosorbent assay second generation (Ortho-Diagnostic System-Raritan, NJ, USA). However, serum positivity for HCV-RNA was confirmed by reverse transcription polymerase chain reaction.^[18] The diagnosis was confirmed by histopathological examination of liver biopsies.

All of them received pegylated interferon- α , 2b as Peginteron (Schering-Plough, Spain) 1.5 $\mu\text{g}/\text{kg}$ by subcutaneous injection once weekly and ribavirin as Ribetol (Schering-Plough, Spain) 200 mg capsules orally (3 capsules a.m. and 3 capsules p.m.) for 25 weeks.^[13-16] Liver and kidney functions, anti-HCV antibody testing, complete blood cell count, and urine analysis were done monthly and when needed, to monitor the antiviral therapy. Patients with contraindications to interferon-ribavirin medications (previous history of failed pegylated interferon- α -2b together with ribavirin treatment, antioxidant supplement intake since at least 1 month, pregnancy, portal hypertension, liver cirrhosis and hepatocellular carcinoma, total serum bilirubin level higher than 2.5 mg/dl, hemoglobin <8.0 g/dl, leucocytic count $<3,000/\text{ul}$, poor nutritional state, systemic infection, concomitant metabolic or autoimmune chronic liver diseases, and DM) were excluded from the present study.

After the antiviral therapy course, serum HCV-RNA testing was done for these patients. Patients who achieved negative HCV-RNA were considered responders and continued a second course (another 25 weeks) of antiviral therapy. On the other hand, patients who showed persistently detectable HCV-RNA with or without normalization of serum alanine aminotransferase (ALT) were considered as non-responders and discontinued the anti-viral therapy.

Laboratory methods

Two fasting blood samples, 6.0 ml each, were withdrawn from every patient: one before the recommended antiviral therapy intake and the second after 25 weeks course of this therapy. Automated complete blood cell counts were done immediately from whole blood sample. Then, the remaining blood was allowed to clot at room temperature and centrifuged at 3000 rpm for 10 minutes. Sera were separated as soon as possible from the clot. 1.0 ml serum from each sample

was put into an Eppendorf tube and stored at -70°C until assayed for the designed oxidant-antioxidant parameters. The remaining serum of each sample was used immediately for determination of serum creatinine, bilirubin, albumin, ALT, and aspartate aminotransferase (AST) concentrations.

Determination of serum oxidant-antioxidant parameters included

- LPO index (MDA, nmol/ml).^[19,20]
- Reduced GSH tripeptide activity (nmol/l).^[21]
- SOD enzyme activity (U/l).^[22]
- Alpha tocopherol vitamin activity (umol/l).^[23]
- Ascorbic acid vitamin activity (umol/l).^[24]

At the same time, 15 healthy subjects (9 males and 6 females) were similarly investigated.

Statistical analysis

The statistical package for social sciences (SPSS) for windows program Version 11 (SPSS Inc., Chicago, USA) was used for all statistical calculations. Results were expressed as mean \pm SD. Comparison of two mean values was done by analysis of variance with *t*-test. The statistical significance between mean values of two groups (unpaired data) was calculated by Mann-Whitney U test. The statistical significance of a difference between mean values of a single group (paired data) was calculated by Wilcoxon Signed Rank test. Correlation between two variables was done using Spearman correlation coefficient (*r*). The level of significance was read at the probability value $P < 0.05$.

RESULTS

There were no statistically significant differences in terms of sex, age, and BMI between CHC patients and healthy subjects. Serum levels of bilirubin, AST, and ALT were significantly higher in CHC patients than in the control group ($P < 0.05$) [Table 1].

Pretreatment serum MDA values were significantly higher in patients with CHC infection than the control group (mean: 5.3 ± 1.7 and 3.1 ± 0.8 nmol/ml, respectively). While pretreatment serum antioxidant levels were significantly lower than control (serum GSH, 54.2 ± 13.0 vs. 67.8 ± 13.5 nmol/l; SOD, 698.5 ± 123.4 vs. 815.2 ± 170.5 U/l; α tocopherol 19.6 ± 5.0 vs. 30.7 ± 5.5 umol/l; and Ascorbate, 50.3 ± 9.6 vs. 69.4 ± 10.7 umol/l, respectively) [Table 2].

Five patients discontinued the antiviral therapy due to bone marrow depression, 35 patients (70%) maintained HCV-RNA positivity and were considered non-responders, 10 patients (20%) had negative HCV-RNA and normal liver enzymes in response to the antiviral therapy course (responders) and continued antiviral therapeutic course.

Pretreatment MDA serum levels were significantly higher in non-responders than the corresponding values in responders (5.9 ± 1.5 vs. 4.8 ± 1.1 nmol/ml, respectively). By 25-week treatment, MDA levels significantly decreased in responders and non-responders (3.6 ± 0.8 and 5.7 ± 2.0 nmol/ml, respectively) [Table 3].

Pretreatment serum antioxidant levels were comparable in responders and non-responders (serum GSH, 50.4 ± 12.9 vs. 48.1 ± 10.5 nmol/l; SOD, 675.9 ± 111.6 vs. 649.7 ± 81.2 U/l; α -tocopherol, 20.9 ± 5.2 vs. 18.3 ± 4.1 umol/l; and Ascorbate, 51.3 ± 9.4 vs. 46.6 ± 10.0 umol/l, respectively). Treatment resulted in a significant decrease of antioxidant serum levels in responders and non-responders [Table 3].

There was significant negative correlation between serum MDA and serum GSH, SOD, α tocopherol, and ascorbic acid concentrations in CHC patients. On the other hand, there was no correlation between the studied parameters and serum bilirubin, albumin, ALT, and AST.

DISCUSSION

Human liver has complex sets of antioxidants (micronutrients, vitamins, and enzymes) that prevent oxidative damage of the life-saving hepatic cell components. Normally, the ROS are generated in small physiological amounts but when hepatic intracellular ROS production exceeds the antioxidant defense, oxidative damage of intracellular content, breakage of DNA strands, and peroxidation of polyunsaturated fatty acids in hepatocyte membrane occur.^[2-5,25] These unfavorable reactions result in decreased liver cell vitality.^[2-5]

Infection by hepatitis C virus is a significant global health problem. The prevalence rate of this infection is as high as 19% in Egypt.^[7] The majority of the infected patients fail to clear the virus and therefore become chronic. The clinical course of CHC infection is highly variable, ranging from mild hepatitis to hepatocellular carcinoma. About 20% of patients with CHC develop liver cirrhosis within 30 years. Once cirrhosis develops, the rate of hepatocellular cancer generation is high.^[26]

LPO is caused by free radicals leading to oxidative destruction of polyunsaturated fatty acids constitutive of cellular membranes. Their destruction leads to the production of toxic and reactive aldehyde metabolites such as MDA and HNE. These highly cytotoxic metabolites, produced in relatively large amounts, can diffuse from their site of origin to attack distant targets and form covalent bonds with various molecules. Therefore, recognition of LPO is of interest, as the deleterious effects of this process, including fibrogenesis, might be prevented by administration of scavenging systems or antioxidants.^[5]

Table 1: Clinical and biochemical characteristics of the study subjects

Parameter	Patient's group	Control group
Age in years	43.8±7.1	36-50 42.5±5.3
Sex		
Male	30	9
Females	20	6
BMI kg/m ²	24.4±3.8	25.1±3.0
Serum bilirubin (mg/dl)	1.8±0.3	0.8*±0.2
Serum albumin (g/dl)	4.0±0.6	4.2±0.4
Serum ALT (U/l)	107.0±11.7	22.9*±6.3
Serum AST (U/l)	89.6±11.6	31.5*±8.0

*Significant difference from the healthy reference group ($P < 0.05$). BMI: Body mass index, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase

Table 2: Serum malondialdehyde, glutathione, superoxide dismutase, a tocopherol, and ascorbate concentrations in the control group and CHC patients group

Parameter	Control group	CHC patient group
Pretreatment MDA nmol/ml	3.1±0.8	5.3±1.7*
Pretreatment GSH nmol/l	67.8±13.5	54.2±13.0*
Pretreatment SOD U/l	815.2±170.5	698.5±123.4*
Pretreatment α tocopherol umol/l	30.7±5.5	19.6±5.0*
Pretreatment ascorbate umol/l	69.4±10.7	50.3±9.6*

*Significant difference from the healthy reference group ($P < 0.001$).

CHC: Chronic hepatitis C virus, MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase

Table 3: Serum malondialdehyde, glutathione, superoxide dismutase, a tocopherol, and ascorbate concentrations in responders and non-responder subgroups

Parameter	CHC patient group	
	Responders subgroup	Non responder subgroup
Pretreatment MDA nmol/ml	4.8±1.1*	5.9±1.5*
MDA after therapy	3.6±0.8**	5.7±2.0**
Pretreatment GSH nmol/l	50.4±12.9	48.1±10.5
GSH after therapy	65.9±14.0**	51.5±11.0**
Pretreatment SOD U/l	675.9±111.6	649.7±81.2
SOD after therapy	790.4±87.9**	665.9±96.7**
Pretreatment α tocopherol umol/l	20.9±5.2	18.3±4.1
α tocopherol after therapy	27.5±6.0**	19.5±5.4**
Pretreatment ascorbate umol/l	51.3±9.4	46.6±10.0
Ascorbate after therapy	65.8±9.9**	44.9±11.5**

*Significant difference between responders and non-responders group

($P < 0.001$). **Significant difference from the corresponding basal value in the same group ($P < 0.01$) CHC: Chronic hepatitis C virus, MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase

In the present study [Table 2], CHC disease was associated with significantly high basal serum MDA level than the

corresponding normal value. Similar results have been reported in other studies.^[19,27] CHC is generally associated with oxidants excess and antioxidant depletion. GSH which is an important intracellular antioxidant was significantly reduced in CHC patients. This was associated with increased oxidized GSH metabolite suggesting an increased GSH turnover.^[2]

In health, plasma erythrocyte Cu-Zn SOD protects hepatocytes against O₂ toxicity through direct deactivation of many ROS.^[28] Being an intracellular component, it has a central role in maintaining cell Redox status. In turn, Cu Zn SOD deficiency can induce widespread oxidative damage and enhance hepatocellular carcinogenesis.^[29,30] The present study serum SOD mean concentration [Table 2] was significantly subnormal in patients with CHC disease before therapy. The basal serum lipid-soluble α -tocopherol and water-soluble ascorbic acid levels were significantly decreased in CHC patients. α -tocopherol is a powerful lipophilic antioxidant which is vital for cell life-maintenance and function. It suppresses LPO in the hepatocyte infrastructures and reverses the detrimental effects of the generated Redox imbalance *in situ* (liver) and other body organs and tissues. Ascorbic acid stimulates α -tocopherol activities. The deficiency of vitamins C and/or E enhances oxidative stress.^[31,32] These antioxidant vitamins maintain eicosapentaenoic acid content in mononuclear cells during antiviral therapy.^[33] Due to the effective role of antioxidants in HCV pathogenesis, it has been proposed in its treatment.^[8] A combination of three potent antioxidants (alpha lipoic acid, silymarin, and selenium) intake induces marked clinical, laboratory, and pathological improvements in chronic HCV-infected patients.^[5,34]

The cure response rate to PEG-IFNs plus ribavirin treatment in this study was about 20% [Table 3]. This relatively insufficient response rate may be due to resistance of the common HCV genotypes in Dakahlia locality of Egypt. The need for development of more efficient genotype drugs is a hope.^[1-16] In our study none of the responders had schistosomiasis. On the other hand, about one half of the non-responder group (35 patients) gave a clinical or laboratory history of schistosomiasis. Therefore, we can assume that portal bilharziasis may exaggerate viral hepatic injury and can suppress the viral response to treatment. This explanation goes hand on hand with that reported by other investigators.^[13-16]

Responders had significantly lower levels of MDA than non-responders. There was no in-between group difference in serum antioxidant levels [Table 3].

Although there was significant negative correlation between serum MDA and serum GSH, SOD, α tocopherol, and ascorbic acid concentrations in CHC patients, no correlation

was found between the studied parameters and serum bilirubin, albumin, ALT, and AST. A noteworthy observation is that patients with lower levels of serum MDA showed better response to antiviral treatment. Serum MDA may be used as a pretreatment predictor of response to antiviral treatment in patients with CHC.

From the present study, it is established that CHC is associated with oxidative stress, as evidenced by increased MDA level with decrease in antioxidant (serum GSH, SOD, α tocopherol, and ascorbic acid). Further study is needed on large sample size for better understanding of the concept so that supplementation with antioxidant vitamins may be helpful in preventing oxidative stress and increasing the efficacy of antiviral treatment in patients' CHC.

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