The Effect of Interferon and Ribavirin on Oxidative Stress in Chronic Hepatitis C Patients

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ABSTRACT

Hepatitis C virus is the most common cause of chronic viral hepatitis. The hepatic damage is due to both the cytopathic effect of the virus and the inflammatory changes. Oxidative stress could play a role in pathogenesis of hepatitis C virus infection. The aim of the present study is to determine oxidant/antioxidant status of patients with chronic hepatitis C and the effect of alpha-interferon plus ribavirin combination therapy on oxidative stress. Twenty patients with chronic hepatitis C and ten healthy controls were included in the study. Erythrocytes reduced glutathione (GSH), superoxide dismutase (SOD) and plasma total sulfhydryl group (tSH) were measured before and after 3 and 6 months of treatment and in control group and correlated with serum AST, ALT and viral load. Our results showed that there was reduction in erythrocyte GSH, SOD and plasma tSH in patients with chronic hepatitis C and they began to rise significantly early after 3 months of treatment and sometimes normalize after 6 months. Also, there were significant negative correlations between these parameters and liver enzymes (ALT and AST) and viral load. The present study showed presence of oxidative stress in chronic hepatitis C patients. Although interferon and ribavirin are not antioxidants, they promote an early inhibition of oxidative stress. This suggests that antioxidant supplementation might be considered in patients with chronic hepatitis C.

INTRODUCTION

Hepatitis C Virus (HCV) is considered the most common etiology of chronic liver disease in Egypt [1]. It is also associated with hepatocellular carcinoma [2]. The net liver damage from HCV infection depends on the balance between the host's antiviral mechanisms and the ability of the virus to subvert them [3].

HCV may cause oxidative stress in infected cells. Several lines of evidence support this connection, including the existence of activated glutathione turnover, the presence of increased levels of lipid peroxidation products and augmented iron stores in the liver[4].

The biological effects of free radicals are controlled by many antioxidants including reduced glutathione (GSH) and β-carotene. GSH detoxifies hydrogen peroxide (H_2O_2) and other organic peroxides to prevent their harmful effects. It also maintains the sulfhydryl (-SH) groups of proteins in the reduced state to protect them from oxidation[5]. GSH is decreased in the blood serum of patients with chronic hepatitis C (CHC) who are not treated with interferon[6].

The level of glutathione peroxidase (GSH-Px) was shown to

be diminished in serum and erythrocytes of children with abnormal liver functions [7] and there was low activity of GSH-Px and superoxide dismutase (SOD) in adults with acute hepatitis B [8].

Lipid peroxidation is caused by free radicals leading to oxidative destruction of polyunsaturated fatty acids which are constituents of cellular membrane[4]. Hydrogen peroxide is not considered as a free radical, but it stimulates damage to the tissues. Superoxide dismutase is an enzyme that catalyzes the dismutation of two superoxide anions (O_2-) into hydrogen peroxide and molecular oxygen then catalase enzyme hydrolyzes H_2O_2 into H_2O and $\frac{1}{2}$ O_2. The activity of these enzymes plays an important role in the process of the disease and the care of viral hepatitis C patients. Superoxide dismutase protects the tissue to a certain degree from the harmful effects of superoxide radicals [9].

Increased SOD activity appears to be an adaptive response to increased generation of superoxide ions [10] and the reduction in the amount of SOD reflects a decrease in the synthesizing capacity of liver, and the antioxidant defense power of the patients with CHC [11].

Sulfhydryl groups occur as non-protein compounds in free cysteine and in protein such as thioredoxin, glutaredoxin and albumin, which is the chief protein constituent of blood plasma [12, 13]. Thiol group (–SH) plays a prominent role in antioxidant reactions, and also in reactions of catalysis, regulation, electron transport and those preserving the correct natural structure of proteins [14]. The redox status of plasma thiols can be a diagnostic indicator of different pathological states [15] and the total sulfhydryl group (tSH) content of a biological sample is a valuable indicator of oxidative stress and of oxidative protein damage [16]. Total plasma sulfhydryl groups (tSH), reflecting the antioxidant capacity of the plasma, were found to be decreased in hepatitis C patients [17].

Because of repeated biopsies are needed to evaluate disease progression[18], many attempts have been made to find serological or cellular markers that help in the clinical management and evaluation of treatment response of chronic hepatitis C (CHC) patients. These include plasma viral load, plasma anti-HCV IgM or other soluble immune factors, hepatic viral RNA[4]. Although interferon-alpha is still used in treatment of chronic viral hepatitis, its effect on oxidants and antioxidants status is not well known and may be significant in deciding on the type of therapy, follow-up and prognosis of patients [4].

The present study was undertaken to evaluate oxidative stress in chronic hepatitis C patients by several potential oxidative stress markers in plasma and erythrocytes and further to evaluate the effect of the treatment on these biomarkers and their correlation with liver enzymes and the viral load.

MATERIALS & METHODS

Patients:
Twenty patients (11 males and 9 females), mean age 45 years, with chronic HCV infection were included in the present study. The patients

group was selected from Liver Unit of Minia Healthy Insurance Hospital. Patients with hepatitis were diagnosed in the Department of Infectious Diseases of that Hospital. The diagnosis of the patients was based on elevated serum transaminases, positivity for anti-HCV antibodies and the presence of HCV-RNA in serum. Also, a per-cutaneous liver biopsy was performed in the patients with chronic hepatitis in the same Unit by standard techniques using an appropriate tissue biopsy needle. The patients with other chronic or autoimmune liver diseases were excluded from the study.

Patients with CHC were started on IFN-α and Ribavirin treatment. They received their therapy once a week for a period of 6 months. Blood samples were drawn before and after 3 and 6 months of treatment.

Control group:

The control group consisted of 10 healthy individuals (6 males and 4 females), mean age 43 years. They were selected from volunteers came to the Liver Unit of Minia Healthy Insurance Hospital. They were seronegative for HCV, HBV. They showed no abnormal laboratory findings including liver function tests. The controls had no history of any liver disease, alcohol or any other chronic drug use.

Laboratory investigations:

Blood samples were drawn from the control and the patients into tubes containing EDTA. They were centrifuged at 4000 g for 10 minutes at 4°C. After separation of the plasma, the buffy coat was removed and the packed cells washed three times with two volumes of isotonic saline. Then, the erythrocytes were lysed with cold distilled water (1:4), stored in a refrigerator at 4°C for 15 minutes. The cell debris was removed by centrifugation (2000 g at 4°C for 10 minutes). The plasma and erythrocytes lysates were stored at -70°C until assayed.

Reduced glutathione and SOD were measured in erythrocyte lysate while tSH was measured in plasma. Data of ALT, AST and viral load are obtained from the Liver Unit.

1. **Measurement of reduced glutathione:** Reduced glutathione was measured using colorimetric assay kit provided by Biodiagnostic, Egypt according to the manufacturer's instruction. The method is based on the reduction of 5, 5′-dithiobis (2-nitrobenzoic acid) with reduced glutathione to produce a yellow colored compound. The values were expressed as mmol/g Hb [19].

2. **Measurement of superoxide dismutase:** Total SOD activity was measured using colorimetric assay kit provided by Biodiagnostic, Egypt as indicated by the manufacture's instruction. The assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. SOD activity is given in U/ g Hb [20].

3. **Measurement of total plasma sulfhydryl groups (t-SH):** t-SH groups were measured using Ellman's reagent 5,5-dithiobis-2 nitrobenzoic acid (DTNB). The thiol-disulfide interchange reaction between DTNB and thiols was the basis of that spectrophotometric assay [21]. An aliquot of plasma (0.20 ml) was mixed in a 10 ml test

tube with 0.6 ml of Tris (0.25 M)-EDTA (20 mM) buffer, followed by addition of 40 µl of 10 mM DTNB and 3.16 ml of absolute methanol. The tubes were capped, and the color was developed for 15-20 minutes, followed by centrifugation at 3000 g for 10 minutes at ambient temperature. The absorbance of the supernatant at 412 nm (A) was subtracted from a DTNB blank (B). Total SH groups were calculated as follow: (A-B) x 1.47 nM. The results are expressed as micromoles t-SH per gram protein after determination of total protein content of the sample with Lowery assay[22].

Statistics Analysis:

All data are presented as mean + SD. Significant difference among data were determined using independent Student's t test with SPSS version 13 and the correlation between the biochemical parameters were performed using Pearson's correlation coefficient (r). P value below 0.05 was considered significant.

RESULTS

Our data are presented in table 1 as mean + standard deviation showing the difference between the four groups in erythrocyte GSH, SOD and plasma tSH.

It was found that the erythrocyte GSH level was significantly decreased in patients of CHC (group II) compared to control group while after treatment (group III and group IV) it is significantly increased compared to group II but it was higher after 6 months of treatment. These findings showed that the treated patients are under the influence of increased oxidative stress.

The level of erythrocyte SOD was found to be significantly lower in hepatitis patients and begins to rise significantly after 3 months and continue rising till 6 months of treatment suggesting that interferon and ribavirin might be effective in the treatment of liver damage by improving the antioxidant system.

The level of plasma tSH was significantly decreased in hepatitis group and an increase was seen after 3 months of treatment reaching normalization after 6 months of treatment suggesting that anti-oxidative capacity of the plasma plays a role in viral hepatitis and its treatment.

Table 1: GSH, tSH concentration and SOD activity in the control group and in CHC patients before and after treatment (mean ±S.D).

Parameter	Group I (Control)	Group II, CHC before treatment	Group III, After 3 months treatment	Group IV, After 6 months treatment
GSH (mmol/g Hb)	6.06 ±0.61	3.37±0.46*	4.25 ± 0.42**	5.03±0.31**
SOD (U/g Hb)	603.3 ± 12.2	473.6±21.4*	536.8 ±19.6**	569.3±16.8**
tSH (µmol/g prot.)	8.96 ± 0.4	7.04 ± 0.3*	8.02 ± 0.3**	8.6 ± 0.4**

* Significant comparing with control group.
** Significant comparing with group II (CHC before treatment).

Table 2: AST, ALT activity and vital load concentrations in the control group and in CHC patients before and after treatment ((mean ±S.D).

Parameter	Group I (Control)	Group II, CHC before treatment	Group III, After 3 months treatment	Group IV, After 6 months treatment
AST (U/ L)	24.3 ±2.9	52.0 ± 20.3*	39.3±23.2**	28.3 ± 6.2**
ALT (U/ L)	28.7±4.2	56.6±18.5*	31.6 ± 11.4**	27.6±6.9**
Viral load IU/ml	[a]Negative	767303.8 ±946485.9 *	Undetectable**	Undetectable**

  * Significant comparing with control group.
 ** Significant comparing with group II (CHC before treatment).
[a] The meaning of "not detected" or "negative" differs, depending on the test used. In this lab, the detection limit for the "quantitative" HCV RNA test by "PCR" is 12 IU/ml.

We found that there is a significant negative correlation between erythrocyte GSH, SOD and plasma tSH and liver function parameters ALT and AST as these parameters highly increased in CHC patients and decreased with the treatment (table 3). Also, there was negative correlation between antioxidant parameters and viral load which was significant with SOD and tSH but insignificant with GSH (r= - 0.30, p=0.053).

Table 3: Correlation between GSH, SOD and tSH with liver parameters (ALT< AST) in the control group and in CHC patients before and after 3, and 6 months treatment and between these parameters and viral load in CHC patients before treatment.

	ALT	AST	Viral load
GSH	N = 70 r= -0.51 p=0.001*	N = 70 r= -0.64 , p=0.0001*	N = 20 r= -0.30 p=0.053
SOD	N = 70 r= -0.56 p=0.0001*	N = 70 r= -0.45 p=0.004*	N = 20 r= -0.52 p=0.001*
tSH	N = 70 r= -0.63 p=0.0001*	N = 70 r= -0.52 p=0.0001*	N = 20 r= -0.51 p=0.001*

  * Significant as correlation is significant at the 0.05 level. N= number of samples

DISCUSSION

HCV is considered the most common etiology of chronic liver disease in Egypt[23]. Although the main role of immunological mechanisms in pathogenesis of the chronic viral hepatitis B and C was demonstrated, researches also concentrate on the problem of oxidative stress in the pathology of the disease[4]. The results of the present study confirm that patients with chronic hepatitis C are in oxidative stress. Although interferon is still used in the treatment of hepatitis, its effects on the oxidant and   antioxidant status

of patients is not well known [11] and although interferon and ribavirin are not antioxidants, their antiviral capacity might reduce viral load and inflammation and perhaps though that mechanism might reduced virus-induced oxidative stress [4].

Our results show that GSH level in patients with CHC was significantly lower compared to those of the healthy control. Such finding was in agreement with **Suarez** and his colleagues who studied a group of 100 individuals with chronic hepatitis C. They demonstrated that GSH was decreased in the blood of patients who was not treated with interferon [24]. Confidently, it is known that GSH, a tripeptide that is synthesized in the liver by enzymatic reaction, is also a significant antioxidant [11]. Therefore, the reduction in the amount of GSH reflects both a decrease in the synthetic capacity of the liver and the antioxidant defense power of patients with viral hepatitis C. In contrast, GSH was significantly higher in blood of patients receiving interferon and ribavirin for 3 and 6 months.

In assessment of the correlation between reduced glutathione and some hepatitis parameters it was found that there is a negative correlation with both ALT and AST activity, while there was insignificant correlation with viral load, that finding may be due to that the number of HCV-RNA copies/ml varied considerably from patient to patient, and in a previous study it was found that there is no correlation between viral load and the level of ALT [25]. Also, it was reported that when a glutathione precursor was used with interferon, such treatment increased

the level of antioxidant enzyme and decreased the activity of ALT [26].

Although some researchers found that SOD may be increased in acute period but the level of enzymes decreased in the chronic period of the disease [9]. Others also found that SOD was significantly decreased in CHC patients before treatment when compared with healthy subjects [4]. That result is consistent with our results as we found that SOD activity was decreased in CHC patients before treatment. Increased lipid peroxidation is caused by the inflammation related to viral infection and decreased the antioxidant level may be an early marker of the oxidative stress [11].

In a previous study it was shown that the average SOD activity in erythrocyte was significantly increased after 48 weeks of treatment with interferon and ribavirin [4], while our results showed that this effect may be earlier as after 3 and 6 months of treatment there was significantly increase in SOD compared to the CHC patients.

Our study showed that there is a negative correlation between erythrocyte SOD and ALT, AST and viral load. This was in reverse with a previous study which showed that the activities of SOD did not correlate with the HCV-RNA levels [27]. However, **Irmisch and his colleagues** concluded the same results as they found that into the various patient groups, liver enzymes activities were associated in a significantly negative way with antioxidants [28].

The third biomarker of oxidative stress studied in our patients was plasma tSH which allows a global

evaluation of the patient's antioxidant defenses [25]. It was found that it is significantly decreased in the patients of CHC and began to rise again after 3 months and continue in rising after 6 months. That result is in agreement with a previous study which reported that values started to normalize from the third month of treatment and were maintained during the follow-up period [25].

By assessment of N-terminal propeptide of procollagen type III (PIIIP), a useful marker of fibrogenesis in the serum of patients [29], it was found that neither grading nor staging were correlated with plasma tSH level [25], however in our study there were significant negative correlation between plasma tSH and liver enzymes and also between tSH and viral load.

To our knowledge it is the first time to describe the correlation between plasma tSH and viral load, AST and ALT. This test is regularly done before treatment but may be a promising one.

As previously reported interferon prevents further development of fibrosis and improves serological markers of fibrogenesis[29,30] and in previous study it had been indicated that the oxidative stress is related with the activation of Hepatic Stellate Cells (HSCs), which are the central mediators in the pathogenesis of liver fibrosis [31], so one of the possible mechanisms of the action of interferon, explaining its anti-fibrotic effect, is by decreasing oxidative stress through the reduction of reactive oxygen species from HSCs.

The present study showed presence of oxidative stress in chronic hepatitis C patients and the effect of interferon and ribavirin on different oxidative markers after 3 and 6 months of treatment which may be helpful in follow-up and prognosis of the patient. Such early normalization of biomarkers may predict future response of the disease. Larger series of patients are necessary to confirm that point and further studies are suggested to evaluate these markers after the arrest of treatment.

Acknowledgment:

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تأثير الانترفيرون والريبافيرين على جهد التأكسد فى حالات الالتهاب الكبدى الوبائى سى

ماجى ماهر رمزى ـ هند محمد عبد الغنى
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يعتبر مرض الألتهاب الكبدى بفيروس سى من الأمراض المسببة للألتهاب الكبدى المزمن فى العالم وهو من أهم أسباب سرطان الكبد. وقد أثبت الانترفيرون فعالية كبيرة فى علاج هذا المرض ويتم استخدامه مع الريبافيرين بجرعات اسبوعية. يهدف هذا البحث الى معرفة تأثير الانترفيرون والريبافيرين على جهد التأكسد وعلاقته بأنزيمات الكبد.

لاجراء هذا البحث تم اختيار ٢٠ مريضا من المصابين بالالتهاب الكبدى سى بالأضافة الى ١٠ أشخاص من الأصحاء كمجموعة ضابطة. وقد تم قياس بعض العوامل الخاصة بجهد التأكسد (سوبر اكسيد ديسميوتيز و الجلوتاثيون المختزل وأيضا مجموعة السلفهيدريل الكلية) قبل العلاج وبعد ٣ و٦ شهور من العلاج ومقارنتهم بالمجموعة الضابطة وايضا تحديد العلاقة مع انزيمات الكبد ومستوى الفيروس فى الدم.

وقد اظهرت النتائج وجود انخفاض ذو دلالة احصائية فى نشاط سوبر اكسيد ديسميوتيز ومستوى الجلوتاثيون المختزل داخل كرات الدم الحمراء وايضا فى مستوى مجموعة السلفهيدريل الكلية فى البلازما عند الاصابة بفيروس سى ، بينما لوحظت زيادة ملحوظة فى هذه العوامل بعد ٣ شهور من العلاج واستمرت هذه الزيادة فى الارتفاع بعد ٦ شهور.

كما وجدت علاقة ارتباط عكسية بين عوامل جهد التأكسد وبين نشاط انزيمات الكبد (الانين ترانزيمينيز واسبارتيت ترانزامينيز) كما لوحظت ارتباط علاقة عكسية بينهم وبين مستوى الفيروس فى الدم.

من هذه الدراسة يمكن الاستنتاج ان الانترفيرون والريبافيرين لهم القدرة على تحسين عوامل جهد التأكسد وقد يعطى هذا مؤشرا لمدى امكانية استخدام هذه العوامل لتقييم الإستجابة للعلاج ومتابعة المرض.