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Reactive oxygen intermediates and serum antioxidative system in patients with chronic C hepatitis treated with IFN-α and thymus factor X

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: In this study, the chemiluminescence (CL) of peripheral blood polymorphonuclear leukocytes (PMNLs) and the serum total antioxidative system (TAS) were assessed in patients with chronic C hepatitis (CCH) before and after 3 and 6 months of treatment with interferon (IFN)-α and thymus factor X (TFX).

Materials and Methods: The study included 26 patients with CCH aged between 25–63 years (mean: 42.67). Combined therapy with IFN-α 2a and a TFX preparation was applied. PMNL metabolic activity was assessed applying the whole-blood CL method. We measured CL response of neutrophils unstimulated and stimulated by opsonized zymosan, N-formyl-methionyl-leucyl-phenylalanine (N-fMLP), and phorbol-myristate-acetate (PMA) without and after priming with tumor necrosis factor α (10 ng/ml). The assessment of serum TAS was performed directly before the beginning of therapy with IFN-α and TFX and after 3 and 6 months of the treatment. A colorimetric method based on the reduction of the cationic radical $ABTS^{\bullet+}$ (cation 2, 2'-azido-bis-[3-ethylobenzothiazolino-6-sulfonate]) in the presence of serum antioxidants was used.

Results: As a result of the treatment with IFN-α and TFX, the formation of free oxygen radicals by resting (unprimed) neutrophils increased statistically significantly both without stimulation and following stimulation by fMLP and PMA. A statistically significant increase in the serum antioxidant capacity was observed, which suggests the induction of compensatory processes.

Conclusion: Increased *in vitro* reactive oxygen species production by both stimulated and unstimulated peripheral blood neutrophils of patients with CCH was observed. Treatment with IFN-α and TFX resulted in a compensatory increase in serum antioxidative capacity.

Key words: chronic hepatitis • HCV • chemiluminescence • neutrophils • free radicals

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INTRODUCTION

Infection with the hepatitis C virus (HCV) is currently one of the most important problems in hepatology. According to a WHO report, in 1997 there were as many as 170 million people infected with HCV[21]. The risk of serious complications, including life-threatening diseases associated with HCV infection, is substantial in infected individuals. Despite the fact that HCV was identified 20 years ago, neither an effective method of treatment nor a vaccination to prevent infection are available. The pathological mechanisms involved in this disease still need to be explained. Several authors suggest the role of free oxygen radicals in liver cell damage. In recent studies, increased lipid, protein, and nucleic acid peroxidation in the blood and liver biopsy specimens from patients with chronic C hepatitis (CCH) has been demonstrated[6, 9, 15, 18]. Decreased levels of reduced glutathione in red blood cells and peripheral blood mononuclear cells as well as increased glutathione turnover have also been reported[9, 15, 19].

It has been shown that treatment with thymic extract, i.e. thymus factor X (TFX), has beneficial effects on the clinical course of chronic active hepatitis B[8]. Moreover, thymic peptide mixtures (Thymosin fraction 5 thymulin) have been proved to stimulate the immune response and enhance phagocytosis as well as the production of interleukin (IL)-1 and oxygen intermediates[3]. The stimulatory effect of thymic extracts in patients with hepatitis C has not been analyzed so far. The above observations suggest the application of thymic extract (TFX) in the supportive treatment of chronic C hepatitis.

Interferon (IFN)-α affects both immune response and the production of free oxygen radicals. Increased reactive oxygen species production in hepatitis C patients can inhibit HCV RNA replication and plays an important role in the suppression of HCV replication[5]. Under the influence of IFN-α, the secretion of other cytokines, e.g. tumor necrosis factor (TNF)-α, increases[11], which stimulates target cells and results in the synthesis of pro-inflammatory cytokines, augmented production of free oxygen radicals, and increased expression of adhesive molecules. Some authors report that in patients with CCH, treatment with IFN-α lowers the level of thiobarbitural acid-reacting compounds and down-regulates the activity of glutathione peroxidase, with a simultaneous increase in sulfuro-hydrogenic groups[15]. To verify the above observations, we decided to analyze the effect of IFN-α treatment on neutrophil oxygen metabolism and serum antioxidative capacity in patients with CCH.

MATERIALS AND METHODS

The study comprised 26 patients with CCH aged between 25 and 63 years (mean: 42.67). Written informed consent was obtained from each patient. Patients with cirrhosis, normal ALT activity, other causes of liver disease, previous immunosuppressive or antiviral treatment, and those who were pregnant were excluded from the study. The characterization of the study group is shown in Table 1. The control group consisted of 19 healthy age-matched subjects.

The diagnosis of chronic hepatitis was based on the results of liver biopsy specimen examination. HCV infection was established based on the presence of viral genetic material detected by the RT-PCR method. Combined therapy using IFN-α 2a (Roferon; Roche, Switzerland) and a TFX preparation (Jelfa Jelenia Góra, Poland) was applied. Roferon was administered subcutaneously 3 times weekly in a single dose of 6 million units. TFX was administered intramuscularly twice weekly in a single dose of 10 mg. The cycle of therapy was continued for 48 weeks. The patients were examined at the beginning of the therapy and after 1, 2, 4, 8, 12, 16, 20, 26, 32, 38, and 48 weeks of treatment. During each control visit, physical examination and basic laboratory tests were performed as follows: peripheral blood image with differential white cell count, platelet number, AST activity, the levels of GGTP, alkaline phosphatase, and bilirubin, and the prothrombin index (to complete the clinical picture only) were determined. During the 7 days prior to the examination no patient received any anti-inflammatory treatment or any other medications which could affect neutrophil activity. During the 4 weeks prior to the examination no patient showed (besides the typical general symptoms associated with IFN-α 2a treatment) any acute symptoms of infection. Blood samples were taken

Table 1. Characterization of the patients with CHC

Parameter	Mean ±SD	Min–max
Age	42.67±10.38	25–63
ALT (U/l)	131.55±77.64	60.00–353.00
AST (U/l)	84.63±59.07	33.00–311.00
GPT(U/l)	72.37±71.70	15.00–330.00
ALP (U/l)	74.54±28.55	44.00–160.00
Bilirubin (mg/dl)	0.93±0.40	0.36–2.25
Total protein (g/dl)	7.35±0.58	6.10–8.40
Albumins (g/dl)	4.19±0.42	3.21–5.25
Gamma globulins (g/dl)	1.36±0.28	0.68–1.85
Prothrombin index (%)	102.41±9.28	83.00–123.00
Fe (μg/dl)	118.45±53.87	39.00–362.00
Histopathology-staging (S)*	2.33±0.68	1.00–3.00
Histopathology-grading (G)*	2.24±0.42	2.00–3.00

* Estimation according Scheuer.

from the elbow vein. Measurements of neutrophil chemiluminescence (CL) were performed on whole-blood samples of each patient to avoid activating separation procedures, directly before the beginning of therapy and in the 3rd and 6th months of treatment. The activity of human neutrophils in the resting state and after priming with TNF-α (10 ng/ml) was assessed. The tests were carried out using the whole-blood CL method with luminol and the following patterns were applied: no stimulators and after stimulation with bacterial peptide fMLP, opsonized zymosane, which mimics bacteria, and PMA (phorbol ester), a receptor-independent transmembrane stimulator. In the priming pattern, the blood samples were incubated for 15 min at 37°C with TNF-α (10 ng/ml) before performing the measurements. CL measurements were performed at 37°C with a MLX Microtiter® Plate Luminometer (Dynex, USA). The CL intensity was measured for 0.2 sec every 2 min and expressed in relative light units (RLU) max. The results were expressed as RLU corrected by whole-blood neutrophil counts and hemoglobin levels according to the formula:

$$\text{CL calculated} = \text{CL measured (RLU max)} \times \times \frac{\text{Hb (\%)}}{\text{WBC } (10^3/100\,\mu\text{l}) \times \text{PMN (\%)}}$$

where: WBC – white blood cells, CL – chemiluminescence, Hb – hemoglobin, PMN – polymorphonuclear leukocytes.

TOTAL ANTIOXIDANT STATUS

Antioxidants were analyzed in the sera. All reagents were provided by Randox Lab. (UK). The examination was performed using a Randox Lab. set on 96--cell transparent plates. Reading the absorbance of the examined serum samples and standards was performed based on a method of one-point measurement by wavelengths of 550 and 630 nm. Antioxidants reduce the radical action of $ABTS^{\bullet+}$ (catio 2.2'-azino-bis-[3-ethylobenzothiazolino-6-sulfonate]) and cause suppression of color production to a degree proportional to their concentration in the sample. The obtained blue-green was measured at 600 nm. The results were expressed in mmol/l. The initial absorbance (after adding chromogen) and absorbance 3 min after adding the substrate (hydrogen peroxide) were measured. The concentrations of antioxidants were calculated based on the pattern and the difference between the absorbance after adding the substrate and the initial absorbance. The results were given in mmol/l.

STATISTICAL ANALYSIS

To assess differences in CL intensity and serum total antioxidant status (TAS) between the CCH patients and the healthy controls we used the Student's *t*-test for normally distributed variables and the Mann-Whitney U-test for variables which were not distributed normally. Differences in CL intensity and serum TAS capacity in CCH patients before treatment and in the 3rd and 6th months of therapy were evaluated statistically by analysis of variance with post hoc comparisons. Statistical significance was defined as a value $p<0.05$.

RESULTS

The above system of analyses enables parallel evaluation of both receptor-dependent and receptor-independent pathways of stimulation[12]. The results obtained are shown in Tables 2, 3, and 4. Long-term therapy of CCH patients resulted in an increase in TAS capacity (Table 4), which probably accounts for the normalization of CL both in the unstimulated pattern and after fMLP stimulation (Table 3). Six--month treatment normalized the CL in almost all the analyzed systems, which would suggest a healing process. The normalization of CL after 6 months of treatment could be explained theoretically by involvement of the TAS increase and the healing processes. Laboratory data, such as platelet number, AST activity, levels of GGTP, alkaline phosphatase, bilirubin, and prothrombin index, showed results typical for CCH treatment with IFN-α (not shown). After 3 months of treatment with IFN-α, the production of reactive oxygen intermediates (ROI) in neu-

Table 2. CL in patients with CCH before treatment compared with healthy controls

	No stimulators		FMLP		Opsonized zymosane		PMA	
	without TNF-α	TNF-α	without TNF-α	TNF-α	without TNF-α	TNF-α	without TNF-α	TNF-α
Before the treatment (n=25)	0.60±0.345*	1.21±0.803*	1.44±0.934*	1.85±1.036*	3.22±1.715	3.38±1.312	2.28±0.956	2.47±0.788
Control group (n=19)	0.33±0.182	0.55±0.260	0.55±0.240	1.08±0.380	3.00±1.103	3.00±0.905	2.21±0.752	2.34±0.856

Mean and SD are shown.
Neutrophils (both NS and after receptor-dependent stimulation with fMLP) of patients with CCH express higher CL values than those of healthy controls; * $p<0.05$.

Table 3. Neutrophil CL intensity in patients with CCH before and during the treatment

	No stimulators		fMLP		Opsonized zymosane		PMA	
	without TNF-α	TNF-α	without TNF-α	TNF-α	without TNF-α	TNF-α	without TNF-α	TNF-α
Before treatment (n=25)	0.60±0.345*	1.21±0.803	1.44±0.934*	1.85±1.036	3.22±1.715	3.38±1.312	2.28±0.956*	2.47±0.788
3 months of treatment (n=23)	1.07±1.011	1.43±0.937	2.37±1.790	2.53±1.796	3.31±2.805	3.36±2.559	3.67±1.869	2.91±1.409
6 months of treatment (n=20)	0.73±0.685	1.25±0.993	1.04±0.943	1.27±0.869	3.45±2.651	3.64±2.324	2.49±1.497	2.96±2.554

Mean and SD are shown.
* $p<0.05$; patients with CCH before treatment compared with the 3rd month of therapy.
The presented results show that a 6-month therapy normalizes neutrophil ROS production measured as CL intensity.

Table 4. Serum total antioxidative system (TAS)

	CCH			
	control group n=19	before treatment n=25	3 months of treatment n=23	6 months of treatment n=20
TAS (λ-550 nm)	1.89±0.094	1.63±0.612*	3.09±1.576	2.70±1.588

Patients with CCH show a lower TAS level before therapy compared with a 3-month therapy; * $p<0.05$. TAS capacity increase is an essential factor in compensating for the increased formation of ROI and could be a useful marker in monitoring results of the treatment.

trophils in the resting state (without priming) increased statistically significantly both without stimulation and after stimulation with fMLP and PMA. A significant increase in serum TAS (Table 4) was also noted, which suggests the existence of induced compensatory processes.

DISCUSSION

Neutrophils eliminate pathogens using an aerobic system and/or oxygen-independent killing systems (defensins)[22]. ROI promote the synthesis of hypochlorus acid, which reacts with primary amines to form relatively stable chloramines. The chloramines promote long-lasting oxidation, which results in nuclear factor-κB inhibitor inactivation and increased release of pro-inflammatory cytokines, which is accompanied by increased cytotoxic activity[1, 10, 14]. The stimulation of neutrophils by IFN-γ or TNF-α induce the receptor and integrin expressions which augment the cell response after agonist stimulation[4, 13]. Increased ROS production was demonstrated in patients with CCH. De Maria et al.[6] observed processes which involved lipid and protein oxidation. In another study, increased levels of lipid peroxidation products and increased activity of superoxide dismutase were found in mononuclear peripheral blood cells from patients with CCH. Simultaneously, glutathione level was decreased and the level of oxidized glutathione was increased in 35% of patients with CCH[15]. Those unfavorable processes are due to increased ROI formation and an insufficient TAS.

In the present study we observed increased production of ROS in patients with CCH in comparison with the healthy controls. However, we found no differences between the values of TAS in the plasma of patients with CCH and those of the control group. Increased ROS production can result from an increased release of pro-inflammatory cytokines such as IFN-α, TNF-α or IL-1. Increased TNF-α level is usually found in the peripheral blood of patients with CCH and is due to the induced immune reactions[11]. Crucial to therapeutic standards is that IFN-α was found to cause an increase in ROI formation *in vitro*. Stimulation of ROI release could account for the inhibition of HCV replication[5, 13]. Theoretically, applying IFN-α would intensify the unfavorable phenomenon of antioxidative stress. However, in several studies, therapy with IFN-α resulted in lowering the increased levels of the compounds which react with thiobarbiturical acid and in diminishing glutathione peroxidase activity with an increase in the total amount of sulfuro-hydrogenic groups[15], which would suggest an inhibitory effect on oxidative stress. Piazzolla et al.[16] found a decrease in ROI formation in patients who were effectively treated with IFN-α. However, ROI production was increased under the influence of adhesion molecules in their study. In the present study we noted an increase in ROI production during treatment with IFN-α and TFX. These results are different from those reported by Piazzolla et al.[16]. The observed differences are probably due to different methods applied in the studies.

Thymic hormones stimulate T lymphocytes to produce cytokines such as IFN-α, IFN-γ, IL-2, and IL-4 and, similarly, the expression of the lymphocyte receptors for IL-2[7]. Adreone et al.[2] examined *in vitro* the effects of one of the thymic proteins, α-1-tymosine (TA-1), on the production of the cytokines and 2'5'-oligoadenyle synthetase by the peripheral blood lymphocytes of patients with HCV infection. In their study, the lymphocytes were incubated in three systems: with TA-1, with IFN-α, and with TA-1 and IFN-α. Incubation

with TA-1 caused an increase in the production of IL-2 and 2'5'-oligoadenyle synthetase and a decrease in the release of Th2 cytokines (IL-4, IL-10).

Incubation with IFN-α caused increased IL-2, IL-4, IL-10, and 2'5'-oligoadenyle synthetase production. However, the incubation of lymphocytes with both of these proteins resulted in a higher production of IL-2 (synergistic effect) and decreased release of Th2 lymphocyte cytokines (IL-4, IL-10).

The statistically insignificant increase in total serum antioxidative capacity during treatment with IFN-α observed in our study is probably a phenomenon secondary to the excessive ROS production. Larrea et al.[11] claimed that antioxidative processes are impaired in patients with CCH. In their study, higher levels of Mn--superoxide dismutase (SOD) were found in peripheral blood lymphocytes, but not in the livers of patients with CCH. There was no correlation found between the Mn-SOD level and the expression of TNF-α mRNA, the level of the viremia, or inflammatory activity in the liver. The authors suggested that the liver may not be protected efficiently enough against oxidative stress and that oxidative stress is involved in the pathogenesis of HCV infection. The fact that lymphocytes and neutrophils also become infected in the course of HCV infection should not be ignored[17]. However, in the study by Toro et al.[20], no differences were found in the production of ROS between HCV-infected neutrophils and neutrophils with no HCV-RNA sequences. Our data are consistent with the results of Little et al.[13] and support the essential role of ROS overproduction in the pathogenesis of HCV infection.

In conclusion, increased *in vitro* ROS production by both stimulated and unstimulated peripheral blood neutrophils of patients with CCH was observed. Treatment with IFN-α and TFX resulted in a compensatory increase in serum antioxidative capacity.

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