Prolidase and Oxidative Stress in Chronic Hepatitis C

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Background: Hepatitis C infection represents a common healthcare issue worldwide. The present trial was designed to investigate the role of prolidase, an enzyme that is significantly involved in the biosynthesis of collagen, and of the oxidative stress that is considered to be involved in the pathogenesis of various diseases, in the chronic hepatitis C infection. The trial was performed to assess the serum prolidase enzyme level and the oxidative–antioxidative status and to determine the relation between the serum prolidase activity and the oxidative stress parameters. **Methods:** A total of 95 individuals, including 55 patients with chronic hepatitis C infection (CHC) and 40 healthy adults, were enrolled in the trial. The values for prolidase, the total antioxidant status (TAS), the total

oxidative stress (TOS), the oxidative stress index (OSI), sulfhydryl (SH), lipid peroxidation LOOH, catalase (CAT), and ceruloplasmin were measured and compared between the patient groups. **Results:** The prolidase, TOS, LOOH, CAT, and the OSI values were higher in the chronic hepatitis C group compared to the control group ($P < 0.001$). The TAS, SH, and ceruloplasmin levels were lower in the CHC group relative to the control group ($P < 0.001$). **Conclusion:** We suppose that the values of prolidase and the oxidative stress are increased while the antioxidant levels are decreased in CHC. As a result, prolidase and the oxidative stress seem to be related with the progression of the disease. J. Clin. Lab. Anal. 26:232–237, 2012. © 2012 Wiley Periodicals, Inc.

Key words: Chronic hepatitis C; prolidase; oxidative stress

INTRODUCTION

Hepatitis C infection represents a common healthcare issue worldwide. The chronic hepatitis C virus infection (CHC) affects 170 million people worldwide. In developed countries, chronic hepatitis C is one of the leading causes of cirrhosis, hepatocellular carcinoma, and liver transplantation (1).

The HCV infection usually exhibits an asymptomatic course. While serologic and molecular tests are used in diagnosing the disease, the most significant prognostic indicator of the disease is the assessment performed by the liver biopsy. Antiviral treatment of chronic hepatitis C aims at viral eradication and/or prevention of the fibrosis progression. In HCV genotype 1 patients, antiviral treatment has been primarily recommended for patients with at least significant fibrosis. HCV infection is curable and therefore such complications may be prevented by successful anti-viral therapy (2).

The liver fibrosis explained by a complex mechanism. It results from the destruction of the hepatic tissue rich in collagen and the excessive accumulation of the extracellular matrix proteins containing collagen. It is known to be formed by the destruction of collagen-rich hepatic tissue and the excessive accumulation of collagen-containing extracellular matrix proteins (3). Collagen is a protein, that is the main component of the connective tissue. Prolidase, an enzyme essential for the collagen biosynthesis, is also significantly involved in the collagen and intracellular protein degradation and shows an increase in activity.

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Received 13 January 2012; Accepted 1 March 2012

DOI 10.1002/jcla.21510

Published online in Wiley Online Library (wileyonlinelibrary.com).

Prolidase is the (EC: 3.4.3.7, iminodipeptidase) enzyme that also degrades the iminodipeptides. These dipeptides are produced upon degradation of the collagen (4).

The amino acids, proline, and hydroxyproline produced by the prolidase enzyme constitute approximately 25% of the collagen tissue and are essential to preserve the connective tissue (5).

The cells continuously produce the free radicals and the reactive oxygen forms as a part of the metabolic process. These free radicals and the reactive oxygen forms are neutralized by a complex-structure antioxidant system. Oxidative stress is the imbalance occurring between the reactive oxygen forms or the free radicals and the antioxidant system, which may lead to an irreversible damage to the significant cell components (6). Various trials demonstrated the role of the oxidative stress in the cellular damage and the damage to the DNA and RNA in cases of chronic viral hepatitis with no clear pathogenesis established (7, 8). In the medical literature, there were no previous trials reporting the activity of serum prolidase in patients with chronic hepatitis C.

This trial was designed to investigate the hypothesis that the serum prolidase activity could contribute to the development of the liver fibrosis. It is known that during the formation of fibrosis in the liver, the connective tissue is destroyed and is replaced by fibrotic scar tissue. We evaluated the usability of oxidative stress and measurement of prolidase enzyme, the amount of which increases in protein destruction, as a noninvasive biochemical marker in the evaluation of fibrosis degree and hepatitis C infection becoming chronic.

MATERIALS AND METHODS

The trial included 40 healthy adults and 55 patients diagnosed with chronic hepatitis C (anti HCV and HCV RNA positive) between 15 and 65 years of age, presenting to the Infectious Diseases and Clinical Microbiology Out-patient Clinic of the Harran University Medical Faculty and Tokat State Hospital.

In patients with anti HCV positive and HCV RNA positive; glucose, ALT, AST, albumin, globulin, and bilirubin values as well as PT, aPTT were measured, and complete blood counts were performed. Hepatobiliary ultrasound was performed for all patients, and the patients who were detected not to suffer from liver cirrhosis were included in the study.

After obtaining a signed informed consent from the patients with positive results for anti-HCV and HCV RNA, liver biopsy was performed. The histopathological evaluation of the biopsy samples was performed according to the Modified Knodell system.

Exclusion criteria

Patients with chronic disorders, including coronary artery disease, diabetes mellitus, hypertension, chronic renal failure, dyslipidemia, and hypertension, and patients with chronic alcohol consumption, smokers, those detected to have anemia and malignity, morbid obese individuals, and pregnant women were excluded from the trial. In addition, patients with liver cirrhosis, irrespective of the etiology were not included in the trial.

Blood samples

A range of 8–10 ml of blood sample was withdrawn from all the participants. The blood samples were centrifuged at 3,500 rpm for 10 min. Sera obtained were transferred into sterile tubes and stored at -80°C until the trial day.

Measurement of the serum prolidase activity

Prolidase activity was assessed by a photometric method devoted to the measurement of the proline levels created by prolidase (9). Plasma samples (100 μl) were combined with 100 μl of serum physiological. Twenty-five microliters of the combination were preincubated with 75 μl of the preincubation solution (50 mmol/l Tris HCl buffer pH 7.0 involving 1 mmol/l glutathione, 50 mmol/l MnCl_2 at 37°C for 30 min. The reaction mixture, which involved 144 mmol/l gly-pro, pH 7.8 (100 ml), was incubated with 100 ml of preincubated sample at 37°C for 5 min in order to stop the incubation reaction, 1 ml glacial acetic acid was added. After the addition of 300 ml Tris HCl buffer, pH 7.8 and 1 ml ninhydrin solution (3 g/dl ninhydrin was melted in 0.5 mol/l orthophosphoric acid) the combination was incubated at 90°C for 20 min and cooled with ice. Absorbance was after that measured at a 515 nm wavelength to determine proline value by the method proposed by Myara et al. (10). This method was a change of Chinard's method (11). Intra-assay and inter-assay coefficient of variations (CV) were under 10%.

Measurement of the oxidative stress

The total oxidative status (TOS), the total antioxidant status (TAS), and the oxidative stress index (OSI) were measured by the method developed by Erel. This method involves the formation of the long-lasting, stabile ABTS radical cation. The color of this characteristically blue-green radical disappears via reduction by the antioxidants. The lightening and/or decolorization efficacy of the antioxidants present in the sample is assessed as the total antioxidant capacity. Trolox, the water-soluble analog of

Vitamin E is used as the standard and the results are expressed in terms of $\mu\text{mol trolox equiv./l}$ (12). The percent ratio of TOS level to the TAC level was accepted as OSI. Oxidative stress index (Arbitrary Unit) = TOS ($\mu\text{mol H}_2\text{O}_2$ equiv./l)/TAC (mmol Trolox equiv./l) (13).

The serum catalase (CAT) activity was determined by Goth's colorimetric method, in which serum was incubated with H_2O_2 , and the enzyme reactions were stopped by the addition of ammonium molybdate. Serum CAT activity was expressed as kU/L (14).

Free sulfhydryl groups of the serum samples were assayed according to the method of Elman (15) as modified by Hu et al. (16). Briefly, 1 ml of buffer containing 0.1 M Tris, 10 mM EDTA, pH 8.2, and 50 μl serum were added to vessels, followed by 50 μl 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) in methanol. Blanks were run for each sample as a test, but there was no DTNB in the methanol. Following incubation for 15 min at room temperature, the sample absorbance was read at 412 nm on a Cecil 3000 spectrophotometer. Sample and reagent blanks were subtracted. The concentration of sulfhydryl groups was calculated using reduced glutathione as free sulfhydryl group standard and the result was expressed as millimolars.

Plasma lipid peroxidation (LOOH) was evaluated by the fluorimetric method (17), based on the reaction between MDA and thiobarbituric acid (TBA). Briefly 50 μl of plasma was added to 1 ml of 10 mmol/l diethylothiobarbituric acid (DETBA) reagent in phosphate buffer (0.1 mol/l, pH 3). The mixture was mixed for 5 sec and incubated for 60 min at 95°C. Samples were placed on ice for 5 min and then 5 ml of butanol was added. The mixture was shaken for 1 min to extract the DETBA-MDA adduct and then centrifuged at $1,500 \times g$ for 10 min at 4°C. Fluorescence of the butanol extract was measured at an excitation wavelength of 539 and an emission wavelength of 553. 1,1,3,3, Tetraethoxypropane (Sigma Chemical Company, St. Louis, MO, USA) was used as the standard solution and the values were presented as mol/l.

Ceruloplasmin levels were assessed by measuring its oxidative activity using p-phenylenediamine as the substrate, according to the method of Sunderman and Nomoto (18).

Statistical analyses

The Chi-square test was used for the categorical variables. The categorical variables were defined as percentages and counts. Kolmogorov-Smirnov test was applied in order to evaluate whether the distribution of continuous variables was normal or not. Two independent sample *t* test or Mann-Whitney *U* test was applied in order to compare the continuous variables between the two groups.

Continuous variables were shown as mean and standard deviation (SD) or as median and interquartile range (Q1–Q3). Pearson or Spearman correlation tests were applied to explore the relationship between the oxidative stress and the other parameters. SPSS software 15.0 for Windows (Chicago, IL) was applied for all statistical analysis. Calculated *P*-values were thought statistically important if they were lower than 0.05.

RESULTS

Of the 55 patients with CHC and the 40 healthy adults, 56 were males (58.9%) and 39 were females (41.1%). All patients were of genotype 1. The characteristics and the laboratory values of the patients are presented in Table 1.

The characteristics and the laboratory values of the patient group and the control group are presented in Table 2. There were no statistically significant differences between the two groups with regard to age and gender (*P* > 0.05 for both).

Prolidase values of patient and control groups are shown in Figure 1.

In cases of CHC, no correlation was detected between the patients' fibrosis levels and the prolidase values and the oxidative stress (*P* > 0.05).

A negative significant correlation was observed between the TAS and CAT, prolidase, TOS, and OSI values ($r = -0.627$, $r = -0.555$, $r = -0.393$, $r = -0.749$, $P < 0.001$); TAS was also negatively correlated with the age ($r = -0.217$, $P < 0.001$). A strong positive correlation was detected between the TOS and SH and ceruloplasmin levels ($r = 0.463$, $r = 0.372$, $P < 0.005$).

While OSI was strongly negatively correlated with TAS and SH ($r = -0.749$, $r = -0.489$, $P < 0.001$) and negatively correlated with ceruloplasmin ($r = -0.277$, $P < 0.005$), it was strongly positively correlated with CAT, prolidase, LOOH, and TOS ($r = 0.389$, $r = 0.467$, $r = 0.731$, $r = 0.874$, $P < 0.001$).

In the group of patients with CHC, HCV was positively correlated with OSI ($r = 0.425$, $P < 0.001$).

TABLE 1. The Characteristics and the Laboratory Values of the Patients

	Patients (<i>N</i> = 55)
Age, mean (SD)	50.69 $\pm$ 10.01
Female, n (%)	24 (43.6%)
ALT, mean (SD)	37.43 $\pm$ 20.12
AST, mean (SD)	30.67 $\pm$ 16.35
HCV RNA [$\times 100,000$ IU/ml], median (IQR)	219 (62.4–670)

ALT, alanine transaminase; AST, aspartate transaminase.

TABLE 2. The Demographic Characteristics and the Laboratory Values of the Chronic Hepatitis C Patients and the Control Groups

	Patients <i>N</i> = 55	Controls <i>N</i> = 40	<i>P</i>
Prolidase, mean (SD)	697.88 (685.04–713.03)	618.41 (598.27–643.91)	<0.001
TAS (μmol Trolox equiv./l), mean (SD)	0.67 ± 01	0.92 ± 0.13	<0.001
CAT (kU/l, median (IQR))	17.30 (14.59–1874)	8.49 (7.68–9.12)	<0.001
SH, mean (SD)	0.22 ± 0.036	0.28 ± 0.028	<0.001
LOOH (mol/l), median (IQR)	8.15 (5.31–9.90)	5.94 (4.98–7.10)	<0.05
TOS (μmol Trolox equivalents/l), median (IQR)	12.06 (8.43–17.24)	9.50 (8.18–10.45)	= 0.001
Ceruloplasmin, mean (SD)	535.79 ± 83.81	632.68 ± 151.71	<0.001
OSI (μmol Trolox equiv./l), median (IQR)	1.80 (1.20–2.69)	1.01 (0.88–1.17)	<0.001

TAS, total antioxidant status; CAT, catalase; SH, sulfhydryl; LOOH, lipid peroxidation; TOS, total oxidative stress; OSI, oxidative stress index.

DISCUSSION

As the first study to demonstrate prolidase activity in hepatitis C infection, this study showed that prolidase and oxidative stress increased in hepatitis C infection, and that prolidase enzyme and oxidative damage may have an association with the HCV infection becoming chronic.

This trial, which was the first trial to demonstrate the prolidase activity, measured the values of oxidative stress in addition to the prolidase values and compared them between the chronic hepatitis C patients and the healthy control individuals. The activity of the prolidase enzyme and the oxidative stress were demonstrated to be increased while the levels of the antioxidant agents were decreased in CHC.

There are certain diseases known to involve an increased prolidase level and oxidative stress, however patients with such diseases were excluded from the trial.

Approximately 3% of the global population is infected by HCV virus. There are six genotypes and subtypes of the hepatitis C virus. The most common is the genotype 1 worldwide. In turkey, the most frequent type of HCV infection is the genotype 1 (1, 19).

The mechanisms responsible for the progression of the liver disease to severe liver injury are still poorly understood (2). Collagen fibrils (specifically the type I and type IV collagen) in space of Disse the liver act as a frame to support the hepatocytes. If this frame structure is damaged, the improvement process results in fibrosis (20, 21).

In the studies conducted, the fibrosis level was evaluated with various biochemical markers, and the procollagen levels were shown to increase in advanced cirrhotic patients. A different study showed that type I and type IV collagen levels increased in alcoholic liver cirrhosis, non-alcoholic steatohepatitis and other chronic liver diseases, and also demonstrated the correlation between fibrosis degree and collagen (22). There is no previous study to measure prolidase in hepatitis C infection. However, the results of this study are similar to those of the other studies, which showed that the prolidase enzyme increased in liver damage.

Prolidase (E.C.3.4.13.9) is a widely distributed cytosolic enzyme involved in the degradation of the collagen structure. The enzyme hydrolyzes imidodipeptides and imidotripeptides with C-terminal proline or hydroxyproline on the helical structure of collagen and releases the two amino acids for collagen resynthesis and cell growth (23).

These imidopeptides originate from the intracellular degradation of procollagen, an extracellular form of collagen and the degradation of other proline- and hydroxyproline-containing proteins and dietary proteins. In addition to other collagenases, prolidase has a role in the final step of the collagen breakdown, and the enzyme may be the rate-limiting factor in the regulation of collagen biosynthesis. Proline and hydroxyproline compose approximately 25% of collagen, which is the most common protein in the human body (24).

Measurement of its activity is generally determined by photometric methods that analyze the level of proline produced by prolidase. However, thin-layer chromatography, paper electrophoresis, amino acid analysis, spectrometry, isotachopheresis, proton nuclear magnetic resonance spectroscopy, mass spectrometry, fluorometry,

Fig. 1. Serum prolidase level of patients and control group

high-voltage electrophoresis, and capillary electrophoresis all have been used for the assessment of prolidase activity (25). This trial used the photometric method to measure the prolidase levels.

There are studies showing an increased prolidase activity in cases of liver damage. An increased prolidase activity was demonstrated upon inducing hepatic damage by administration of CCl₄ to the mice (26). The four trials evaluating the prolidase activity in steatohepatitis, alcoholic liver disease, and the chronic liver disease reported an increase in the prolidase activity in the presence of hepatic damage (27–30). These studies demonstrated that the pathogenesis of these diseases could be elucidated by the collagen metabolism. However, our trial is the first to measure the prolidase levels in patients with CHC.

To define in simple terms, oxidative stress is the imbalance between the body's antioxidant defense and the production of the free radicals, which result in the peroxidation of the cellular lipid layer (10).

In cases of chronic hepatitis, oxidative stress was demonstrated to be involved in the development of fibrosis, cirrhosis, and hepatocellular carcinoma. The molecular mechanism of HCV pathogenesis remains unclear. However, oxidative stress has emerged as a key player in the development and the progression of many pathological conditions, including HCV-induced pathogenesis of liver (8).

Oxidative stress was measured by various biochemical markers in cases of chronic hepatitis C infection and shown to be involved in the pathogenesis and progression of the disease (31). The measurement of the total antioxidant status may yield more valuable information compared to the individual measurements; therefore, the use of total antioxidant capacity measurement to detect the antioxidative status becomes increasingly common. In the present trial, a simple, inexpensive, and convenient method developed by Erel was employed (12, 13). This trial used this method to measure the OSI, TOS, and TAS levels.

Additionally, ceruloplasmin, LOOH, SH, and the antioxidant SH, CAT enzymes were compared between the CHC and the control groups. All the markers of oxidative stress were significantly high in the CHC patients while the antioxidant markers were decreased in the presence of the disease.

In addition, the oxidative stress was detected to increase as the level of HCV RNA, a significant indicator of the disease. In the literature, there are no previous trials investigating the association between HCV RNA and the oxidative stress.

The level of hepatic fibrosis is established by the histological evaluation of the liver biopsy material. All the patients underwent liver biopsy. The degree of fibrosis was determined by Knodell scoring. HAI was assessed on the

biopsy material. This trial investigated the correlation of the oxidative stress with fibrosis and HAI. No correlation was detected between the fibrosis and HAI, which was assessed on the biopsy material, and prolidase and the oxidative stress. We suppose that the homogeneity of the patient groups, the exclusion of the patients with liver cirrhosis from the trial, and the presence of moderate chronic active hepatitis in all patients may have contributed to this result. Studies to be performed in a larger patient population with varying degrees of fibrosis are required.

Conflict of Interest: None to declare.

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