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Levels of Malondialdehyde, Myeloperoxidase and Nitrotyrosine in Patients with Chronic Viral Hepatitis B and C*

Stężenie aldehydu dimalonowego, mieloperoksydazy i nitrotyrozyny u chorych na przewlekłe wirusowe zapalenie wątroby B i C

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Abstract

Objectives. Oxidative stress is one of the potential biochemical mechanisms involved in the pathogenesis of chronic viral hepatitis The aim of the present study was to determine levels of oxidative stress in a large group of chronic viral hepatitis (CVH) patients who had not received antiviral treatment, and to assess the relationship between these parameters and viral load, fibrosis score and necro-inflammation of the liver.
Material and Methods. Two hundred CVH patients and 107 healthy subjects were included in this study. Malondialdehyde, myeloperoxidase and nitrotyrosine levels were determined.
Results. Malondialdehyde levels were significantly higher in the CVH patients than in the control group ($p < 0.05$), whereas myeloperoxidase activities were significantly lower ($p < 0.001$). There was no statistically significant difference between nitrotyrosine levels of the patients and the controls ($p > 0.05$). Additionally, no significant correlation was shown between these markers and viral load, necro-inflammation and fibrosis of the liver in chronic viral hepatitis patients.
Conclusions. The data from this study demonstrate that there is a disturbance in oxidative balance in patients with chronic viral hepatitis, but this imbalance was not correlated with viral load, necro-inflammatory activity or fibrosis of the liver (**Adv Clin Exp Med 2012, 21, 1, 47–53**).

Key words: chronic viral hepatitis, malondialdehyde, myeloperoxidase, nitrotyrosine.

Streszczenie

Wprowadzenie. Stres oksydacyjny jest jednym z potencjalnych mechanizmów biochemicznych w patogenezie przewlekłego wirusowego zapalenia wątroby.
Cel pracy. Określenie poziomu stresu oksydacyjnego w dużej grupie chorych na przewlekłe wirusowe zapalenie wątroby (CVH), którzy nie otrzymali leczenia przeciwwirusowego oraz ocena relacji między tymi parametrami i wiremią, stopniem włóknienia i martwiczego zapalenia wątroby.
Materiał i metody. 200 chorych na CVH i 107 zdrowych osób włączono do badania. Ustalono stężenie aldehydu dimalonowego, mieloperoksydazy i nitrotyrozyny.
Wyniki. Stężenie aldehydu dimalonowego było znacząco większe u pacjentów chorych na CVH niż w grupie kontrolnej ($p < 0,05$), a aktywność mieloperoksydazy była istotnie mniejsza ($p < 0,001$). Nie stwierdzono statystycznie istotnej różnicy między stężeniem nitrotyrozyny w grupie badanej i kontrolnej ($p > 0,05$). Nie wykazano także istotnej korelacji między tymi znacznikami a wiremią, martwiczym zapaleniem wątroby i włóknieniem wątroby u chorych na przewlekłe wirusowe zapalenie wątroby.
Wnioski. To badanie pokazuje, że równowaga oksydacyjna u pacjentów z przewlekłym wirusowym zapaleniem wątroby jest zaburzona, ale ten brak równowagi nie jest skorelowany z wiremią, martwiczym zapaleniem wątroby ani włóknieniem wątroby (**Adv Clin Exp Med 2012, 21, 1, 47–53**).

Słowa kluczowe: przewlekłe wirusowe zapalenie wątroby, aldehyd dimalonowy, mieloperoksydaza, nitrotyrozyna.

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Chronic viral hepatitis (hepatitis B and C) infections are major worldwide problems, frequently progressing to cirrhosis, liver failure or hepatomas [1]. The pathogenic mechanisms of liver injury and fibrosis in chronic viral hepatitis (CVH) are unclear, but are reported to include immunologic liver damage, direct cytotoxicity mediated by different viral products and induction of oxidative stress. The evidence for oxidative stress as a pathogenic mechanism is suggested by increased levels of lipid peroxidation (LPO) products such as malondialdehyde (MDA) in the serum and liver of patients with CVH [2].

One of the oldest hypotheses of reactive oxygen-induced cell injury.The peroxidative destruction mechanisms of polyunsaturated fatty acids have been extensively studied *in vitro*. A role for this mechanism in the liver *in vivo* is based on the following two observations: an increase in the parameters of LPO and the protective effect of antioxidants in combination with reduced LPO. In the activated stellate cells LPO products induce fibrosis by enhancing collagen gene expression. Therefore, LPO products may be important for signaling molecules [3].

One of the mechanisms for nitric oxide-induced cytotoxicity is explained, at least in part, by its reaction with superoxide to form peroxynitrite (PYT) during inflammation. PYT is a powerful oxidant that may damage DNA and membrane lipids. It can modify proteins at the methionine, tyrptophan, cysteine, and especially tyrosine residues. Nitration at the ortho position of tyrosine yields nitrotyrosine (NTY) as a stable end product after interaction with cellular proteins. Thus, the *in vivo* generation and biological activity of PYT is evidenced by the presence of NTY within cells from injured tissues [4].

As Liu et al reported, "Nitration of tyrosine, in both protein-bound form and free amino acid form, can readily occur in cells under oxidative/nitrative stress". Under these conditions, formation of NTY has been detected in a wide range of pathophysiologic conditions, including atherosclerosis, stroke, and pulmonary injury [5]. As Garcia-Monzon et al also noted: "In addition to serving as a biomarker of oxidative/nitrosative stress, elevated levels of NTY have been shown to cause DNA damage or trigger apoptosis" [4].

Myeloperoxidase (MPO) is a lysosomal heme protein, and as Arnhold wrote, it "is released from stimulated polymorphonuclear leukocytes at sites of inflammation... [Oxidative products] formed by MPO are involved in numerous processes of tissue damage. [One of the them,] hypochlorous acid is known to oxidize at a significant rate sulfhydryl and thioether groups of proteins.... MPO products are also involved in initiation of lipid peroxidation.... Nitration of free and protein-bound tyrosine correlates well with myeloperoxidase activity under inflammatory conditions" [6].

The aim of the present study was to investigate the role that hepatic oxidative stress may play in the pathogenesis of liver injury in CVH-B and CVH-C infections. The study entailed measuring plasma MDA, MPO and NTY levels and evaluating the association between these parameters and biochemical and histological findings in a large group of CVH patients who had received no antiviral treatment.

Material and Methods

The Study Population

The study was conducted at Gaziantep University Medical Faculty's Department of Infectious Disease and Department of Biochemistry, between January 2006 and January 2008. The study population consisted of 98 CVH-B subjects (66 males and 32 females; mean age 31.38 ± 11.45 years); 102 CVH-C subjects (38 males and 64 females; mean age 39.34 ± 12.66 years) and 107 healthy controls (58 males and 49 females; mean age 33.50 ± 9.09 years). The study protocol was carried out in accordance with the Helsinki Declaration as revised in Tokyo 2004. All of the study participants were informed regarding the study protocol and their written consent was obtained. The investigation was approved by the Ethical Committee of Gaziantep University.

Exclusion Criteria

Patients with other liver diseases were excluded by appropriate serologic testing and clinical history. Patients infected with both CVH-B and CVH-C viruses were excluded from the study, as were those who had received any antiviral/immunomodulatory treatment. Further exclusion criteria included concurrent diseases, severe lipid metabolism disorders, total serum bilirubin levels higher than 2 mg/dL and/or the use of medications capable of interfering with free radical production, such as nonsteroidal anti-inflammatory drugs, vitamins and iron-containning drugs. None of the subjects had smoked cigarettes or consumed alcohol in the previous 10 years.

Initial Evaluation

The diagnostic criteria for viral hepatitis were based on elevated liver enzymes (aminotransferases) for at least 6 months, and included positivity of

Table 1. Clinical characteristics of the patient and control groups

Tabela 1. Kliniczna charakterystyka grupy badanej i kontrolnej

Characteristics (Cecha)	CVH-B (n = 98)	CVH-C (n = 102)	Control (Grupa kontrolna) (n = 107)	p
Age – years (Wiek – lata)	31.38 ± 11.45	39.34 ± 12.66	33.50 ± 9.09	NS
Gender (Płeć)	32F/66M	64F/38M	49F/58M	NS
BMI – kg/m^2	24.23 ± 3.48	27.96 ± 5.65	25.26 ± 4.78	NS

Data are expressed as the mean ± SD.

Dane są wyrażone jako średnia ± SD.

both the hepatitis B surface antigen (HBsAg) and HBV DNA in the serum of patients with CVH-B, and the presence of antibodies to both HCV (anti-HCV) and HCV RNA by reverse transcription polymerase chain reaction (PCR) in the serum of patients with CVH-C. Findings of CVH were supported by histopathologic evaluations based on the Ishak modification of Knodell Histological Activity Index (HAI) scores. The control group consisted of healthy individuals with normal medical histories, physical examinations and blood biochemistry; none had positive CVH-C antibodies or CVH-B serum markers.

Sample Preparation

Blood samples were collected on the day of the liver biopsy. The blood was centrifuged at 900 × g for 10 minutes and the plasma samples were stored at –80 °C until analysis, as described below.

Malondialdehyde Analysis

The concentrations of lipid peroxidation were determined by estimating MDA using the thiobarbituric acid test. Calculations were performed from the standard curve with 1,1,3,3-tetramethoxypropane for calibration at 532 nm [7].

Nitrotyrosine Analysis

The NTY levels were detected in plasma samples using the Bioxytech sandwich ELISA immunoassay (OxisResearch, USA) according to the manufacturer's instructions.

Myeloperoxidase Analysis

Serum MPO protein concentration was measured quantitatively with the MPO Enzyme Immunoassay (ELISA) kit (Immundiagnostik AG, Bensheim, Germany), as described by Zelzer et al:

"Briefly, a rabbit anti-MPO peroxidase-labeled antibody was used for detection prior to the addition of tetramethylbenzidine as a substrate. After the acidic stop solution had been added to terminate the reaction, the intensity of the yellow color (change from blue-to-yellow) was directly proportional to the concentration of the MPO in the sample. A dose-response curve of the absorbance unit (optical density [OD] at 450 nm) versus the concentration was generated using the values obtained from the standard. The presence of MPO in the sample of patient was determined directly from this curve" [8].

Liver Histology

Liver biopsies were performed on the day the blood samples were collected. Liver histologic evaluation was performed by an experienced liver pathologist without knowledge of the background and clinical conditions of the patients. Needle biopsies were scored for inflammation and fibrosis using the Ishak modification of Knodell HAI scores [9].

Statistical Analysis

Statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to compare more than two groups with Tukey's test as a *post hoc* test. Spearman's coefficient of correlation was calculated. Statistical differences were considered significant at p values of 0.05.

Results

The clinical characteristics of the patients and the control group are presented in Table 1. There were no significant differences between age, gender and body mass index (BMI) of the patients and the control group ($p > 0.05$).

Table 2 shows the laboratory findings obtained for the patients with CVH. As noted in an earlier publication [10], except for alanine transaminase (ALT), the laboratory data were not significantly different between the two groups. CVH-B patients had significantly higher ALT and ESR levels compared to the CVH-C subjects ($p < 0.05$). No significant differences were observed in the liver histology grading and staging scores between the CVH-B and CVH-C patients.

The oxidant status of patients with CVH and the control group is presented in Table 3. Plasma MDA levels were significantly higher in the CVH-B and CVH-C patients than the controls ($p < 0.05$ and $p < 0.001$, respectively), while the MPO level was significantly lower ($p < 0.001$). There were no statistically significant differences between the NTY levels of the patients with CVH and the controls.

In patients with CVH, Spearman's correlation analysis showed no significant correlation between plasma MDA, MPO, NTY, viral load (HBV-DNA and HCV-RNA), fibrosis score and HAI ($p > 0.05$). In the patients with CVH-B, serum ferritin levels were significantly correlated with the fibrosis score and HAI (r: 0.328 and $p < 0.05$; and r: 0.294 and $p < 0.05$, respectively), but there was no correlation in the CVH-C patients.

Discussion

In the cases of CVH-B and CVH-C infections, as Fujita et al. noted, the "underlining mechanisms by which hepatitis viruses cause liver cell injury are largely unknown. Oxidative stress is one of the most probable mediators, because patients with chronic HCV infection show an increase in serum or liver content of oxidative stress markers, such as lipid peroxidation products" [11] including MDA and 4-hydroxynonenal. In the present study, MDA levels were significantly higher in subjects with CVH-B and CVH-C infections ($p < 0.05$ and $p < 0.001$, respectively). No significant difference in MDA levels was detected between the two patient groups ($p > 0.05$). These results are in accordance with the findings of other investigators [12–17]. Indeed, it has been reported that the amount of reactive oxygen species in the livers of healthy subjects was significantly lower than in the livers of patients with hepatitis B or hepatitis C infections [17].

It has been suggested that increased LPO in the livers of patients with CVH-B and CVH-C initiates a cascade leading to active fibrogenesis. Oxidative stress and reactive aldehyde products induce activation of hepatic stellate cells. As Yadav et al. wrote: "Activation of hepatic stellate cells is a critical step in liver fibrosis because activation markedly stimulates collagen production by these cells" [2].

Moreover, in the present study MDA levels were not correlated with biochemical parameters, necro-inflammatory activity, fibrosis score or viral load in subjects with CVH-B and CVH-C infections. Previous studies have reported similar findings of non-correlation between MDA levels and liver histology [18, 19].

Although increased LPO in the form of MDA has been reported previously, no study has investigated the levels of MPO and NTY in the plasma of patients with CVH-B and CVH-C. The present study demonstrated that plasma MPO levels were significantly lower in subjects with CVH-B and CVH-C than the controls ($p < 0.001$). There was no significant difference between the MPO levels of the two patient groups ($p > 0.05$). Bekhett et al. reported that tissue MPO activity increases significantly in patients with CVH-C than the controls, whereas plasma MPO activity does not [20]. To the current authors' knowledge this is the first study investigating plasma MPO levels in patients with CVH-B.

As Arnhold wrote, MPO is a "multifunctional enzyme involved in both host defense and tissue damage at inflammatory sites. It produces not only oxidative equivalents, but contributes also to the regulation in general response to invading microorganisms" [6].

Arnhold also wrote: "Azurophilic granules of [polymorphonuclear leukocytes (PMNs)] contain a huge amount of [MPO]. Together with the membranous NADPH oxidase, MPO is involved in the formation of reactive oxygen species and oxidation of biological material together with the membranous NADPH oxidase" [6] Citing Babior et al [21] and Segal and Abo [22], Arnhold continued: „In stimulated PMNs, NADPH oxidase reduces molecular oxygen to superoxide anion radical. This species and its dismutation product, hydrogen peroxide, are substrates for [MPO]" [6].

As Kothari et al. wrote, "plasma MPO concentrations may be a marker of the neutrophil proliferation and severity of inflammation" [23]. Citing Cuzzocrea et al. [24], Van der Vliet et al. [25] and Baldus et al [26], Arnhold wrote: "Nitration of free and protein-bound tyrosine correlates well with myeloperoxidase activity under inflammatory... In inflammatory models, the immunoreactivity of MPO strongly co-localizes with the formation of nitrotyrosine in subendothelial and epithelial tissue regions" [6]. In the present study, the correlations between plasma MPO levels and MDA, and between NTY levels and the liver histology findings, were not statistically significant ($p > 0.05$).

As Dikici et al wrote: „In viral hepatitis, the virus also infects the peripheral lymphocytes. The infected lymphocytes produce interferon to stimulate healthy cells against viruses" [27]. Although IFN-α is not an anti-oxidant, the antiviral capacity of IFN-α might reduce viral load, inflammation and virus-induced oxidative stress through this mechanism [13, 28].

The findings in the current study demonstrated that there were no statistically significant differences between the plasma NTY levels of CVH patients and those of the controls. Additionally, no significant difference was found in NTY levels between the types of hepatitis. It has been reported that the intrahepatic amount of NTY was significantly higher in viral liver disease than in non-viral liver disease and also that intrahepatic accumulation of NTY was positively related to the severity of liver damage [4]. In the present study, it was demonstrated that plasma NTY levels did not correlate with HAI, fibrosis score or viral load. Plasma NTY levels do not reflect NTY accumulation in the hepatic tissue.

Iron is the ideal metal catalyst for the generation of reactive oxygen species (ROS) and other free radical intermediates, as well as for the induction of LPO [29].

As Fujita et al. wrote: „Hepatic iron deposits have been associated with the degree of liver inflammation and damage in HCV-infected liver tissues. Moreover, a close correlation between the amount of iron accumulation and hepatic fibrosis has also been reported in [CVH-C]" [11, 30]. It has been clearly demonstrated that hepatic iron is responsible for liver damage through ROS formation, leading to LPO and alteration of the cellular membrane. Fujita et al. went on: „Therefore, iron may cause liver tissue injury by increasing the formation of toxic hydroxyl radicals leading to progressive liver inflammation, fibrosis, and increased risk for developing liver cancer in [CVH-C]" [11, 30]. In the present study, the serum ferritin level

Table 2. The laboratory findings of subjects with chronic viral hepatitis

Tabela 2. Wyniki laboratoryjne chorych na przewlekłe wirusowe zapalenie wątroby

	CVH-B Mean ± SD	CVH-C Mean ± SD	Reference ranges (Wartości referencyjne)
WBC ($\times 10^3/\mu L$)	6.91 ± 1.64	6.98 ± 2.24	3.98–10.04
Hb (g/dL)	14.97 ± 1.59	14.32 ± 1.53	12–16
PLT ($\times 10^3/\mu L$)	236.45 ± 59.47	226.03 ± 79.78	150–450
PT (second)	13.84 ± 1.09	13.49 ± 1.14	10–14
aPTT (second)	30.86 ± 5.46	31.15 ± 4.15	23–35
INR	1.08 ± 0.11	1.05 ± 0.11	0.8–1.2
ALT (U/L)	118.38 ± 114.65	78.87 ± 53.57*	8–41
AST (U/L)	76.12 ± 87.78	61.66 ± 39.75	8–38
Albumin (g/dL)	4.12 ± 0.45	4.13 ± 0.47	3.4–5.5
ESR (mm/hour)	1.35 ± 8.00	17.76 ± 13.72*	1–18
Serum ferritin (ng/mL)	150.03 ± 451.13	103.20 ± 91.84	5–150
Viral load			
HBV DNA (KIU/ml)	22.87 ± 51.36		
HCV RNA (KIU/ml)		973.69 ± 4.32	
Liver histology			
Histologic activity index (HAI)	4.67 ± 2.61	5.74 ± 2.87	
Fibrosis staging (Stopień zwłóknienia)	1.88 ± 1.56	2.38 ± 1.36	

* $p < 0.05$.

* $p < 0{,}05$.

Table 3. Oxidant status of patients with chronic viral hepatitis B and C and the control group

Tabela 3. Stan oksydacyjny chorych na przewlekłe wirusowe zapalenie wątroby B i C i osób zdrowych z grupy kontrolnej

	CVH-B	CVH-C	Control (Grupa kontrolna)
MDA – nmol/mL	11.56 ± 2.21*	13.83 ± 2.49**	3.89 ± 0.28
MPO – ng/mL	33.74 ± 2.23**	34.52 ± 2.38**	57.96 ± 5.13
NTY – nM	17.68 ± 0.91	18.14 ± 0.67	19.27 ± 1.07

Data are presented as the mean ± SEM.
*$p < 0.05$ vs. controls.
**$p < 0.001$ vs. controls.

Dane są wyrażone jako średnia ± SEM.
*$p < 0.05$ vs. grupa kontrolna.
**$p < 0.001$ vs. grupa kontrolna.

was significantly correlated with the fibrosis score and HAI in patients with CVH-B (r: 0.328 and $p < 0.05$; r: 0.294 and $p < 0.05$, respectively), while no correlation was found in patients with CVH-C. Fujita et al. proposed that „iron reduction therapy is a safe and potentially promising therapeutic modality for patients with CVH-C" [11, 30]. In view of the data from the current study, the same therapy may be suggested for patients with CVH-B.

Nutritional factors also significantly impact oxidative stress, and analyzing both serum levels and intake of micronutrient antioxidants might have been helpful additions to this study.

In conclusion, the data from the current study demonstrate that in CVH patients a disturbance of oxidative balance exists, with a significant increase in the LPO product MDA. Additionally, a significant decrease was observed in plasma MPO levels in patients with CVH-B and CVH-C infections as compared to the control group. However, this difference was not correlated with viral load, necroinflammatory activity or fibrosis score in the liver. No significant differences were noted in the CVH patients' NTY levels as compared to the control group.

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