A new marker for lipid peroxidation: Serum paraoxonase activity in non-alcoholic steatohepatitis

Yeni lipid peroksidasyon belirleyicisi: Non-alkolik steatohepatitte serum paraoxonase aktivitesi

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Background/aims: *Relationship between hepatic antioxidant paraoxonase 1 (PON1) activity, lipid peroxidation and liver injury was investigated in patients with non-alcoholic steatohepatitis.* ***Methods:*** *A total of 23 patients with non-alcoholic steatohepatitis (15 males, 8 females; mean age: 40.30±7.67 yrs) and 23 healthy controls (14 males, 9 females; mean age: 39.70± 8.78 yrs) were enrolled in the study. Serum paraoxonase 1 activity and levels of a well-known lipid peroxidation marker, serum malondialdehyde, were determined.* ***Results:*** *Serum paraoxonase 1 activity decreased significantly in non-alcoholic steatohepatitis compared to the control group (p<0.01). Serum malondialdehyde levels were significantly higher in patients with non-alcoholic steatohepatitis as compared with the control group (p<0.05). No statistically significant correlations were found between serum paraoxonase 1 activities and the grade-stage of non-alcoholic steatohepatitis, serum lipid levels or serum malondialdehyde levels (p>0.05).* ***Conclusions:*** *Increased lipid peroxidation may be either a cause or a result of liver injury in patients with non-alcoholic steatohepatitis. Although serum paraoxonase 1 activity does not reflect the degree of liver damage in non-alcoholic steatohepatitis, reduced paraoxonase 1 activity, especially in the presence of mild disease, could be interpreted as a biochemical marker of the lipid peroxidation.*

Key words: Non-alcoholic steatohepatitis, lipid peroxidation, paraoxonase 1, malondialdehyde

Amaç: *Non-alkolik steatohepatit olgularında karaciğer kökenli antioksidan paraoxonase 1 aktivitesi ile lipid peroksidasyonu ve karaciğer hasarı arasındaki ilişkinin ortaya konulması amaçlandı.* ***Yöntem:*** *Non-alkolik steatohepatit tanısı almış 23 olgu (15 erkek, 8 kadın; yaş ortalaması: 40.30±7.67 yıl) ve 23 kontrol (14 erkek, 9 kadın; yaş ortalaması: 39.70± 8.78 yıl) çalışmaya dahil edildi. Serum paraoxonase 1 aktivitesi ve lipid peroksidasyon belirleyicisi olarak kabul edilen serum malondialdehid düzeyleri ölçüldü.* ***Bulgular:*** *Serum paraoxonase 1 aktivitesi non-alkolik steatohepatit grubunda kontrol grubuna göre istatistiksel olarak anlamlı derecede düşük bulundu (p<0.01). Serum malondialdehid düzeyleri non-alkolik steatohepatit grubunda kontrol grubuna göre istatistiksel olarak anlamlı derecede artmış olarak bulundu (p<0.05). Serum paraoxonase 1 aktivitesi, hastalığın derecesi, serum lipid düzeyleri ve serum serum malondialdehid düzeyleri arasında istatistiksel olarak anlamlı bir ilişki gösterilemedi (p>0.05).* ***Sonuç:*** *Artmış lipid peroksidasyonu non-alkolik steatohepatit'te ortaya çıkan karaciğer hasarının bir sonucu veya nedeni olabilir. Sonuç olarak, her ne kadar serum paraoxonase 1 aktivitesi ile hastalığın evresi arasında doğrudan bir ilişki olmasa da non-alkolik steatohepatit'in erken evresinde dahi serum düzeylerinin anlamlı derecede azalması, paraoxonase 1 aktivitesinin lipid peroksidasyonunun biyokimyasal belirleyicisi olarak kabul edilebileceğini düşündürmüştür.*

Anahtar kelimeler: Non-alcoholic steatohepatitis, lipid peroxidation, PON1, MDA

INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a form of chronic hepatitis with histological features of alcohol-induced liver disease that occurs in individuals who do not consume significant amounts of alcohol (1). Female gender, obesity, hyperlipidemia and diabetes mellitus are well-known risk factors of NASH (1). However, NASH is not necessarily a disease of patients that have one or more risk factors. In fact, NASH has been reported in those without any of the above-mentioned risk factors (2). The pathogenesis of NASH remains unclear, although two pathways of injury are implicated: increased oxidative stress and lipid peroxidation associated with increased fat deposition in the liver, and tumor necrosis factor endotoxin-mediated injury (3-10). A role for lipid peroxidation in NASH

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has been suggested by recent studies showing its presence in both animal models of non-alcoholic fatty liver and humans with steatosis of different etiologies (3-13). The proinflammatory and profibrogenic properties of lipid peroxidation end products, malondialdehyde (MDA) and 4-hydroxynonenal, potentially account for all of the typical histological features observed in this disorder (1, 3, 4). Paraoxonase (PON) is an ester hydrolase that catalyzes the hydrolysis of some xenobiotics, such as organophosphates, unsaturated aliphatic esters, aromatic carboxylic esters, and possibly, carbamates. The paraoxonase gene family contains at least three members, PON1, PON2 and PON3. Several studies have shown that PON1 has antioxidant properties especially against low-density lipoprotein (LDL) oxidation (14-16). The liver plays a key role in the synthesis of serum PON1 (15, 17-19). It was shown that PON1 levels decreased in chronic liver disease and the authors put forward that serum PON1 activity measurement could significantly improve the current efficiency of a laboratory's evaluation of patients with suspected chronic liver disease (19). Liver enzyme elevations, especially mild to moderate increases in serum alanine aminotransferase (ALT) levels and infrequently in serum aspartate transaminase (AST) and g-glutamyl transpeptidase (GGT) levels, were detected in patients with NASH (1). However, there is no correlation between the enzyme levels and the severity of histopathological impairment. Ultrasonographic findings are not correlated with liver enzyme levels or histological findings. Liver biopsy is the gold standard for diagnosis at present (1-10). As mentioned above, measurement of PON1 activity may contribute to the diagnosis of patients with suspected liver disease. In addition, PON1 is accepted as an antioxidant enzyme (14-16). In light of these data, this study was designed to investigate (a) the oxidative and antioxidative status by measuring serum MDA levels and PON1 activity, (b) whether or not PON1 activity can be a non-invasive method of estimating the severity of liver disease, and (c) the relation between PON1 activity and MDA, ALT, GGT, and AST levels, or histopathological findings in patients with NASH.

MATERIALS AND METHODS

A total of 23 patients with NASH (15 males, 8 females; mean age: 40.30±7.67 years) and 23 healthy controls (14 males, 9 females; mean age:

Table 1. The demographic variables and body composition in the two groups

Variable	NASH	Control	p
Gender (M/F)	9/14	8/15	NS
Age (yr)	40.3±7.7	39.7±8.8	NS
BMI, kg/m²	29.32±4.25	25.36±5.15	<0.01

Data are mean±standard deviation, NS=nonsignificant

39.70± 8.78 years) were enrolled in the study (Table 1). NASH patients were admitted or referred to our department with liver enzyme elevations, which were found by coincidence. For the diagnosis of NASH and to rule out other possible liver diseases, all patients with NASH underwent a detailed clinical and laboratory evaluation. Patients and controls with possible ethanol ingestion, a previous or current history of gastrointestinal surgical procedures and protein malnutrition and a history of corticosteroid use were excluded from the study. Laboratory studies were obtained at the time of referral and these included serum liver tests (ALT, AST, alkaline phosphatase, total bilirubin, albumin, and total protein levels), hepatitis B and C serology (hepatitis B surface antigen, antibody to hepatitis B surface antigen, antibody to hepatitis B core antigen and antibody to hepatitis C virus), autoimmune serology (anti-mitochondrial antibody, anti-nuclear antibody, anti-smooth muscle antibody, and anti-liver/kidney microsomal antibody), studies of iron metabolism (fasting serum iron, transferrin saturation, and ferritin levels), and ceruloplasmin levels. Serum glucose, cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels were also obtained. Abdominal ultrasonography was performed in all cases. Control cases had normal ultrasonographic findings. NASH was definitively diagnosed in all patients by histopathological examination following liver biopsy. A combination of hepatocellular steatosis, ballooning and disarray, acinar, or portal inflammation, and fibrosis in histopathological examinations was graded as grade I (mild), grade 2 (moderate), and grade 3 (severe), and fibrosis was staged as stage one to four as suggested by the necroinflammatory grading and staging system for steatohepatitis (20). Body mass index (BMI) was calculated as weight (in kilograms) divided by the square of height (in square meters) in all patients and controls. Cases were classified into three groups as normal weight group (BMI <25 kg/m²), overweight group (BMI≥25 - <30 kg/m²) and obese group (BMI≥30 kg/m²) (21).

Informed consent was obtained from the patients prior to the study. Erciyes University Ethical Committee approved the study protocol and the procedures; these were in accordance with the Helsinki Declaration of 1975. All blood samples were collected in the morning after an overnight fast, and serum samples were stored at - 70°C until assay for PON1 and MDA.

Assay of Paraoxonase Activity

We measured the rate of hydrolysis of paraoxon by monitoring the increase of absorbance at 405 nm and at 25°C. The basal assay mixture included 1.0 mM paraoxon and 1.0 mM CaCl$_2$ in 0.05 M glycine buffer, pH 10.5. One unit (IU) of paraoxonase activity is defined as 1μmol of p-nitrophenol formed per min, and activity was expressed as U/L of serum (22).

Measurement of Serum MDA Concentration

Serum MDA levels were measured according to a method described elsewhere (23). The principle of the method was based on the spectrophotometric measurement of the color occurring during the reaction of thiobarbituric acid with MDA. Concentration of thiobarbituric acid reactive substances (TBARS) was calculated by the absorbance coefficient of MDA-thiobarbituric acid complex and expressed in nmol/ml. As a standard, MDA bis (dimethyl acetal)-TBA (thiobarbituric acid) complex was used.

Statistical Analysis

All results are expressed as mean values with their standard deviations (± SD). Mann-Whitney U, χ^2 and Fischer exact tests were used to compare the differences in values between the groups. Spearman's correlation analysis was performed. All analyses were two-tailed and were conducted using computer-based statistical software (SPSS® for Windows® 9.0); p value less than or equal to 0.05 was accepted as statistically significant.

RESULTS

The results of the main parameters studied in the groups are summarized in Table 2. Hypertriglyceridemia was detected in two (8.7%) subjects in the control group and in four (17.4%) patients in the NASH group (p>0.05). Six patients (26.1%) and two (8.7%) subjects in the control group had hypercholesterolemia (p>0.05). Among 23 patients in the NASH group, 10 (43.5%) were overweight and 10 obese (43.5%), while in the control group,

Table 2. The results of main biochemical tests in the two groups

|	NASH	Controls	p
PON1 activity (U/L)	186.38±138.76	248.36±90.79	<0.01
MDA (nmol/ml)	1.56±0.93	1.04±0.43	<0.05
ALT (U/L)	89.30±37.02	22.13±8.44	<0.001
AST (U/L)	57.83±45.81	22.78±8.97	<0.001
GGT (U/L)	70.95±40.02	20.13±5.54	<0.001
Glucose (mg/dl)	94.26±11.79	75.78±12.17	<0.001
Total cholesterol (mg/dl)	195.87±40.81	175.17±42.18	NS
Triglyceride (mg/dl)	153.22±61.32	159.87±45.36	NS
LDL cholesterol (mg/dl)	125.36±34.83	135.26±38.22	NS
HDL cholesterol (mg/dl)	41.35±11.06	39.65±12.98	NS

Data are mean±SD, NS=nonsignificant

eight subjects (34.8%) were overweight and four (17.4%) obese (p<0.05). The steatohepatitis group had higher BMI (p<0.01). None of the patients or the subjects in the control group had morbid obesity, and none of the patients had a diagnosis of diabetes mellitus. On the other hand, mean serum glucose level was higher in the NASH group than in the control group (94.26±11.79 mg/dl vs. 75.78±12.17mg/dl; p<0.001). Serum PON1 activities were found to be significantly lower in the NASH group than in the control group (p<0.01; Figure 1). Serum MDA levels were significantly higher in patients with NASH as compared with the control group (p<0.05; Figure 2).

Histopathological examinations revealed that 19 (82.6%) of the NASH patients had grade 1 and four (17.4%) had grade 2 necroinflammatory activity. Three patients (13%) had stage one and two patients (8.7%) had stage two fibrosis. No
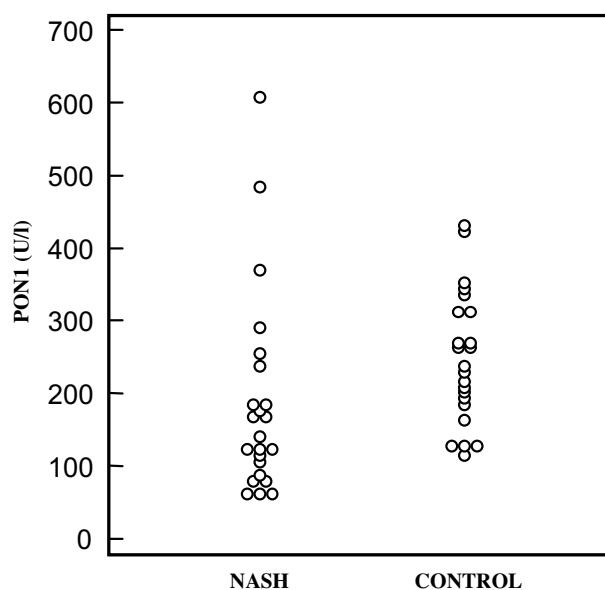
Figure 1. Serum PON1 activities in the two groups
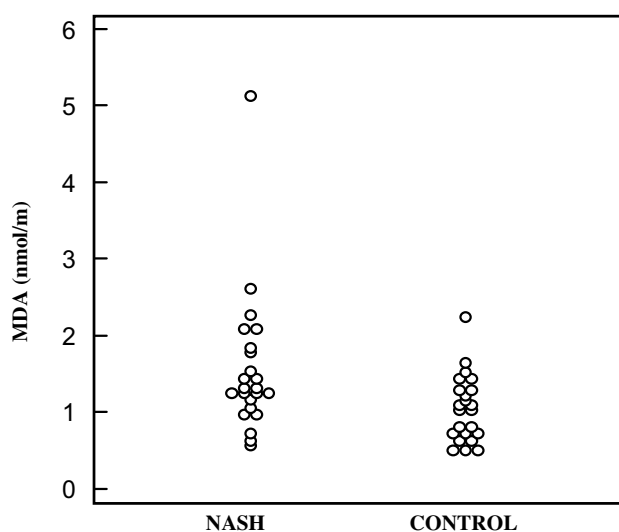
Figure 2. Serum MDA activities in the two groups

statistically significant correlations were found between serum PON1 activities and the grade-stage of NASH, serum lipid levels or serum MDA levels (p>0.05).

DISCUSSION

The pathogenesis of NASH is poorly understood. The exact stimulus that causes steatosis to progress to steatohepatitis and fibrosis is unclear. Recently, increased free fatty acid levels, increased mitochondrial fatty acid β oxidation, increased hepatic lipid peroxidation and the presence of peripheral insulin resistance were identified in patients with either fatty liver alone or NASH without cirrhosis (1, 3-13). MDA and 4-hydroxynonenal (HNE) are well known by-products of lipid peroxidation (1, 3, 4). Reactive oxygen species, which are formed due to increased microsomal ω-oxidation, peroxisomal β-oxidation, and mitochondrial β-oxidation, trigger lipid peroxidation; this in turn causes cell death and release of MDA and HNE (3, 4, 6). In this study, significantly higher levels of MDA were measured in patients with NASH than in controls. Increased serum MDA level is one of the well-known indicators of lipid peroxidation, but there is insufficient data about serum PON1 levels in patients with NASH. Reduced serum PON activities have previously been reported in diabetes mellitus, chronic renal failure and rheumatoid arthritis (24-28). The present study revealed that serum levels of PON1 activity were reduced in patients with NASH. The mechanism of decrease in serum PON1 activity in patients with NASH is

unclear. Serum PON1 activity is generally considered to vary in response to the consumption of PON1 for the prevention of oxidation (29). The decrease in serum PON activity in NASH patients might have resulted from increased inactivation of PON1 according to increased generation of reactive oxygen species in NASH (29, 30). There is increasing evidence that several cytokines mediate hepatic inflammation, apoptosis, and necrosis of liver cells and fibrosis. Among the various cytokines, the proinflammatory cytokine tumor necrosis factor-α (TNF-α) has emerged as a key factor in various aspects of liver disease. TNF-α mediates not only the early stages of fatty liver disease but also the transition to more advanced stages of liver damage (5). It has been shown that proinflammatory cytokines such as interleukin-1 and TNF-α down-regulated mRNA expression of PON1 in HepG2 cells (31). This cytokine-mediated reduction of PON1 production by the liver might be responsible for the decreased serum PON1 activity in NASH patients. On the other hand, decreased serum PON1 activity could be accepted as another evidence of increased lipid peroxidation, since it was shown that a decrease in liver microsomal PON1 activity is an early biochemical change related to lipid peroxidation and liver injury observed in rats with CCl4-induced cirrhosis (32). It was suggested that these changes result in the conversion of HDL from an anti-inflammatory/antioxidant complex into a proinflammatory/prooxidant complex (32). In a recent study, decreased PON1 activity in sera of patients with chronic liver disease was suggested to be related to the degree of liver damage (19). In our study, a great majority of NASH patients (82.6%) had grade 1 necroinflammatory activity and 18 of 23 (78.2%) patients had no fibrosis. Although NASH patients had minimal disease activity, decreases in PON1 activities were evident. Nevertheless, we could not determine a statistically significant correlation between grade and stage of NASH and serum PON1 and MDA levels.

Increased MDA level, a well-known lipid peroxidation marker, and reduced serum PON1 activity reflect increased oxidative damage in patients with NASH. We conclude that increased lipid peroxidation may be either a cause or a result of liver injury in patients with non-alcoholic steatohepatitis, and that serum PON1 activity does not reflect the degree of liver damage in non-alcoholic steatohepatitis.

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