Nitric Oxide and Nitrotyrosine Levels in Relation with Oxidative Stress-Related Markers in Non-Alcoholic Fatty Liver Disease

Non-Alkolik Yağlı Karaciğer Hastalığında Oksidatif Stres Belirteçleri ile İlişkili Olarak Nitrik Oksit ve Nitrotirozin Düzeyleri

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ABSTRACT Objective: Non-alcoholic fatty liver disease (NAFLD) is recognized as one of the most common liver diseases when viral hepatitis and heavy alcohol consumption are excluded. Although the pathogenesis of NAFLD is still poorly understood, a crucial role has been proposed for oxidative stress. Nitric oxide, through the formation of peroxynitrite, has the potential to augment the oxidative stress seen in NAFLD. Therefore, we aimed to investigate the possible contribution of nitric oxide and peroxynitrite to the pathogenesis of this disease. **Material and Methods:** Thirty-eight NAFLD patients [8 hepatosteatosis, 30 non-alcoholic steatohepatitis (NASH)] and nine healthy subjects were included in this prospective study. Nitrite/nitrate, malondialdehyde, superoxide dismutase, catalase, glutathion and nitrotyrosine (peroxynitrite marker) levels were measured in blood samples. **Results:** Nitrite/nitrate, malondialdehyde, catalase, superoxide dismutase, and glutathion levels in NAFLD patients were higher than those in control subjects. On the other hand, the levels in hepatosteatosis group were not different from those in NASH group. In addition, there were no differences in nitrotyrosine levels between the groups investigated. In addition, nitrite/nitrate levels showed significant correlations with malondialdehyde, superoxide dismutase, catalase and glutathion levels. In contrast, no correlations were found between nitrite/nitrate and nitrotyrosine and between malondialdehyde and nitrotyrosine levels. **Conclusion:** Nitric oxide and reactive oxygen species seem to have a parallel role in the pathogenesis of NAFLD.

Key Words: Nitric oxide; oxidative stress; nitrotyrosine; liver diseases

ÖZET Amaç: Non-alkolik yağlı karaciğer hastalığı (NAYKH), viral hepatitler ve ağır alkol tüketimi bir kenara bırakılırsa en sık görülen karaciğer hastalıklarından biri olarak kabul edilmektedir. Her ne kadar NAYKH patogenezi tam olarak anlaşılamamış olsa da oksidatif stres için önemli bir rol biçilmiştir. Nitrik oksidin peroksinitrite dönüşerek NAYKH'de görülen oksidatif stresi arttırma potansiyeli vardır. Bundan dolayı nitrik oksit ve peroksinitritin bu hastalığın patogenezine olası katkısını araştırmayı planladık. **Gereç ve Yöntemler:** Bu prospektif çalışmaya 37 NAYKH hastası [8 hepatosteatozis, 30 non-alkolik steatohepatitis (NASH)] ve dokuz sağlıklı gönüllü dâhil edildi. Nitrit/nitrat, malondialdehid, süperoksid dismutaz, katalaz, glutatyon ve nitrotirozin (peroksinitrit belirteci) düzeyleri kan örneklerinde ölçüldü. **Bulgular:** Nitrit/nitrat, malondialdehid, süperoksid dismutaz, katalaz ve glutatyon düzeyleri NAYKH hastalarında kontrol grubuna göre yüksekti. Öte yandan hepatosteatozis hastalarının değerleri NAYKH hastalarınkinden farklı değildi. Ek olarak, gruplar arasında nitrotirozin düzeyleri açısından fark yoktu. Ayrıca nitrit/nitrat düzeyleri malondialdehid, süperoksid dismutaz, katalaz ve glutatyon düzeyleri anlamlı pozitif korelasyon gösterdi. Aksine nitrit/nitrat ve nitrotirozin arasında herhangi bir korelasyona rastlanmadı. **Sonuç:** Nitrik oksit ve serbest oksijen radikalleri NAYKH'nin patogenezinde paralel rol oynuyor gibi görünmektedir.

Anahtar Kelimeler: Nitrik oksit; oksidatif stres; nitrotirozin; karaciğer hastalıkları

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Non-alcoholic fatty liver disease (NAFLD) is recognized as one of the most common liver diseases when viral hepatitis and heavy alcohol consumption are excluded. NAFLD includes a broad spectrum of fat-induced liver injury, namely hepatosteatosis, non-alcoholic steatohepatitis (NASH), and cirrhosis. The term NASH was first introduced by Ludwig and colleagues in 1980, which occurred predominantly in obese, diabetic females who denied alcohol consumption.[1] The mechanisms underlying the pathogenesis of NAFLD are still poorly understood. Nevertheless, there exists a hypothesis named "two-hit", where steatosis being related to the first hit and steatohepatitis to the second.[2,3] In this context, accumulation of fat in the liver due to several factors such as obesity and insulin resistance has been proposed to constitute the first hit.[2] As regards to the second hit, several factors have been postulated to have a role in the progression from steatosis to steatohepatitis, including increased cytokine activity, oxidative stress, and mitochondrial dysfunction.[4] In this regard, both animal and human studies have demonstrated an association between oxidative stress and NAFLD.[5-7]

From a molecular point of view, both reactive oxygen species (ROS) and reactive nitrogen species (RNS) are prone to contribute to the oxidative stress seen in chronic liver diseases. Among the ROS, superoxide anion (O_2^-) seems to be a key starting point of oxidative stress, even though it is not a potent oxidant *per se*.[8] On the other hand, several other and more toxic ROS can be generated by means of distinct mechanisms such as Fenton reaction.[8] In addition, peroxynitrite ($ONOO^-$) is a strong and more toxic radical, which exists due to the reaction of O_2^- and nitric oxide (NO).[8] Thus, NO may augment cytotoxicity of oxidative stress through this particular reaction. The source of NO within the liver may be inducible NO synthase (iNOS) since oxidative stress has been shown to, directly or through the induction of transcription factors, regulate iNOS expression.[8] In support of this, iNOS expression, in connection with nitrotyrosine ($ONOO^-$ marker), in the liver samples of patients with severe NASH revealed an increase in comparison to those with mild NASH, suggesting that NO-mediated oxidative injury may be involved in the etiology of NASH.[9]

In view of the compelling data collected so far, we aimed to investigate the potential contribution of oxidative stress and NO to the pathogenesis of NAFLD. Accordingly, we first measured nitrite/nitrate (NOx) levels as an indicator of NO formation. Secondly, malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and glutathion (GSH) levels were analyzed to evaluate oxidative status. Finally, nitrotyrosine were measured so as to assess the possible interaction of NO and O_2^-.

MATERIAL AND METHODS

SUBJECTS

This was an observational prospective study in where standard medical procedures performed on patients were not altered. Nine healthy subjects volunteered served as the control group, the data of which was used in another study carried out in cirrhotic patients (an as yet unpublished study). Control subjects were accepted as healthy after a detailed clinical and biochemical evaluation of the liver, heart and kidney, thereby excluding any disease state including infection. The patients were selected from those applying to the gastroenterology outpatient clinic of our hospital. Patients suspected to have NAFLD were further analyzed through a detailed clinical, biochemical and ultrasonographic examination. Afterwards, biopsies were taken from patients to confirm the disease and classify the patients into hepatosteatosis and NASH. Patients were accepted to have hepatosteatosis upon the histological criteria that is the presence of macrovesicular steatosis without any necro-inflammatory changes and to have NASH upon the presence of macrovesicular steatosis with lobular inflammation with or without hepatocytes necrosis. Other causes of liver disease had been excluded by history, family interview, laboratory data, liver histology, and hepatobiliary ultrasound in all patients. Thereafter patients with a history of alcohol consumption (>20 g, daily), hepatocellular carcinoma and drug usage that would cause steatosis (corticoste-

roids, high dose estrogens, methotrexate, tetracycline, calcium channel blockers, or amiodarone) were not included in the study. Ultimately, thirty-eight patients with NAFLD could be collected and 8 patients were classified as hepatosteatosis and 30 as NASH. The study protocol conforms to the ethical guidelines of the declaration of Helsinki and approved by the ethics committee of the Faculty. A written consent was obtained from all the subjects included in the study.

METHODS

During the 7 days before the collection of blood samples, all subjects had a particular protocol in order to rationalize NOx measurement. For this, patients received a restricted diet with lowered nitrate, sodium (7 g/day) and protein (60-70 g/day) content. In addition some of the drugs that would influence the measurements were restricted; these were diuretics, vasoactive agents, antibiotics and lactulose. Finally patients were hindered to smoke cigarette during 7 days proceeding the experiment day. After a fasting state, all blood samples were collected at 9 AM in the supine position after an appropriate bed rest. Blood samples for routine biochemical study were collected in tubes without heparin and made according to standard hospital procedures.

NOX MEASUREMENT

Blood samples in silicone-coated glass tubes with no additive were used for NOx assessment, achieved by Griess reagent. Deproteinization of serum samples was accomplished by ultra filtration membrane. Nitrite concentration was assayed directly in supernatants. However, previous reduction of nitrate to nitrite by nicotinamide adenine dinucleotide phosphate in the presence of the enzyme nitrate reductase was obligatory to assess the concentration of nitrate. The nitrite formed reacts with N-(1-naphthyl)-ethylene-diamide dihydrochloride and sulfanilamide to give a red-violet diazo dye. The diazo dye is measured on the basis of its absorbance in the visible range at 540 nm. The result is calibrated from the calibration curves constructed using standard solutions of sodium nitrite and sodium nitrate. Data were obtained upon addition of the nitrite and nitrate concentrations and expressed in micromoles per liter serum.

MDA MEASUREMENT

MDA was measured according to procedure of Ohkawa et al.[10] The reaction mixture contained 0.1 ml of sample, 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% aqueous solution of TBA. The mixture pH was adjusted to 3.5 and volume was finally made up to 4.0 mL with distilled water and 5.0 mL of the mixture of n-butanol and pyridine (15:1, *v/v*) were added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm. The results of MDA were expressed as nmol/mL.

CAT ACTIVITY

CAT activities were determined by measuring the decrease in hydrogen peroxide concentration at 230 nm by the method of Beutler.[11] Assay medium consisted of 1 M Tris HCl-5 mM Na_2EDTA buffer solution (pH 8.0), 1.0 M phosphate buffer solution (pH 7.0), and 10 mM H_2O_2. CAT activity was expressed as U/mg protein.

SOD ACTIVITY

SOD activity was measured according to the method described by Fridovich.[12] This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye which was measured at 505 nm. Assay medium consisted of the 0.01 M phosphate buffer, CAPS (3-cyclohexilamino-1-propanesulfonicacid) buffer solution (50 mM CAPS, 0.94 mM EDTA, saturated NaOH) with pH 10.2, solution of substrate (0.05 mM xanthine, 0.025 mM INT) and 80 U/L xanthine oxidase. SOD activity was expressed as U/mg protein.

GSH LEVELS

GSH levels were determined by measuring a highly colored yellow anion formed by the reduction of

DTNB [5, 5'-Dithiobis (2-nitrobenzoic acid)] with nonprotein sulfhydryl compounds of tissue samples by the method of Beutler.[13] The optical density of yellow anion was measured at 412 nm within the first 10 minutes of color development. Entire procedure was carried out at room temperature. The levels of GSH were calculated as μmol/mg protein.

PROTEIN MEASUREMENT

Total protein contents were determined by the method of Lowry, using bovine serum albumin (Merck-Darmstadt, Germany) as standard.[14]

NITROTYROSINE ELISA

Nitrated plasma proteins were assayed by sandwich enzyme-linked immunosorbent assay (ELISA) described in detail elsewhere.[15,16] In brief, 96-well plates were coated with 1 mg/mL monoclonal antibody to nitrotyrosine in PBS (100 mL/well) overnight at 4°C. After blocking with 1% bovine serum albumin (BSA) (150 mL/well) and washing, nitrated proteins were added as antigen (100 mL/well) for 1 h. The plates were then incubated with biotinylated HM11 (Hbt) diluted 1:1000 in PBS containing 0.1% BSA (100 mL/well) for 1 h. Thereafter, 0.1 mL of streptavidin ± horseradish peroxidase conjugate was added to each well, and the plate was incubated for 3 h at 37°C. The plates were washed three times with washing buffer to completely remove any reagents not bound to the solid phase. Finally, 0.1 mL of O-pheylenediamine substrate solution was added to each well to develop a yellowish color. The enzymatic reaction was stopped by addition of 0.1 mL/well of 2 Normal H_2SO_4. The amount of nitrotyrosine was measured as absorbance at 490 nm using an ELISA plate reader. The use of HM11 as an antibody resulted in an ELISA with a detection limit of ~0.2 nmol/l nitrotyrosine.

STATISTICAL ANALYSIS

Data are presented as mean ± S.D. and P values less than 0.05 were accepted statistically significant. Spearman Rank Correlation test, Mann Whitney U test and Kruskal-Wallis test were performed where appropriate.

RESULTS

The clinical and biochemical characteristics of the study groups are presented in Table 1. A detailed statistical analysis was performed and only glycemia and protein levels were found to be higher in patients than control. Similarly, NOx levels were higher when a comparison was made between control group and NAFLD group (Table 2). However,

TABLE 1: Clinical and biochemical characteristics of subjects.

	Control (n=9)	HS (n=8)	NASH (n=30)
Gender (M/F)	6/3	4/4	15/15
Age (years)	40±9	43±8	52±10
Cholesterol (mg/dL)	170±24	213±55	222±52
Triglyceride (mg/dL)	162±32	164±105	247±92
Glycemia (mg/dL)	86±2	121±59a	166±70[a,b]
AST (U/l)	27±8	30±13	60±18
ALT (U/l)	32±6	43±29	51±20
ALP (U/l)	153±23	164±81	196±68
GGT (U/l)	24±11	28±12	86±43
Protein (g/dL)	6.6±0.4	7.3±1.3	21±11[a]
Albumin (g/dL)	4.3±0.4	4.7±0.3	5.3±0.7
Bilirubin (mg/dL)	1.03±0.27	0.7±0.3	1.9±0.9
Type II diabetes	-	3 (37.5%)	9 (30%)
Dyslipidemia	-	5 (62.5%)	19 (63%)

HS: Hepatosteatosis, NASH: Non-alcoholic steatohepatitis, ALT: Alanin aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, GGT: γ-glutamyltranspeptidase. Results are presented as mean ± SD or number and (percentage). [a]p <0.05 versus control group, [b]p <0.05 versus HS.

TABLE 2: Comparison of NOx, MDA, SOD, CAT, GSH and NTY levels in study groups.

	Control (n=9)	HS (n=8)	NASH (n=30)
NOx (≒mol/l)	0.17±0.07	0.48±0.12*	0.44±0.14*
MDA (nmol/ml)	2.00±0.16	2.90±0.70*	2.72±0.85*
SOD (U/mg)	1.78±0.47	2.99±0.39*	3.13±0.48*
CAT (U/mg)	1.78±0.34	2.54±0.54*	2.52±0.80*
GSH (≒mol/mg)	1.27±0.16	2.61±0.43*	2.50±0.47*
NTY (nmol/l)	11.40±1.28	10.66±0.80	11.38±1.54

HS: hepatosteatosis, NASH: non-alcoholic steatohepatitis, NOx: nitrite/nitrate, MDA: malondialdehyde, SOD: superoxide dismutase, CAT: catalase, GSH: glutathione, NTY; nitrotyrosine. Results are presented as mean ± SD. * p <0.05 versus control group.

TABLE 3: Correlation analysis of NOx, MDA, SOD, CAT, GSH and NTY levels in study groups.

	MDA	SOD	CAT	GSH	NTY
NOx	r= 0.755*	r=0.715*	r= 0.743*	r= 0.788*	r= -0.107
	p=0.0001	p=0.0001	p=0.0001	p=0.0001	p=0.474
MDA		r= 0.460*	r= 0.491*	r= 0.563*	r= 0.030
		p=0.001	p=0.0001	p=0.0001	p=0.841
SOD			r= 0.491*	r= 0.667*	r= 0.126
			p=0.0001	p=0.0001	p=0.398
CAT				r= 0.542*	r= -0.248
				p=0.0001	p=0.092
GSH					r= -0.207
					p=0.163

NOx: Nitrite/nitrae, MDA: Malondialdehyde, SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione, NTY; Nitrotyrosine,
* statistically significant correlation.

the levels in hepatosteatosis group were not different from those in NASH group (Table 2). Although no differences were seen between MDA, SOD, CAT and GSH levels in hepatosteatosis and NASH groups, these values were higher than those in control group (Table 2). On the other hand, there was no difference in nitrotyrosine levels between the groups investigated (Table 2). In addition, all these parameters were compared between the patients with or without diabetes mellitus; there was no difference between them (data not shown). A similar approach was done for dyslipidemia and no significant difference was observed between the patients with dyslipidemia and without dyslipidemia (data not shown). In further analysis, NOx levels showed significant and positive correlations with MDA, SOD, CAT and GSH levels (Table 3). In contrast, no correlations were found between nitrotyrosine and NOx and between nitrotyrosine and MDA levels (Table 3). A detailed correlation analysis among the variables is summarized in Table 3.

DISCUSSION

In the present study, MDA levels were higher in patients when a comparison was made versus the control group. Similarly, serum levels of oxidized LDL and thiobarbituric acid-reacting substances were found to be elevated in NASH group with respect to control or steatotic group.[17,18] Although systemic levels of these parameters may not entirely reflect the oxidative status of the liver, these results support the hypothesis that oxidative stress plays a prominent role in the etiology of NASH. Accordingly, studies utilizing liver samples demonstrate similar results.[5,19] Furthermore, both liver and plasma antioxidant capacities were reduced in patients with NASH in a study, supporting the notion that systemic imbalance in oxidative status may reflect the derangement seen in the balance between oxidant and antioxidant systems in the liver.[20] On the other hand, in another similar study results indicated that peripheral values may not reflect the oxidative status within the liver.[21] In fact, investigations conducted so far have documented conflicting results regarding oxidative status of human body. In an interesting study, the composition of diet was assumed to be the possible reason of the inconsistent results obtained in distinct studies since impaired glutathione metabolism had been found to be related to the nutrient intake.[22] In view of this, the results obtained in the present study may be more reliable since an important potential bias, namely the food intake in the period preceding the blood sampling is lacking in our study.

To elucidate the potential mechanisms responsible for oxidative stress in NASH is not simple since the potential confounding factors like diabetes and obesity may involve in the etiology of NASH. These factors may readily influence the levels of oxidative stress markers, thereby complicating the elucidation of the origin of oxidative stress. In other terms, does NASH make the difference between the groups or is it obesity/diabetes responsible for this? Considering our results diabetes was present in approximately 30% of our patients, which may significantly affect MDA levels in the bloodstream. However, when our patients were divided into 2 groups as those with and without diabetes and results were re-evaluated, there were no differences between 2 groups, discarding the possibility that diabetes influences oxidative stress parameters. On the other hand, we have, unfortunately, no records on how many of our patients were obese, another confounding factor with the potential to interfere with oxidative status. Nevertheless, 5 out of 9 and 19 out of 30 patients with hepatosteatosis

and NASH, respectively, presented dyslipidemia in our study. The data from patients with and without dyslipidemia were further investigated and we could not find any difference between 2 groups. Overall, our results suggest that NAFLD, rather than diabetes and dyslipidemia, may be responsible for the increased MDA levels. In harmony with our conclusion, in a study discarding these potential confounding factors both liver and peripheral lymphocyte CYP2E1 activities, an important candidate for oxidative stress, were determined to be increased in nondiabetic patients with NASH.[23]

Another explanation for the increased generation of ROS/RNS would be the deficiency occurred in the antioxidant defense mechanisms such as SOD, CAT, and GSH. Interestingly, there were no attenuations in the levels of these molecules in the present study. Rather, it was determined that SOD, CAT and GSH levels were elevated. These results were partly in parallel with the study published by Koruk et al.[24] In contrast, these parameters and systemic antioxidant capacity were reduced in a study conducted on NAFLD patients in a previous study.[20] This increase observed in our study may be explained by a compensatory response to increased oxidants.

A potential modulator that would either decrease or increase oxidative stress, is NO. In this context, NO levels were higher in patients with steatosis and with NASH than those in healthy controls in the present study. Accordingly, Koruk et al showed that NO levels were elevated in patients with NASH in comparison with healthy controls.[24] However, another group showed controversial results wherein NO levels were decreased in NASH patients with respect to healthy controls.[24] These controversial results might result from methodological differences concerning the excluding criteria of the subjects. Relevantly, we and Koruk et al did not exclude the patients with diabetes mellitus or dyslipidemia in contrast to Baskol study.[24,25] These concomitant factors could be responsible for the elevation of NO seen in our NASH patients; however, there were no differences between patients with or without diabetes mellitus or dyslipidemia when a further analysis were performed, discarding the former possibility. Indeed, in another study, including subjects with diabetes mellitus, obesity and hyperlipoproteinemia, NO levels did not show any difference between NASH and steatosis groups.[26] Nevertheless, diabetes mellitus rate was the only variable that differed between groups, being higher in NASH group than the others.[26] The absence of a healthy control group of this study complicates the interpretation of NO results with respect to those obtained abovementioned studies. Overall, we must stress that only our results referring NO seems to be reliable since all other studies, utilizing griess reaction that allows the measurement of nitrite/nitrate (end-products of NO metabolism), lack a restricted protocol which allows to avoid measurement errors. All these factors (see methods section) have the potential to impair nitrite/nitrate measurements.

Supporting the interaction of NO and ROS, oxidative stress end-products are known to induce NF-kB-mediated NO synthesis.[8] Indeed, iNOS expression, in connection with nitrotyrosine, in the liver samples of patients with severe NASH revealed an increase in comparison with those with mild NASH, suggesting that NO-mediated oxidative injury may be involved in the etiology of NASH.[27] In order to identify whether NO augments oxidative stress, we analyzed the relationship between NO and MDA and found a significant positive correlation between these molecules, supporting a pro-oxidant behavior of NO in NAFLD. In fact, NO has been found to display both pro-oxidant and anti-oxidant effects and the underlying oxidative status of the tissue has been suggested to be the key determinant of the direction of this action.[28] Relevantly, NO, as a free radical, is capable of reacting with other ROS, thereby leading the formation of more or less toxic radicals in the microenvironment. One of the main molecules interfering with NO is superoxide anion, which, in turn, favors the generation of peroxynitrite anion.[28] Indeed, peroxynitrite anion seems to mediate cytotoxic effects of NO in liver pathologies.[29] Because of this, we aimed to evaluate if this reaction occurs in NAFLD through the assessment of nitrotyrosine levels, which is accepted to be the specific marker of peroxynitrite anion.[30] However, there was no difference in nitrotyrosine

levels between control and NAFLD groups, partially discarding the possible reaction between NO and superoxide anion in favor of peroxynitrite generation. In contrast, 3-nitrotyrosine immunostainings were shown to be increased in NAFLD patients in comparison with control subjects in previous studies.[27,31] Alternatively, it is possible that other radicals other than $ONOO^-$, including nitrite, dinitrogen trioxide, nitrogen dioxide and nitryl cation, may take place in the pathogenesis of NAFLD in relation with NO.[28,32]

In conclusion, our results demonstrate that ROS and NO seem to have a prominent and parallel role in the pathogenesis of NAFLD. Although our results do not favor a role for $ONOO^-$ formation, it is impossible with our data to exclude the possibility that this molecule may be formed in the liver.

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