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Advanced oxidation protein products, total thiol levels and total oxidant/antioxidant status in patients with nash

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Original Article

ABSTRACT

Background/Aims: In this study we aim to evaluate the relationship of advanced oxidation protein products (AOPP), total thiol, total antioxidant status (TAS), total oxidant status (TOS) levels and oxidative stress index (OSI) in patients with nonalcoholic steatohepatitis (NASH).

Materials and Methods: A total of 28 patients with NASH and 19 age-and-gender-matched healthy subjects were enrolled in the study as control group. Plasma AOPP and thiol levels were determined by spectrophotometric methods. Plasma TAS and TOS levels were measured using commercially available kits and OSI was calculated.

Results: Plasma AOPP (256.7 vs. 125.8 µmol/L) and TOS levels (8.9 vs. 5.9 µmol H2O2 equiv/L) were higher in patients with NASH than the controls ($p<0.019$ and $p<0.041$ respectively). Plasma total thiol levels (235.0 vs. 291.6 µmol/L) were lower in patients with NASH than the controls ($p<0.001$). TAS levels (1.14 vs. 1.14 mmol Trolox equiv/L) were not significantly different in patients with NASH than the controls ($p>0.900$). OSI values (8.0 vs. 5.5) were higher in patients with NASH than the controls ($p<0.039$).

Conclusion: Our findings indicate that oxidative stress increases in patients with NASH as shown by increases in TOS level. We think effective antioxidant therapy to inhibit protein oxidation and also to increase of TAS and total thiol levels may be a therapeutic option in patients with NASH who have under increased oxidative stress.

Keywords: NASH, AOPP, total thiol, TAS, TOS, OSI

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the important liver diseases in Western societies. NAFLD is a spectrum of disorders beginning with simple fatty liver and progressing nonalcoholic steatohepatitis (NASH) and cirrhosis (1). In the last decade, an increasing amount of studies have linked NASH and oxidative stress parameters. Moreover recent studies have suggested that oxidative stress may also contribute to clinical progression from simple fatty liver to NASH (2).

Oxidative stress is characterized by imbalance between oxidant-producing systems and antioxidant defense mechanisms, resulting in excessive formation of reactive oxygen species (ROS). Excessive accumulation of ROS can damage biomolecules, including lipids, proteins and nucleic acids (3).

In 1996, a new oxidative stress biomarker, referred to as advanced oxidation protein products (AOPP), was detected in the plasma of chronic uremic patients (4). Advanced oxidation protein products (AOPP) are one of the biochemical parameters indicative of oxidation stress. AOPP are proteins, predominantly albumin and its aggregates damaged by oxidative stress. They contain quantities of dityrosines which allow crosslinking, disulfide bridges and carbonyl groups, and are formed mainly by chlorinated oxidants-hypochloric acid and chloramines (5). Protein oxidation is currently considered to be an important factor in a variety of diseases such as Alzheimer's disease, cancer, diabetes and cirrhosis (6).

Thiol groups are important members of the antioxidant team and have been shown to destroy ROS and other free radicals by enzymatic and non-enzymatic mecha-

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nisms (7). Total thiol groups of proteins are mainly responsible for their antioxidant response, and they can serve as a sensitive indicator of oxidative stress (8).

The measurement of different antioxidant molecules separately is not impractical. Because the effects of antioxidants can be additive and measuring individual antioxidants separately is time consuming and labor intensive, a measurement of the combined activities of all antioxidants or the total antioxidant status (TAS) is often used to estimate the overall antioxidant status (9). Total oxidant status (TOS) is measured to determine a patient's overall oxidation state (10). Furthermore, the oxidative stress index (OSI), which is calculated as the ratio of TOS to TAS, may be a more accurate index of oxidative stress in the body because it is a comprehensive measurement of TAS and TOS.

To the best of our knowledge, AOPP, total thiol, TAS and TOS levels have not previously been studied together in patients with NASH. Therefore in this study we aim to evaluate the relationship of AOPP, total thiol, TAS, TOS levels and OSI index in patients with NASH.

MATERIALS AND METHODS

Subjects

A total of 28 patients with NASH (mean age: 44.8±12.7 years; 14 female and 14 male) and 19 age-and-gender-matched healthy subjects (mean age: 41.1±11.3 years; 7 female, 12 male) were enrolled in the study as control group. NASH patients were admitted or referred to our department with liver enzyme elevations, which were found by coincidence. NASH was definitively diagnosed in all patients by histopathological examination following liver biopsy (11). 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. Presence of concomitant conditions such as diabetes mellitus, renal insufficiency, advanced atherosclerosis, and malignancy have been investigated and it was confirmed that no such concomitance was present in patients and controls in this study.

Serum alanine-aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), total bilirubin (Total-Bil), total Cholesterol (Total-C), triglycerides (TG), HDL-Cholesterol (HDL-C), and LDL-Cholesterol (LDL-C) levels were measured in both groups. None of the patients received any form of supplemental therapy, such as folic acid, antioxidants or oligoelements. All of the laboratory measurements were performed at the biochemistry laboratory of the Medical Faculty of Erciyes University. The study protocol was approved by Erciyes university ethical committee.

Samples

All blood samples were collected in the morning after an overnight fast, and serum samples stored at - 40 ^{0}C until assays for AOPP, total thiol, TAS and TOS.

Assay of AOPP levels

Spectrophotometric determination of AOPP levels was performed by Witko's method (12). Samples were prepared in the following way: Two hundred microliters of serum were diluted 1/5 in PBS; 10 µl of 1.16 M potassium iodide were added to each tube, followed by 20 µl of acetic acid. The absorbance of the reaction mixture was read immediately at 340 nm, against a blank, containing 1000 mL of PBS, 10 mL of potassium iodide and 20 mL of acetic acid. Chloramine T solution (0-100 µmol/L) was used as calibrator (4). The chloramine T absorbance at 340 nm is linear within a range of 0-100 µmol/L, and AOPP concentrations were expressed asµmol/L of chloramine T equivalents.

Assay of total thiol levels

A spectrophotometric assay based on 2,2-dithiobisnitrobenzoic acide (DTNB or Elman's reagent) is used for total thiol assay (13). An aliquot of serum is mixed with Tris-EDTA buffer, than DTNB is added. After 15-minute incubation at room temperature, the absorbance is measured at 405 nm. A reagent blank without sample and a sample blank with methanol instead of DTNB were prepared in a similar manner. GSH (50-100 µmol/L) solution is used as calibrator. Total thiol levels were expressed as µmol/L.

Assay of total antioxidant capacity

Total antioxidant status levels were measured using commercially available kits (Rel Assay). The method is based on the bleaching of characteristic color of a more stable ABTS (2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid]) radical cation by antioxidants (14). The results were expressed as mmol Trolox (Rel Assay) equivalent/L.

Assay of total oxidant capacity

Total oxidant status levels were measured using commercially available kits (Rel Assay). In the new method, oxidants present in the sample oxidized the ferrous ion-o-dianisidine complex to ferric ion (10). The oxidation reaction was enhanced by glycerol molecules abundantly present in the reaction medium. The ferric ion produced a colored complex with xylenol orange in an acidic medium. The color intensity, which could be measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (µmol H_2O_2 equivalent/L)

Determination of oxidative stress index (OSI)

The ratio of TOS to TAC was accepted as the oxidative stress index (OSI). The OSI value was calculated according to the following formula: OSI (arbitrary unit) = TOS (µmol H_2O_2 Eq/L)/TAS mmol Trolox Eq/L) (15).

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Statistical analysis

SPSS 15.0 (Statistical Packages for Social Sciences; SPSS Inc., Chicago, Illinois, USA) program was used for statistical analysis of data. Kolmogorov-Smirnov test was used to reveal the distribution pattern of data. Parametric (Student t test) and non-parametric (Mann-Whitney U) tests were used if the data meet the requirements of the tests. Data were expressed as mean±SD. A p value of less than or equal to 0.05 was accepted as statistically significant. In addition, we used Pearson's correlation analysis to determine whether significant correlations existed between chosen variables.

RESULTS

Demographic characteristics of the groups are shown in Table 1. There was no statistically significant difference of age (44.8 vs. 41.1 year) and sex distribution between the control and NASH groups (p>0.05). Serum ALT (85.6 vs 17.6 U/L), AST (54.4 vs. 17.4 U/L), GGT (96.9 vs. 17.1 U/L), Total-C (199.0 vs. 167.3 mg/dL), TG (145.3 vs. 98.2 mg/dL), and LDL-C (123.8 vs. 101.2 mg/dL) levels were higher in patients with NASH than the control group. HDL-C (44.2 vs. 43.7 mg/dL) and Total-Bil levels (0.7 vs. 0.6 mg/dL) were not different between groups (Table 1).

Plasma AOPP levels (256.7 vs. 125.8 µmol/L) were higher in patients with NASH than the controls (p<0.019). Plasma total thiol levels (235.0 v.s 291.6 µmol/L) were lower in patients with NASH than the controls (p<0.001). Plasma TOS levels (8.9 vs. 5.9 µmol H_2O_2 equiv/L) were higher in patients with NASH than the controls (p<0.041). TAS levels (1.14 vs. 1.14 mmol Trolox equiv/L) were not significantly different in patients with NASH than the controls (p>0.900). OSI values (8.0 vs. 5.5) were higher in patients with NASH than the controls (p<0.039) (Table 2). The coefficients of variation of the methods were 3.32 for the AOPP levels and 2.98 for the thiol levels.

Table 1. Demographic and clinical characteristics of the groups

Parameters	Patients with NASH	Healthy group	p
n	28	19	
Age (year)	44.8±12.7	41.1±11.3	0.302
Weight (kg)	90.9±22.2	72.3±13.3	0.002*
Height (m)	1.6±0.18	1.7±0.1	0.09
BMI (kg/m2)	32.2±6.16	24.4±3.2	0.001*
TG (mg/dL)	145.3±67.8	98.2±34.1	0.003*
LDL-C (mg/dL)	123.8±35.0	101.2±17.8	0.006
HDL-C (mg/dL)	44.2±11.1	43.7±10.3	0.884
Total-C (mg/dL)	199.0±39.5	167.3±24.7	0.002*
AST (U/L)	54.4±27.8	17.4±3.9	0.001*
ALT (U/L)	85.6±63.4	17.6±8.9	0.001*
GGT (U/L)	96.9±132.2	17.1±6.4	0.001*
Total-Bil (mg/dL)	0.7±0.3	0.6±0.3	0.180

BMI:body mass index; TG: trigliserid; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; Total-C: total cholesterol; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gama glutamyl transferase; Total-Bil: total bilirubine *Statistically significant after Bonferroni's correction

Advanced oxidation protein products levels were negatively correlated with total thiol levels (r=-0.342, p<0.019) (Table 3). OSI values were negatively correlated with TAS levels (r=-0.393, p<0.007) and positively correlated with TOS levels (r=0.942, p<0.001) (Figure 1-3). We could not find any statistically significant correlations between oxidative stress parameters and parameters of liver histology.

DISCUSSION

The pathogenesis of NASH is still poorly understood. Liver biopsy is still gold standard diagnostic method for NASH. However liver biopsy is an invasive method. Therefore non invasive methods are necessary for diagnosis of NASH. New non invasive diagnostic modalities such as biochemical markers are emerging nowadays (16-18).

An increasing number of published studies have pointed towards increased importance of the role of oxidative stress in NASH (19). ROS and lipid peroxidation cause direct damage to hepatocytes by affecting membranes, proteins and DNA. Consequences of oxidative stress include lipid peroxidation in

Table 2. Plasma AOPP, Total thiol, TAS, TOS levels and OSI index in patients with NASH and control groups.

Parameters	Patients with NASH	Healthy group	p	p‡
n	28	19		
AOPP (µmol/L)	256.7±205.7	125.8±40.6	0.018	0.021
Total Thiol (µmol/L)	235.0±47.8	291.6±39.9	0.001*	0.001*
TAS (mmol Trolox equiv/L)	1.14±0.23	1.14±0.25	0.900	0.342
TOS (µmol H_2O_2 equiv/L)	8.9±5.0	5.9±1.5	0.001*	0.002*
OSI Index	8.0±5.2	5.5±1.7	0.039	0.002*

NASH: non alcoholic steatohepatitis; AOPP: advanced oxidation protein products; TAS: total antioxidant status; TOS: total oxidant status; OSI: oxidative stress index

‡Adjusted by BMI, HOMA, TG, LDL, Col, GGT, ALT, AST. *Statistically significant after Bonferroni's correction. Power of the performed tests were 0.604, 0.398, 0.706, 0990, 0.800 for TOS, OSI, AOPP, total thiol and TAS after α=0.05 respectively.

Table 3. Correlation analysis of parameters in NASH.

Parameters	r	p
TOS-OSI	0.942	0.001*
AOPP-Total Thiol	-0.342	0.019
OSI-TAS	-0.393	0.007*

*Statistically significant after Bonferroni's correction.
AOPP: advanced oxidation protein products; TAS: total antioxidant status; TOS: total oxidant status, OSI: oxidative stress index

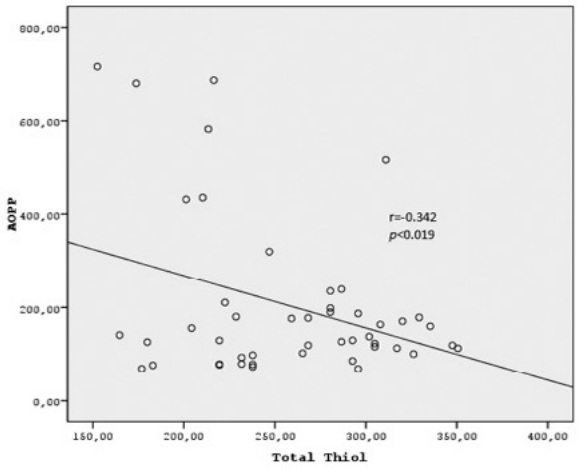

Figure 1. Correlation between AOPP and total thiol levels.

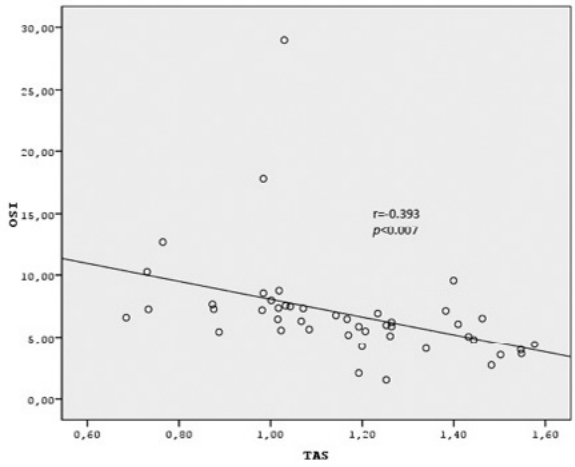

Figure 2. Correlation between OSI and TAS levels.

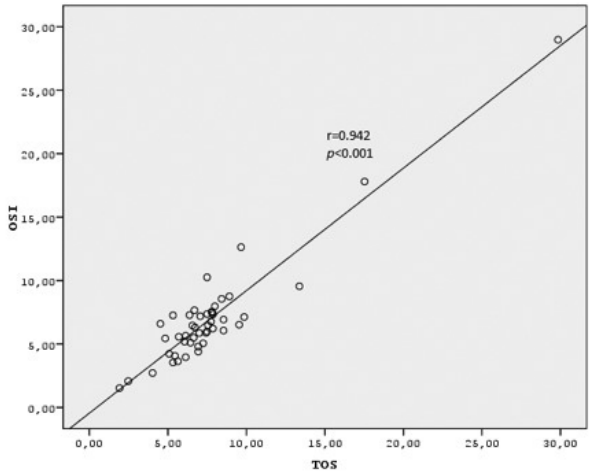

Figure 3. Correlation between OSI and TOS levels.

cell membranes, satellite cell activation in the liver leading to liver fibrosis, chronic inflammation, and apoptosis (20). Besides membrane damage caused by lipid peroxidation, also protein damage is one of the consequences of oxidative stress. The imbalance between the rate of free radical production and the antioxidant defense causes cellular damage resulting in protein oxidation. Proteins are susceptible to oxidant-mediated injury, forming cross-linkage and aggregation products that are resistant to proteolysis (21).

Advanced oxidation protein products have been accepted as novel markers of oxidant-mediated protein damage (4,5). In literature there is not any study about AOPP levels in patients with NASH. We found increased AOPP levels in patients with NASH than the control groups. According to our AOPP results, evidence of increased protein oxidation was demonstrated in patients with NASH. To our best knowledge, there are no studies that measure the AOPP and thiol levels in patıents with NASH. AOPP (mainly reduced thiol groups, which can be formed in an environment rich in chlorinated oxidants) levels were found to be elevated in patients with NASH. Based on our AOPP results, evidence of increased protein oxidation was demonstrated in patients with NASH. AOPP result from the interaction between oxidants and plasma proteins. AOPP is not only a marker of protein oxidation but also act as inflammatory mediators (22). Increased plasma AOPP levels may be related to hepatic inflammation in NASH.

Total thiols are composed of both intracellular and extracellular thiols. Intracellular thiols such as glutathione and thioredoxin play an important role in maintaining the highly reduced environment inside the cell (23). Extracellular thiols are protein bound and are mainly disulfide proteins due to the oxidative environment. Total thiol status in the body, especially thiol groups present on protein are considered as major plasma antioxidants in vivo and most of them are present over albumin, and they are the major reducing groups present in our body fluids (24).

Total thiol groups are very susceptible to oxidation and considered as one of the most important plasma sacrificial antioxidants. When the cells are exposed to oxidative stress, thiol groups are the first antioxidants that are consumed (25). We found decreased plasma total thiol levels in patients with NASH. Thiol is known as a strong antioxidant and this decrease could possibly be in response to the continuous production of ROS which need thiol for detoxification. However, according to our results the thiol levels in NASH patients is apparently insufficient to compensate for oxidative stress. In our study, AOPP levels were negatively correlated with total thiol levels. According to this correlation, increased AOPP levels could decrease antioxidant total thiol levels.

Most studies measured only one or several antioxidant substances and enzymes separately. We also previously studied different antioxidant enzymes separately and we found decreased antioxidant enzymes activities in patients with NASH (26). However, the measurement of different antioxidant molecules separately was not only impractical but also held no clinical significance. Because the effects of antioxidants can be additive and measur-

ing individual antioxidants separately is time consuming and labor intensive, a measurement of the combined activities of all antioxidants as TAS is often used to estimate the overall antioxidant status (27). We studied TAS instead of individual antioxidant compounds such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase.

In this study TAS levels were not different in patients with NASH. Relative antioxidant activities of individual antioxidants and their estimated contributions to TAS were reduced glutathione and protein sulphydryl groups (52.9 %), uric acid (33.1 %), vitamin C (4.7 %), Total-Bil (2.4 %), vitamin E (1.7 %) and others (5.2 %) (10). We found decreased total thiol levels as an acts antioxidant, BUT we did not found any difference in TAS levels in patients with NASH. The reason of these results may be other components which compose of TAS we mentioned above. There are very few studies about TAS levels in patients with NASH and different results were found. Park et al. (28) reported that increased TAS levels in NASH patients. Chitapanarux et al. (29) found that increasing TAS levels after oral supplementation with whey proteins on patients with NASH. Musso et al. (30) showed that a significant reduction in TAS levels in patients with NASH. Horoz et al. (31) found that TAS levels were lower in patients with NASH than the controls. As we mention above there is discrepancy between literatures, so we think that further studies are needed on this topic.

In our study, in addition to the TAS, we measured serum TOS levels of which the main components are hydrogen peroxide and lipid hydro peroxide, and calculated OSI values (11). According to our literature search, this is the first study about TOS levels in patients with NASH. We found increased plasma TOS levels in patients with NASH than those of controls. We also found that the OSI values were significantly higher in patients with NASH. OSI, the ratio of the total plasma TOS level to TAS, is an indicator of oxidative stress, reflecting the redox balance between oxidation and antioxidation. We found that OSI values were negatively correlated with TAS levels and positively correlated with TOS levels. These correlations also indicate increased oxidative stress in NASH. Horoz et al. reported that OSI levels were increased in patients with NASH and correlated with fibrosis scores. But they calculated OSI with a different method from us which the ratio of the total peroxide to the total antioxidant status (31).

In conclusion our findings indicate that oxidative stress increases in patients with NASH as shown by increases in TOS level. In particular, AOPP levels, which estimate protein oxidation, are increased. Therefore, prevention of protein oxidation via oxidative stress could be used as powerful tool for the establishment of pathogenesis of NASH. So that, we think effective antioxidant therapy to inhibit protein oxidation and also to increase of TAS and thiol levels may be a therapeutic option in patients with NASH who have under increased oxidative stress.

Conflict of Interest: No conflict of interest was declared by the authors.

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