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□ ORIGINAL ARTICLE □

The Serum Oxidative/Anti-oxidative Stress Balance Becomes Dysregulated in Patients with Non-alcoholic Steatohepatitis Associated with Hepatocellular Carcinoma

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Abstract

Objective Oxidative stress is associated with the progression of chronic liver disease. Non-alcoholic fatty liver disease (NAFLD) is also an oxidative stress-related disease. However, the oxidative/anti-oxidative balance has not been fully characterized in NAFLD. The objective of the present study was to investigate the balance between oxidative stress and the anti-oxidative activity in NAFLD, including non-alcoholic steatohepatitis (NASH)-related hepatocellular carcinoma (HCC).

Patients We recruited 69 patients with histologically proven NAFLD without HCC (NAFLD; n=58), and with NASH-related HCC (NASH-HCC; n=11). The 58 NAFLD patients included patients with non-alcoholic fatty liver (NAFL; n=14) and NASH (n=44).

Methods The serum levels of reactive oxygen metabolites (ROM) and anti-oxidative markers (OXY) were determined and then used to calculate the oxidative index. The correlations among such factors as ROM, OXY, oxidative index, and clinical characteristics were investigated.

Results In NAFLD, ROM positively correlated with the body mass index (BMI), hemoglobin A1c (HbA1c), C-reactive protein (CRP), and the histological grade or inflammatory scores, while only high HbA1c and CRP levels were significant factors that correlated with a higher ROM according to a multivariate analysis. OXY positively correlated with the platelet counts, albumin, and creatinine levels, while negatively correlating with age. However, it improved after treatment intervention. The oxidative index positively correlated with BMI, CRP, and HbA1c. The NASH-HCC patients exhibited a lower OXY than the NASH patients, probably due to the effects of aging.

Conclusion Oxidative stress correlated with the levels of NASH activity markers, while the anti-oxidative function was preserved in younger patients as well as in patients with a well-preserved liver function. The NASH-HCC patients tended to be older and exhibited a diminished anti-oxidative function.

Key words: antioxidant, non-alcoholic steatohepatitis, oxidative stress

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease and is a representative problem associated with the increasing prevalence of metabolic syndrome (1). Most patients with NAFLD exhibit non-progressive simple fatty liver, namely non-alcoholic fatty liver (NAFL). Non-alcoholic steatohepatitis (NASH) is a more severe form of NAFLD that is broadly defined by the presence of steatosis with inflammation and progressive fibrosis that ultimately leads to cirrhosis and hepatocellular carcinoma (HCC) (2, 3). However, some patients with NAFL develop NASH through mechanisms that are still poorly understood (4, 5)

NAFL and NASH are recognized as different diseases because they are likely to have different genetic backgrounds and lipid contents, although the presence of a fatty liver is a common feature. A recent genome-wide association study (GWAS) identified patatin-like phospholipase 3 (*PNPLA3*) as a key gene in the development of NASH (6). Patients harboring the risk allele of *PNPLA3* have been reported to have progressive disease. The toxic lipids observed in NASH and the non-toxic lipids in NAFL (simple steatosis) may differ (7). Antisense treatment for diacylglycerol acyltransferase 2 (DGAT2), which catalyzes the final step in hepatocyte triglyceride biosynthesis, reduces the hepatic triglyceride content. Conversely increased levels of hepatic free fatty acids, lipid oxidant stress, lobular necroinflammation, and fibrosis have been reported in a mouse NASH model (7). This result indicates that hepatic free fatty acid is a harmful oxidative stress-inducing lipid, while triglyceride is comparatively less harmful. These genetic and lipid characteristics suggest that the pathogeneses of steatosis in simple fatty liver and NASH are different, and that disease-specific treatments are therefore required.

Oxidative stress appears to be responsible for initiating necroinflammation. Reactive oxygen species (ROS), which are generated by the free fatty acid metabolism in microsomes, peroxisomes, and mitochondria, comprise an established source of oxidative stress. As mitochondria make up the most important cellular source of ROS, mitochondrial dysfunction may thus play a central role in the progression of NASH (4).

The standard treatment for NASH is supplementation with the representative antioxidant vitamin E, according to the recommendation of the American Association for the Study of Liver Diseases (AASLD) (8). Controversy surrounds the various antioxidant therapies because ROS play vital roles in living organisms. Antioxidants have chemical activities *in vitro*; however, such activities have not yet been confirmed *in vivo* (9). Many cerebrovascular and mortality clinical studies have reported the administration of vitamin E to be associated with unfavorable outcomes (5). Therefore, the concept of controlling oxidative stress with vitamin E requires re-evaluation. We have previously reported that oxidative stress increases in hepatitis C virus-infected patients, while the anti-oxidative activity decreases in hepatitis C virus-related hepatocellular carcinoma patients (10). There are no comparable data for NASH; therefore, we performed an oxidative/anti-oxidative balance analysis including these patients.

The objective of the present study was to investigate the balance between oxidative stress and the anti-oxidative activity in patients with histologically proven NAFL and NASH, as well as in patients suspected of having NASH-related HCC (without non-cancerous histological data). The serum levels of reactive oxygen metabolites (ROM) have been determined to be a marker of circulating ROS (11, 12). The OXY-adsorbent test was also performed in order to evaluate the corresponding anti-oxidative status (OXY) (13). We investigated the possible correlations among ROM and OXY values and the clinical parameters and clinical course of NASH.

Materials and Methods

Subjects

The study comprised 3 groups, with the first group consisting of 14 patients with NAFL (NAFL group) and the second group consisting of 44 patients with NASH (NASH group), both confirmed via histological interpretation of liver biopsy specimens. The third group consisted of 11 patients with diagnoses suggestive of NASH-related HCC (NASH-HCC group). The NASH-HCC patients had no non-cancerous liver biopsy findings (except for one patient) and were diagnosed to have neither hepatitis B nor C viral markers, no anti-nuclear antibodies or anti-mitochondrial antibodies, and no history of >20 g/day alcohol intake, but did have a history of obesity [body mass index (BMI) >25; according to the obesity criteria for Japan]. One patient in the NASH group and two patients in the NASH-HCC group used insulin for diabetic therapy, and no patients were treated with anti-oxidants such as Vitamin E.

The objective of the first study was to identify any correlations between oxidative stress-related markers and the clinical characteristic data in NAFLD. The objective of the second study was to characterize the oxidative stress balance in NAFL, NASH, and NASH-HCC. The serum levels of ROM and OXY were determined (see below), and an oxidative index was used to define the balance between ROM and OXY. The correlations between ROM, OXY, oxidative index, and clinical characteristics were assessed for all patients. The third study was a follow-up study of 12 NAFLD patients (1 NAFL, 11 NASH). They were followed for a median of 70 months after liver biopsy, and their serum was collected for comparison with the pre-intervention levels.

All of the patients were recruited at the Clinic of Gastroenterology and Hepatology, Okayama University Hospital, from August 2009 to December 2013. Healthy volunteers consisted of patients with no systemic diseases and no laboratory data abnormalities based on the findings of a public

medical checkup who were admitted to the Preventive Dentistry Clinic. The study was approved by the Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (Approval number 1635). After obtaining written informed consent, a detailed medical questionnaire was completed by either doctors or dentists.

Blood sample collection and preparation

Fasting blood samples were collected from all of the patients. The serum was collected at the time of admission or at the outpatient clinic, meaning that no intervention had been performed before specimen collection. Immunoreactive insulin (IRI) and homeostasis model assessment of insulin resistance (HOMA-IR) were measured, except for in patients under insulin treatment (1 for NASH and 2 for NASH-HCC). If not assayed immediately, the serum aliquots were stored at -80℃ until a subsequent analysis. The samples were used to obtain biochemical data, including the serum levels of ROM and OXY.

Measurement of the serum ROM and OXY levels

Measurement of the serum ROM levels was performed using a spectrophotometer (Diacron International, Grosseto, Italy), as reported previously (11). The total serum anti-oxidant capacity was determined via the OXY-adsorbent test using a spectrophotometer (Diacron International) (13). This test evaluates the capacity of serum to prevent the occurrence of massive oxidative activity in a hypochlorous acid (HClO) solution. The total anti-oxidant capacity was expressed in terms of the HClO (μmol) consumed by 1 mL of sample (μmol HClO/mL).

Calculation of oxidative/anti-oxidative balance

The balance between oxidative stress and anti-oxidative activity was calculated as an oxidative index. To incorporate parameters with differing measurement units, the standardized values of ROM and OXY were assessed using the formula developed by Vassale et al. (14):

$$sv\text{-}var = (v\text{-}var - m\text{-}var) / sd\text{-}var$$

In this formula, *sv-var* represents the standard value of a given parameter, *v-var* corresponds to its original value, and *m-var* and *sd-var* are the mean and standard deviation of the parameter, respectively. The oxidative index was calculated by subtracting the OXY standardized variable from the ROM standardized variable.

Measurement of the serum reduced glutathione and superoxide disumutase (SOD) levels

The serum reduced glutathione and SOD levels were measured using a plate reader (Thermo Fisher Scientific, Waltham, MA, USA) with a QuantiChrom™ Glutathione Assay Kit (Bioassay Systems, Hayward, CA, USA) and a DetectX© Superoxide Dismutase Colorimetric Activity Kit (Arbor Assays, Ann Arbor, MD, USA).

Liver biopsy interpretation

Liver histology data were available for all 14 patients with NAFL and 44 patients with NASH. The liver tissue specimens were fixed with 10% formalin and embedded in paraffin. Cross-sections (5 μm) were cut and stained with Hematoxylin and Eosin (H&E) and Azan. All of the liver specimens were assessed by two hepatologists (TY and FI) blinded to the study groups.

Three classification systems were adopted. The first was a system reported by Matteoni et al. that categorized the samples into four stages: type 1, steatosis alone; type 2, steatosis with lobular inflammation; type 3, steatosis with hepatocyte ballooning; type 4, type 3 plus either Mallory hyaline bodies or fibrosis (2). Types 1 and 2 are regarded as NAFL, while types 3 and 4 are regarded as NASH. The second system was a system reported by Brunt et al. that categorized the activity (grade 1, mild; grade 2, moderate; grade 3, severe) and staging (stage 1, zone 3 peri-cellular fibrosis; stage 2, fibrous progression to portal tract; stage 3, bridging fibrosis; stage 4, cirrhosis) (15). The third system was the NAFLD Activity Score (NAS) reported by Kleiner et al, which represents the sum of the scores for steatosis (0-3), lobular inflammation (0-3), and hepatocellular ballooning (0-2) (16). The sum of these scores is used to categorize the patients as NAFL-NAS (score 0-2), borderline-NAS (score 3-4), or NASH-NAS (score >4).

Statistical analysis

Statistical analysis was conducted using the JMP software package (Version 11.0.0, SAS Institute Inc., Cary, NC, USA). Continuous variables were expressed as a median value (interquartile range), and the Mann-Whitney *U*-test or the chi-squared test was used to compare parameters. For multiple group comparisons, the Steel-Dwass test was conducted. Spearman's rank sum correlation coefficients were used to determine the relationship among the clinical characteristic data and oxidative stress-related markers. A logistic regression analysis was used to perform a multivariate analysis by stratifying the variables that were found to be significantly correlated in a univariate analysis. The distribution in the patient groups of oxidative stress-related markers was compared using the chi-squared test. Statistical significance was set at $p<0.05$.

Three types of logistic models were investigated for NAFL vs. NASH and NASH vs. NASH-HCC, while calculating the adjusted odds ratios and 95% confidence intervals (CIs). Statistically significant factors identified in a univariate analysis, including age, platelet counts, prothrombin time international ratio (PT-INR), aspartate aminotransferase (AST), and the homeostasis model assessment of insulin resistance (HOMA-IR), were selected for the multivariate analysis to differentiate NAFL and NASH. For NASH vs. NASH-HCC, age, platelet counts, PT-INR, albumin, C-reactive protein (CRP), ALT, serum SOD, and OXY were significantly different in the univariate analysis and thus

Table 1. Baseline Patient Characteristics.

	HV (n=15)	NAFL(n=14)	NASH(n=44)	NASH-HCC(n=11)
Age	65 †	44 ‡	56 ‡	68
Sex (M/F)	4/11	7 / 7	16 / 28	4 / 7
Current smoker / not current-smoker	-	3 / 11	2 / 39	1 / 10
BMI (kg/m^2)	22.4 †‡	26.0	27.6	27.9
Platelet(10^3/μL)	22.0 ‡	25.4 †‡	21.7 ‡	14.0
Total bilirubin (mg/dL)	-	0.66	0.65	0.84
Albumin (g/dL)	-	4.5 †‡	4.3 ‡	3.5
PT-INR	-	0.90 †‡	0.97 ‡	1.04
AST(IU/L)	-	41 †	61	49
ALT(IU/L)	13 †‡	59 ‡	78 ‡	38
T-Cho (mg/dL)	-	207	185	162
LDL-Cho (mg/dL)	-	106	115	75
HDL-Cho (mg/dL)	-	57	49	55
Triglyceride (mg/dL)	-	171	140	105
Creatinine (mg/dL)		0.72	0.635	0.69
HbA1c(%)	-	5.8	5.9	5.8
FPG (mg/dL)	-	101	105	108
IRI (μU/mL)	-	6.95 †‡	14.4	14.7
HOMA-IR	-	1.68 †‡	3.64	3.96
CRP (mg/dL)		0.075 ‡	0.15 ‡	0.39
Ferritin (ng/m*)	110.9 †	170.6	243.3	116.7
Reduced glutathione (μM)		24.5	19.8	18.4
Serum SOD (U/mL)		0.136 ‡	0.136 ‡	0.156
Brunt				
Grade (1/2/3)	-	(14/0/0) †	(20/18/6)	-
Stage (1/2/3/4)	-	(14/0/0/0) †	(12/9/13/10)	-
NAS (1/2/3/4/5/6/7/8)		(5/3/4/2/0/0/0/0)	(0/2/6/13/17/3/2/1) †	
steatosis score (0/1/2/3)		(1/5/5/3)	(0/19/17/8) †	
Inflamation score (0/1/2/3)		(9/5/0/0)	(5/27/10/2) †	
balooning score (0/1/2)	-	(12/2/0)	(2/16/26) †	-

HV: healthy volunteer, NAFL: non-alcoholic fatty liver, NASH: non-alcoholic steatohepatitis,
NASH-HCC: NASH related hepatocellular carcinoma, BMI: body mass index, AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: gamma glutamyltransferase, T-Cho: total cholesterol,
HbA1c: hemoglobin A1c, FPG: fasting plasma glucose, IRI: immune-reactive insulin,
HOMA-IR: homeostasis model assessment of insulin resistance
†p<0.05 vs. NASH, ‡p<0.05 vs. NASH-HCC

were selected for the multivariate analysis. Each serum marker was divided into two groups according to the median value in NASH-HCC patients. For the follow-up data analysis, the Wilcoxon signed-rank test was adopted. Any variables yielding $p<0.05$ were considered to be statistically significant.

Results

Baseline characteristics of the groups

The clinical characteristics of the study groups are shown in Table 1. The NASH patients tended to be older than the NAFL patients and had lower platelet counts, higher AST levels, and higher HOMA-IR, indicating insulin resistance. NASH-HCC patients tended to be older than NASH patients and had lower platelet counts and lower ALT levels, indicating progressive fibrosis with diminished hepatitis activity.

Oxidative stress-related markers and clinical characteristics

The oxidative stress marker ROM positively correlated with BMI, hemoglobin A1c (HbA1c), CRP, histological activity, and inflammation and ballooning scores of NAS, and it also tended to be higher in women than in men (Table 2). OXY positively correlated with the platelet counts, albumin, and creatinine and negatively correlated with age. As in chronic liver disease, low platelet counts reflect the progression of liver fibrosis, and low serum albumin levels reflect an ameliorated liver function. The present data suggest that low OXY levels correlate with aging and the progression of liver fibrosis, which thus is associated with a diminished liver reservoir function. The oxidative index positively correlated with BMI and HbA1c and negatively correlated with creatinine, and it also tended to be higher in women than in men. Serum reduced glutathione levels correlated with ROM and the oxidative index, suggesting that glutathione was induced as an anti-oxidant. A logistic regression analysis revealed ROM to be higher in patients with increased HbA1c and CRP levels or decreased glutathione levels. In addition, ROM tended to correlate with histological inflammation. OXY had no statistical correlation with any markers. These data suggest that high ROM levels correlated with the diabetic conditions associated with active hepatitis in NAFLD. Although ROM and OXY are well known to correlate with aging, our present healthy volunteers did not show any correlations.

Table 2. Correlation between Oxidative Stress Related Markers and Clinical Factors in NAFLD.

	ROM			OXY			Oxidative index		
	Spearman's rho	p	logistic regression	Spearman's rho	p	logistic regression	Spearman's rho	p	logistic regression
Age	0.072	0.591		**-0.305***	**0.019**	0.269	0.232	0.079	
BMI (kg/m^2)	**0.377***	**0.003**	0.225	-0.192	0.147		**0.466***	**<0.001**	**0.002**
Platelet (10^3/μL)	0.009	0.944		**0.326***	**0.012**	0.208	-0.182	0.170	
Total bilirubin (mg/dL)	-0.206	0.119		-0.110	0.409		-0.135	0.312	
Albumin (g/dL)	-0.009	0.942		**0.291***	**0.026**	0.246	-0.205	0.122	
PT-INR	-0.078	0.558		-0.073	0.584		-0.030	0.820	
AST (IU/L)	0.218	0.099		-0.092	0.489		0.223	0.091	
ALT (IU/L)	0.236	0.073		0.017	0.899		0.214	0.106	
T-Cho (mg/dL)	0.030	0.819		0.000	0.999		-0.004	0.972	
LDL-Cho (mg/dL)	-0.008	0.956		-0.123	0.447		0.089	0.581	
HDL-Cho (mg/dL)	-0.089	0.515		-0.007	0.956		-0.036	0.791	
Triglyceride (mg/dL)	-0.026	0.842		0.172	0.196		-0.135	0.310	
Creatinine (mg/dL)	**-0.292***	**0.025**	0.422	**0.385***	**0.002**	0.060	**-0.497***	**<0.001**	0.099
HbA1c (%)	**0.283***	**0.031**	**0.029**	-0.181	0.172		**0.345***	**0.007**	0.125
FPG (mg/dL)	0.210	0.116		0.012	0.928		0.119	0.374	
IRI (μU/mL)	0.202	0.133		0.036	0.788		0.096	0.478	
HOMA-IR	0.204	0.129		0.029	0.829		0.099	0.465	
CRP (mg/dL)	**0.304***	**0.021**	**0.033**	-0.074	0.58		**0.289***	**0.029**	0.453
Ferritin (ng/mL)	**-0.284***	**0.035**	0.213	0.092	0.501		-0.221	0.103	
Reduced glutathione (μM)	**-0.386***	**0.002**	**0.032**	0.170	0.205		**-0.428***	**<0.001**	**0.001**
Serum SOD (U/mL)	0.005	0.675		-0.117	0.384		0.081	0.546	
Brunt									
histological grade	**0.280***	**0.033**		-0.074	0.579		0.217	0.100	
histological stage	0.091	0.492		-0.215	0.104		0.121	0.362	
NAS									
steatosis score	0.080	0.549		0.180	0.175		-0.072	0.588	
Inflamation score	**0.292***	**0.025**	0.078	-0.148	0.266		0.218	0.099	
balooning score	**0.285***	**0.029**	0.976	-0.078	0.559		0.254	0.053	
Sex (F)	-	**0.002**	0.052		0.157			**0.003**	0.525

BMI: body mass index, AST: aspartate aminotransferase, ALT: alanine aminotransferase, γ-GTP: gamma glutamyltransferase, T-Cho: total cholesterol, HbA1c : hemoglobin A1c, FPG: fasting plasma glucose, IRI: immune-reactive insulin, HOMA-IR: homeostasis model assessment of insulin resistance, SOD: superoxide dismutase, NAS: NAFLD Activity Score
The Mann-Whitney *U*-test was used to compare parameters in Sex.

Oxidative stress-related markers in NAFL, NASH, and NASH-HCC

ROM was higher in NASH than in healthy volunteers, and it tended to be higher in NASH than NAFL (Figure A), but it was higher in patients with NASH-NAS than NAFL-NAS (Figure B). OXY levels were significantly higher in NAFLD than in healthy volunteers and NASH-HCC, while the oxidative index was not significantly different among the patient groups. To define the impact of oxidative stress in NAFL, NASH, and NASH-HCC, the oxidative stress-related markers and clinical characteristics were compared using a multivariate analysis (Table 3). The results of the multivariate analysis indicated that an elevated HOMA-IR was the only characteristic factor that differentiated NASH from NAFL. NASH-HCC patients tended to be older than NASH patients.

To determine whether oxidative stress markers normalize after treatment for NASH (diet, exercise, and/or drugs), we identified 12 NAFLD patients for a follow-up analysis. The serum ROM did not significantly change, but OXY improved after 70 months of follow-up with treatment intervention (Table 4). The patients treated with pioglitazone gained weight but exhibited a reduction in ROM and an elevation of OXY.

Discussion

In the present study, the serum ROM in NAFLD patients correlated with HbA1c, CRP, and a decrease in the reduced glutathione levels, and it also tended to correlate with histological hepatic inflammation, suggesting that diabetic patients with active hepatitis exhibit high levels of oxidative stress. The anti-oxidative activity was attenuated in elderly patients, as well as in patients with lower platelet counts and lower serum albumin levels, suggesting the presence of advanced cirrhosis in elderly patients. NAFL was characterized by a lack of insulin resistance compared with NASH or NASH-HCC. NASH-HCC was characterized by ROM levels that were comparable to NASH, with a relatively reduced anti-oxidative capacity, indicating the presence of a defective antioxidant capacity in elderly patients. Follow-up experiments revealed the effectiveness of treatments for improving OXY.

ROM is considered to be a reliable indicator of circulating ROS (11, 12). It has been reported that ROS induces the progression of HCC (17), thereby inducing the synthesis and activation of a large number of cytokines and growth fac-

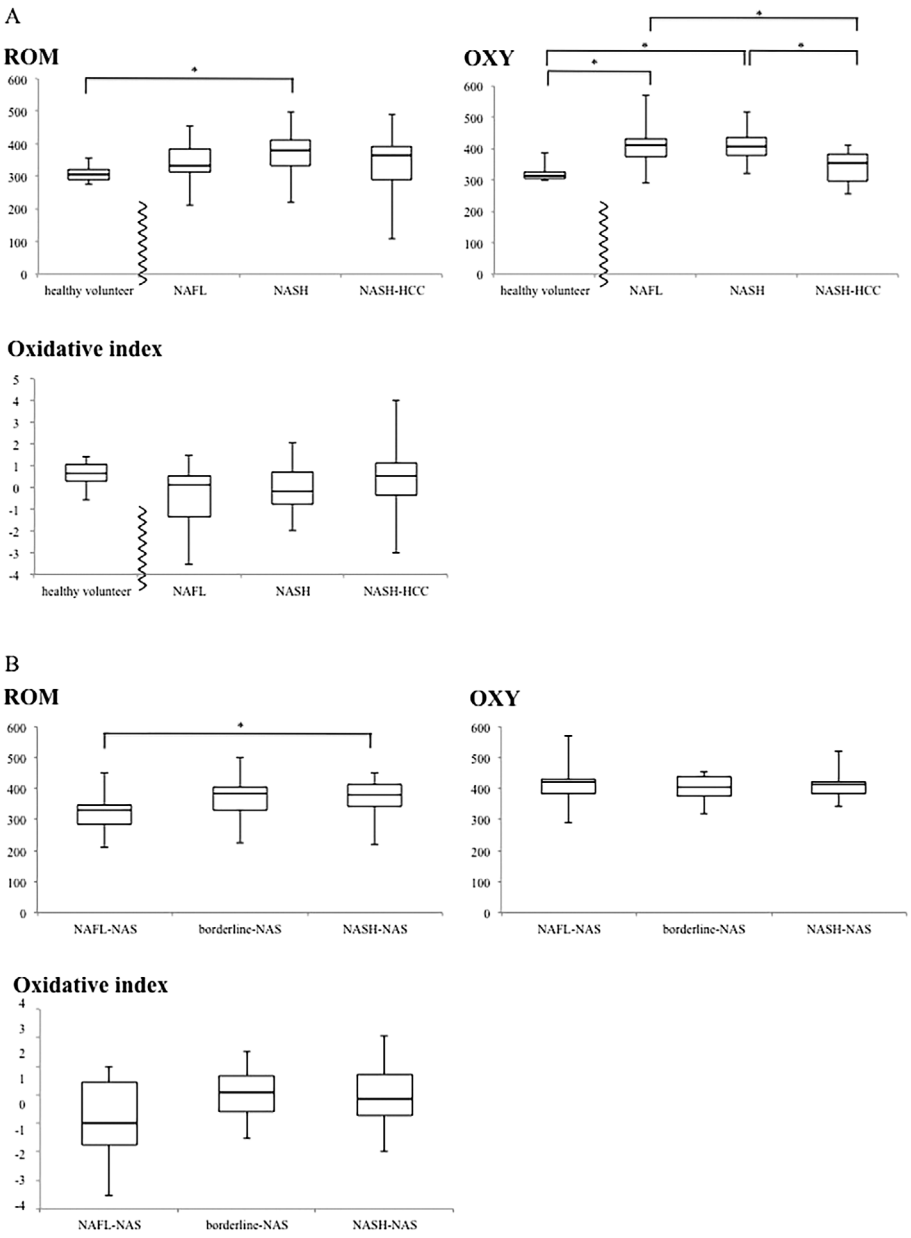

Figure. **(A) The distribution of ROM, OXY, and the oxidative index in the patient groups. These data were analyzed using the Steel Dwass test for any between-group differences. Box plots show the median, lower, and upper quartile ranges, and the minimum and maximum of all data. The ROM levels were significantly higher in NASH than in the healthy volunteers. The OXY levels were significantly higher in NAFL and NASH than in the healthy volunteers. NASH-HCC had lower OXY levels than NAFL or NASH. No significant differences in the oxidative index were observed among the groups. *p<0.05. (B) ROM, OXY, and the oxidative index in NAFLD patients were categorized according to the NAS score. The ROM levels were higher in NASH-NAS than in NAFL-NAS.**

tors, which in turn lead to malignant transformation (18). The results of the present study suggest that oxidative stress plays a strong role in active hepatitis associated with a poorly controlled diabetic condition. In obese or type 2 diabetes patients, the accumulation of oxidative damage markers and deficient antioxidant defenses in various tissues are widely accepted (19). Obesity and diabetes have additive effects on mitochondrial oxidative stress in isolated mitochondria from adipose tissue (20). ROM has also been shown to negatively correlate with the accepted anti-oxidative stress marker, namely a reduced glutathione level, which is vital in maintaining hemoglobin in a reduced state and thereby protecting cells from oxidative damage. The present results suggest that obesity and a diabetic state can thus affect hepatic mitochondrial oxidative stress, thereby resulting in elevated hepatitis activity.

The antioxidant activity has also been shown to correlate with obesity and diabetes (20). However, the present results indicate that the antioxidant capacity was significantly lower in elderly patients with advanced fibrosis than in patients

Table 3. Multivariate Analysis for Differential Diagnosis of NAFL, NASH, and NASH-HCC.

A: NAFL vs. NASH	Multivariate analysis		
	odds ratio	95%CI	p
platelet (<23)	1.84	0.35-10.19	0.460
PT-INR (≥0.96)	3.87	0.80-22.72	0.091
Albumin (<4.4)	3.20	0.65-19.49	0.153
AST (≥55)	1.13	0.22-6.45	0.883
HOMA-IR (≥3.09)*	**6.64**	**1.25-49.28**	**0.024**

B: NASH vs. NASH-HCC	Multivariate analysis		
	odds ratio	95%CI	p
sex (male)	1.26	0.10-12.76	0.839
age (≥68) *	**18.90**	**2.11-315.70**	**0.007**
platelet (<14.0)	3.32	0.31-93.28	0.345
PT-INR (≥1.04)	5.50	0.57-69.36	0.137
Alb (<3.5)	13.33	0.91-405.31	0.057
ALT (<38)	1.24	0.09-12.08	0.295
CRP (≥0.39)	2.81	0.35-23.24	0.316
OXY (<355.1)	3.90	0.28-58.48	0.295

Table 4. Follow-up Characteristics of Twelve NAFLD Patients.

	day of biopsy	follow up	p
Months	-	70	-
treatment (Pioglitazone/Metformin/Vitamin E)	(1/1/0)	(3/4/1)	-
age	61	65	-
BMI (kg/m^2)	26.9	28.5	0.05
AST (IU/L)	55	42	**0.04**
ALT (IU/L)	68	45	**0.02**
HbA1c (%(mmol/mol))	5.9(41)	6(42)	0.26
albumin (g/dL)	4.4	4.4	0.89
ROM (CARR U)	391	352	0.08
OXY (μmol HClO/mL)	410.5	455.6	**<0.01**

BMI: body mass index, AST: aspartate aminotransferase,
ALT: alanine aminotransferase, HbA1c: hemoglobin A1c,
ROM: reactive oxygen metabolities, OXY: OXY-adsorbent test

with obesity or diabetes. This observation is probably due to the fact that liver fibrosis progression strongly reduces the anti-oxidative reservoir, as the liver function decreases in patients with low platelet counts, fibrosis, and low serum albumin levels. In addition, NASH-HCC patients tended to be older and have lower serum albumin levels than other patients. The antioxidant system is accepted as an important pathway for detoxifying ROS-induced cell damage and facilitating patient recovery. Antioxidant-related transcription factors, such as AMP-activated protein kinase (AMPK) or nuclear factor erythroid-derived 2, like 2 (Nrf2), control the expression of several antioxidant genes and are potential treatment targets for counteracting oxidative stress (21, 22). AMPK is a highly conserved heterodimeric serine-threonine kinase that serves as an energy sensor in eukaryotic cells and bridges the metabolism to carcinogenesis (23). The activation of AMPK suppresses cell proliferation in non-malignant and malignant cells via the regulation of the cell cycle, apoptosis, autophagy and the inhibition of fatty acid synthesis (24). Phospho (p)-AMPK is down-regulated in HCC tissues from patients, and a low p-AMPK expression correlates with a poor prognosis, indicating the importance of AMPK signaling in HCC (25). This phenomenon might be one reason for the lower antioxidant capacity in NASH-HCC patients observed in the present study. Another antioxidant, transcriptional factor Nrf2, binds to Kelch-like ECH associating protein 1 (Keap 1), which is located in the cytoplasm in an inactive form (26). In response to oxidative stress, the critical cysteine residues in Keap 1 are oxidized, thereby resulting in conformational changes and the translocation of Nrf2 to the nucleus. Nrf2 then binds to the anti-oxidant response elements (AREs) of anti-oxidative target genes, such as glutathione reductase, thioredoxin, or superoxide dismutase (27). However, Nrf2 activation has also been shown to impair liver regeneration by activating the genes involved in cell-cycle control and apoptosis (28).

The oxidative index, the balance of the oxidative to anti-oxidative reservoir function, was not elevated in NASH-HCC, contrary to our expectations. The reason for this is not clear, but as ROM was elevated in active hepatitis and the background ALT levels of NASH-HCC were lower than NAFL or NASH, the ROM in NASH-HCC was not high, thus resulting in no increase in the oxidative index even though OXY was low in NASH-HCC.

Insulin resistance is accepted as an independent risk factor for NAFLD severity (29). Adipose and hepatic insulin resis-

tance progressively increases across the NAFLD stages even in non-obese, non-diabetic, and normolipidemic patients. The oral glucose tolerance test (OGTT) has been used to identify an impaired pancreatic β-cell function in patients with NASH, but not in those with simple steatosis (30). Visceral fat induces the production of several fat-associated cytokines and induces inflammation, resulting in insulin resistance and other organ inflammation, including NASH (31). Obese patients with insulin resistance were found to have adipose tissue with a greater number of $CD4^+$ T cells with induced IL-17 and IL-22, but not IFN-gamma or IL-13 (31). IL-17 and IL-22 lower the insulin-mediated muscle cell glucose uptake and the insulin-mediated suppression of hepatocyte glucose production. The prominence of $CD4^+$ T cell infiltration is one of the characteristics of NASH, while an increased number of macrophages in the liver and adipose tissue is an early phenotypic marker of such diseases as NAFL or simple obesity (32).

There are several determinants of the antioxidant capacity, such as the SOD activity, thioredoxin concentration, glutathione, and OXY. OXY has been assessed in various chronic viral liver diseases (10). We previously reported that an HCV-positive status correlates with a lower OXY. Furthermore, the markers for the liver reservoir function (e.g., lower albumin) or liver fibrosis (e.g., lower platelet counts) also correlate with a lower OXY. In HCV-positive patients, the HCC-positive status and reduced serum albumin levels correlated with lower OXY values. Comparable reductions in OXY were observed in HCV- and NASH-related HCC. The mitochondrial anti-oxidative enzyme manganese superoxide dismutase (MnSOD) was reported to be lower in both the human male NAFLD liver and male high-fat-diet-fed diabetic mice than in human females and normal female mice, respectively (33). A GWAS analysis revealed PNPLA3 to be a gene that is consistently associated with advanced NASH; however, many other modifier genes remain unidentified. *SOD2*, encoding MnSOD, is an additional candidate gene that has been shown to correlate with advanced NASH (34, 35). As the OXY levels represent the serum capacity for neutralizing oxidative stress, the previously mentioned local changes in the antioxidant capacity might result in reduced OXY levels in the NASH-HCC patients investigated in this study.

At present, antioxidant treatment with vitamin E is recommended by the AASLD for NASH at any stage of disease. From the results of the present study, antioxidant treatment might be suitable for active NASH with elevated ROM; however, NASH-related HCC patients might not be suitable for antioxidant therapy. In this case, the up-regulation of OXY might be needed to support the mitochondrial function. In our present study, most of the administered treatments were insulin sensitizers which were associated with an improvement of OXY. Such treatment approaches may therefore be beneficial for preventing the development of HCC.

In conclusion, oxidative stress was higher in NAFLD patients with strong hepatic inflammation and poorly controlled diabetes. The anti-oxidative activity (assessed as OXY) was lower in NASH-related HCC patients than in other patients, probably due to the elderly age of these patients. The oxidative-to-anti-oxidative ratio was elevated in obese NAFLD patients. Diabetic NAFLD patients with active hepatic inflammation might thus be good candidates for providing standard anti-oxidant treatment, while NASH-HCC patients might benefit from agents that support the mitochondrial function in elderly individuals.

The authors state that they have no Conflict of Interest (COI).

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