Oxidative Stress and Lipid Peroxidation in NAFLD with and without Type 2 Diabetes Mellitus

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ABSTRACT

Objective: To compare superoxide dismutase 1 (SOD 1) and malondialdehyde (MDA) levels along with biochemical parameters in patients of non-alcoholic fatty liver disease (NAFLD) with and without Type 2 diabetes mellitus.

Study Design: Cross-sectional comparative study.

Place and Duration of the Study: Centre for Research in Experimental and Applied Medicine, AMC, in collaboration with the Department of Radiology, Combined Military Hospital, Rawalpindi, from February to November 2022.

Methodology: Two hundred and ten patients were selected by non-probability purposive sampling and divided into 3 groups. Healthy individuals were labelled as Group I, Group II included patients of NAFLD without diabetes mellitus, and Group III had patients of NAFLD with diabetes mellitus. Fasting blood glucose levels and lipid profile were measured. ELISA (enzyme-linked immunoassay) was done for the assessment of SOD 1 and MDA levels. The data was analysed by version 22.0 of SPSS and expressed in mean ± SD and percentage. One–way ANOVA was done for all groups and grade comparison was followed by the post-hoc Tukey test.

Results: When compared to control groups, the mean SOD 1 level in diseased groups was significantly lower ($p<0.001$). There was a statistically significant difference between each group ($p<0.001$). Mean levels of MDA were significantly increased in diseased groups as compared to controls with a statistically significant difference between all groups except between Group II and III.

Conclusion: In patients having NAFLD with and without diabetes mellitus, SOD 1 levels were considerably lower compared to controls whereas MDA levels were significantly higher. This decrease in SOD 1 and raise in MDA levels was indicative of increased oxidative stress in patients and can be viewed as a biomarker for oxidative stress.

Key Words: *NAFLD, ELISA, Oxidative stress, SOD 1, MDA, Lipid peroxidation*.

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INTRODUCTION

The term "non-alcoholic fatty liver disease" (NAFLD) was first used by Ludwig in 1980 to describe a range of liver conditions caused by more than 5% of hepatocytes having steatosis without drinking alcohol.[1] NAFLD represents a continuum with severity progression from steatosis leading to non-alcoholic steatohepatitis (NASH) and finally cirrhosis.[2] With a 25% global incidence, NAFLD is the most common chronic liver disease and is now considered as a pandemic.

NAFLD is often linked to insulin-resistance, Type 2 diabetes mellitus and metabolic syndrome with obesity as one of the significant risk factors. More than 50% of people with Type 2 diabetes mellitus (DM2) have NAFLD.[3] With a significant bidirectional relationship in disease progression, NAFLD is a potential risk factor for DM2.[4] The complicated etiopathogenesis of NAFLD includes factors such as age, gender, ethnicity, nutrition, metabolic condition, genetic predisposition, and epigenetics.[5]

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Although the mechanism for pathogenesis is still not clear, oxidative stress has a crucial role in the onset and progression of non-alcoholic fatty liver disease, causing cellular malfunction, inflammation, and induction of apoptosis.[6,7] Oxidative stress (OS) refers to a situation in which the ratio of oxidant species to antioxidant systems is skewed in favour of oxidants.[8] This imbalance affects the activities of several cellular components, including organelles, proteins, lipids and membranes, hence, disrupting signalling and redox control. This affects the pathophysiology of numerous chronic diseases, including NAFLD.[9]

Antioxidant systems can be enzymatic or nonenzymatic with superoxide dismutase 1-3 (SOD), catalase and peroxiredoxin 1-6 among the examples of enzymatic systems and glutathione, Vitamin E and C, and bilirubin as a few examples of nonenzymatic compounds.[10] Antioxidants diminish oxidative stress by either hindering their synthesis or by neutralising the reactive species. Superoxide dismutase (SOD), the most potent endogenous antioxidant, serves as the first line of enzymatic defence.[11]

The effects of oxidative stress are implicated on the lipid membranes and are expressed by the levels of malondialdehyde as the marker of lipid peroxidation. Therefore, the objective of the study was to measure the levels of SOD and MDA as well as other biochemical parameters in patients with and

without DM2 to compare the oxidative stress and resulting lipid peroxidation in the two diseased groups and the control group.

METHODOLOGY

This cross-sectional comparative study was conducted at the Centre for Research in Experimental and Applied Medicine, Army Medical College (AMC), in collaboration with the Department of Radiology of Combined Military Hospital, Rawalpindi from February to November 2022. The study was carried out in accordance with the rules of the Declaration of Helsinki (version 2013), after a formal approval from the Medical College Ethical Review Committee. Sample size was calculated at 210 by using OpenEpi calculator taking into account prevalence of NAFLD at 14% in Pakistan,[12] setting confidence interval at 95% and error at 5%. Samples were collected using non-probability purposive sampling technique.

The enrolled 210 individuals were allocated into three groups of 70 each; Group 1 = control, Group 2 = diagnosed NAFLD patients without DM2, and Group 3 = diagnosed NAFLD patients with DM2. Inclusion criteria was set as diagnosed cases of NAFLD with and without diabetes mellitus. Age and gender were matched among the groups within the range of 35-65 years. Exclusion criteria ruled out the individuals suffering from NAFLD due to alcohol consumption along with those suffering from chronic illnesses other than hypertension and taking medication for a period of more than six months. Patients younger than 30 and older than 65 were not included in the study. Prior to inclusion, each subject provided a written informed consent. A structured proforma was used to gather each study participant's general information, including name, gender, age, smoking/addiction, personal history and any other medical history. Weight and height were measured to calculate body mass index (BMI).

Diabetes was classified using the diagnostic criteria by World Health Organisation set in 1999, while NAFLD was determined using the 2017 American Society of Liver Diseases diagnostic criteria. The patients were predominantly evaluated utilising real-time sonography after a 6- to 8-hour fast. Most of the time, supine postures and right anterior oblique views were utilised. On the basis of the ultrasound characteristics such as brightness of parenchyma, liver-to-kidney contrast, vascular blurring of portal or hepatic vein and gallbladder wall definition, fatty liver was determined by classified radiologists at Combined Military Hospital. Grades 0 to 3 or mild, moderate, or severe served as convenient labels for qualitative grading (with 0 being normal). Blood tests were then recommended for patients who met the criteria for the two diseased groups. Five ml of blood were taken under aseptic conditions from the individuals' anti-cubital vein after 10 hours of fasting. From this, 3ml was obtained in vacutainers with serum gel separator to do ELISA for superoxide dismutase and malondialdehyde levels and 2ml for other biochemical parameters including BSF, lipid profile, LFTs and blood complete picture. The serum was isolated from the blood after the blood had been centrifuged at 3000 rpm for 20 minutes and kept at -20°C. Using the Human SOD 1 (Soluble, superoxide dismutase 1) ELISA Kit with the catalogue number E-EL-H1113 from Elabscience, serum superoxide dismutase 1 levels were assessed. Malondialdehyde levels were determined using the MDA (malondialdehyde) ELISA Kit, E-EL-0060 (Elabscience®) catalogue number.

The data was evaluated using SPSS, version 22.0, which is a statistical package for social sciences. Quantitative data was expressed as means ± SD while categorical data was expressed in the form of frequency and percentages. Comparison of SOD 1 and MDA among the groups and grades was done by statistical formula ANOVA (one-way) which was followed by a post-hoc Tukey test. Pearson's r (Pearson coefficient) was applied to show relationship of SOD 1 and MDA with other biochemical parameters. Statistics were considered significant at a p-value of 0.05 or lower.

RESULTS

Out of the 210, 110 (52.4%) subjects were males and 100 (47.6%) were females, aged 30-65 years. The mean BMI values among Group I, II, and III were 24.25 ± 3.06, 29.80 ± 4.92, and 29.44 ± 3.79, respectively. These results displayed a significant difference statistically between the diseased groups and controls (p<0.001). Of the 70 patients included in Group II, 46(65.7%) had Grade 1, 17(24.3%) had Grade 2 and 7(10.0%) had Grade 3 fatty liver; and in Group III, 37(52.9%) had Grade 1 fatty liver, 27(38.6%) had Grade 2 and 6(8.6%) had Grade 3 fatty liver disease. Total cholesterol (TC) levels were more in Group II (4.63 ± 0.86) as compared to Group I (4.27 ± 0.55) and Group III (4.54 ± 0.82), having p-value of 0.014*. Mean values of triacylglycerol (TAG) in Group I, II and III were 1.26 ± 0.30, 3.16 ± 3.20, and 3.14 ± 1.64, respectively (p=0.001*). When compared with Group I (1.06 ± 0.20), the mean values of high-density lipoprotein cholesterol (HDL-c) were significantly lower (p=0.001*) in Group II (0.92 ± 0.19) and Group III (0.93 ± 0.24). The values of the mean ±SD of low-density lipoprotein cholesterol (LDL-c) in Group II (2.84 ± 0.64) and III (2.64 ± 0.78) were significantly raised (p=0.001*) as compared to that of Group I (2.23 ± 0.30). When TAG, HDL-c, and LDL-c were compared between the control and diseased sets using the post-hoc Tuckey test, the difference was significant. However, TC of controls (Group I) as compared to diseased groups (Group II and III) showed no significant difference.

The mean levels of SOD 1 and MDA are presented in Figure I. This study showed serum SOD1 levels to be lowest in Group III which is NAFLD with DM2. SOD 1 levels were decreased in Group II as well as compared to the controls, and this result was statistically significant (p<0.001*).

Table I: Groups' comparison by post-hoc Tukey test, preceded by ANOVA.

Parameters	Group I *vs.*		Group II *vs.*
	Group II	Group III	Group III
SOD(pg/ml)	<0.001*	<0.001*	<0.001*
MDA (ng/ml)	<0.001*	<0.001*	0.414
TC(mmol/L)	0.015*	0.082	0.783
TAG(mmol/L)	0.001*	0.001*	0.998
HDL-c(mmol/L)	0.001*	0.001*	0.978
LDL-c(mmol/L)	0.001*	0.001*	0.156

**Significant result (≤0.05).*

Table II: Mean values (±SD) SOD 1, MDA levels, and lipid profile across different grades of NAFLD.

Parameters	Controls (none) Mean ± SD	Grade I (Controls) Mean ± SD	Grade II (NAFLD without DM2) Mean ± SD	Grade III (NAFLD with DM2) Mean ± SD	Significance (p-value)
SOD(mg/ml)	4047.19± 976.76	1983.75 ± 557.81	1451.22 ± 258.43	1080.48 ± 121.80	<0.001*
MDA (ng/mL)	363.31 ± 91.62	801.15 ± 793.34	886.78 ± 696.46	1091.56 ± 698.97	<0.001*
TC(mmol/L)	4.2729 ± 0.55	4.6393 ± 0.882	4.4509 ± 0.840	4.7615 ± 0.45	0.014*
TAG(mmol/L)	1.2654 ± 0.309	3.4705 ± 3.123	2.8225 ± 1.164	2.2323 ± 0.993	<0.001*
HDL-c(mmol/L)	1.0651 ± 0.208	0.9451 ± 0.226	0.8732 ± 0.198	0.9862 ± 0.242	<0.001*
LDL-c(mmol/L)	2.2321 ± 0.301	2.8342 ± 0.676	2.5764 ± 0.843	2.7469 ± 0.497	<0.001*

**Significant result (≤0.05).*

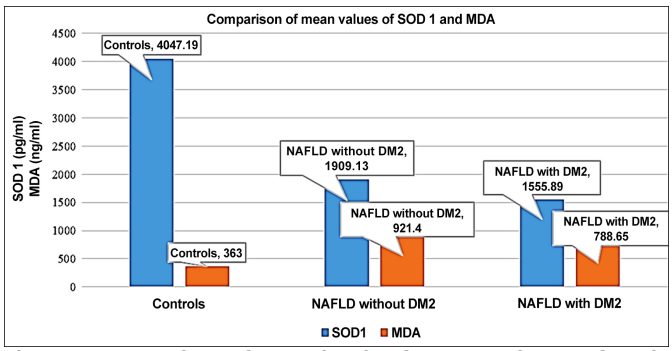

Figure 1: Comparison of mean levels of SOD 1 and MDA of study groups (p-value <0.001*).

This study showed serum MDA levels were highest in Group II which is NAFLD without DM2. MDA levels were raised in Group III as well as compared to the controls, and this result was statistically significant (p<0.001*).

Post-hoc analysis of SOD 1, MDA, and biochemical parameters among the groups revealed differences among all the study groups to be significant statistically, as indicated in Table I.

Mean values ± SD of SOD 1, MDA and biochemical parameters were also compared across the different grades of NAFLD by ANOVA followed by pots-hoc Tukey test. The mean values are expressed in Table II.

SOD 1 had negative and significant Pearson correlation with BMI (r = -0.481, p = <0.001*), blood glucose fasting (r = -0.410, p = <0.001*), LDL-c (r = -0.291, p = <0.001*) and TAG (r = -0.315, p = <0.001*), and non-significant relationship with TC. However, with HDL-c, it had positive and statistically significant relationship (r = 0.233, p = 0.001*). MDA exhibited significantly positive correlation with BMI (r = 0.292, p = <0.001*) while its Pearson correlation with other biochemical parameters were insignificant.

DISCUSSION

Since the prevalence of DM2, obesity and metabolic syndrome have increased, NAFLD has emerged as the most prevalent chronic hepatic disease worldwide.[13] By impacting a number of ROS-generating pathways, hepatic lipid excess causes an increase in the synthesis of oxidants. Elevated oxidative stress, which is critical in the development of non-alcoholic steatohepatitis from simple steatosis, seems to be one of the most significant variables in the evolution of NAFLD liver damage.[14] The measurement of antioxidant status and the biomarkers that indicate the impact of oxidative stress on the body are two ways to assess oxidative stress. Lipid peroxidation being the most pronounced effect of oxidative stress is assessed by malondialdehyde.[15] In this study, levels of superoxide dismutase have been assessed to ascertain the oxidative stress status in diseased groups. To determine ROS-mediated damage to cell membranes, malondialdehyde was analysed in the current study.

According to the study's findings, SOD 1 levels significantly dropped in both the diseased groups with significant rise in the levels of malondialdehyde, implying oxidative stress in both the diseased groups. This outcome is in line with the results of Arya *et al.*, who explained the reduction in the levels of SOD by MDA *via* the mechanism of molecular docking. In addition to this, the results of his study also showed nonalcoholic fatty liver group had significantly higher ALT, AST, and BMI values than the control group analogous to authors' results.[16]

In another study carried out on the Pakistani population to assess the total antioxidant capacity, a significant decrease in the levels of antioxidant enzymes namely SOD, CAT, GSH along with non-enzymatic antioxidants Vitamin E and C was depicted. Levels of MDA and NO (nitric oxide) were however raised significantly, thus, reinforcing the fact that increase in reactive species leading to the decline of the antioxidant titer causes a rise in MDA, a marker of lipid peroxidation.[17]

Considering how important oxidative stress is in the genesis of NAFLD, it is possible to assume that patients who have liver fibrosis and higher levels of liver enzymes also have greater levels of pro-oxidative and other harmful variables. Aberrant circulating oxidative stress indicators, such as elevated malondialdehyde and superoxide dismutase activity, were noticed in a study that was conducted to investigate the potential antioxidative capacity of HDL-c with atherosclerosis among the patients who had NAFLD. Patients who had NAFLD had lower HDL-c than the control group. In comparison with the controls, the levels of AST, ALT, and GGT were considerably higher in patients who had NAFLD, and results were significant. MDA levels were raised whereas the antioxidant enzyme SOD was observed to be decreased in patients of NAFLD when compared to control.[18] These results are in congruence with the outcomes of this study.

In a study conducted recently by Asghari *et al*., findings congruous to the results of the current study regarding the levels of MDA were observed. However, the outcome of SOD was found to be in contradiction with the authors' findings. This surge in the levels of superoxide dismutase can be attributed to the compensatory mechanism employed by the first-line antioxidant defense enzyme SOD to counteract the superfluous production of reactive oxygen species in patients of NAFLD. Fluctuations in SOD levels might be a consequence of the variations in the grades of fatty liver and the duration of the disease.[19] The same trend was represented by Świderska *et al.* who carried out a comprehensive study to evaluate the association between the body's enzymatic along with the non-enzymatic antioxidants, redox homeostasis and products of oxidative damage in individuals having NAFLD. Several markers were assessed in the serum samples through different stages of the disease to assess the difference between early and late NAFLD. In comparison with the controls, both the early and advanced NAFLD groups had significantly ($p < .001$) higher levels of GPx, SOD, GR, GSH, AGE, MDA, TOS, and RNA/DNA oxidative damage.[20]

The present study reported that biochemical parameter levels, ALT, AST, ALP, TAG, LDL-c, were significantly elevated in patients having NAFLD regardless of diabetes mellitus in comparison to controls with HDL-c decreased in the diseased groups. The two diseased groups, however, did not differ statistically significantly from one another. These results are consistent with a research conducted in Iran where these parameters were also deranged in NAFLD as compared to the controls.[21]

Sanju and his colleagues' study found a correlation between AST, ALT, and ALP in AFLD and NAFLD, with a significant rise in the levels of hepatic enzyme. These findings resonated in this study with a significant mean difference across the groups reflecting increase in liver enzymes.[22] Therefore, disease initiation and progression in NAFLD is an interplay of the oxidative stress, defence markers, and oxidative damage products.

A larger cohort could have been included to generalise the results of the study for a larger population such as city-wise. In this study, lifestyle effects were also not considered which play a part in the NAFLD status.

CONCLUSION

In the current investigation, SOD 1 levels were significantly decreased in diseased groups which are NAFLD with and without diabetes mellitus indicating an upsurge in the oxidative stress. The enzymic defense system of the body, foremost of which is superoxide dismutase, tends to fight off the excess reactive oxygen species, and hence are consumed in the process. Hence, among the factors contributing to the pathophysiology of NAFLD, disturbance in the antioxidant defence system holds significance. Malondialdehyde levels used to measure lipid peroxidation, an indication of oxidative stress, revealed substantial impact among the diseased groups. MDA count was higher in the diseased groups when paralleled to controls. Increase in MDA levels corresponding to increased grades stressed upon increase in lipid peroxidation and progression of the disease.

ETHICAL APPROVAL:
The Ethical Review Committee of Army Medical College gave its approval (ERC/ ID/ 181) on 13th January 2022. The research was initiated in February 2022.

PATIENTS' CONSENT:
Well-informed and written consent was taken from each patient before including them in this research.

COMPETING INTEREST:
The authors declared no competing interest.

AUTHORS' CONTRIBUTION:
AJ: Conception and design, data analysis, sampling, manuscript writing.
KM: Review of content.
AR: Proofreading of manuscript.
AM: Sampling and laboratory investigations.
SK: Data interpretation, result compilation.
ZAB: Wet lab procedure assistance.
All authors approved the final version of the manuscript to be published.

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