Patients with Nonalcoholic Fatty Liver Disease (NAFLD) have Higher Oxidative Stress in Comparison to Chronic Viral Hepatitis

Amit Kumar*, Arun Sharma*, Ajay Duseja*, Ashim Das**, Radha K. Dhiman*, Yogesh K. Chawla*, Krishan K. Kohli†, Anil Bhansali‡

*Departments of *Hepatology, **Histopathology, †Biochemistry, and ‡Endocrinology, Postgraduate Institute of Medical Education and Research, Sector 12, Chandigarh 160012, India*

Introduction: **Oxidative stress and cytokines play an important role in the pathogenesis of nonalcoholic fatty liver disease (NAFLD). We compared the presence of oxidative stress and cytokines in 25 patients with NAFLD with 25 age, sex and BMI-matched patients with chronic viral hepatitis (CVH) and 25 healthy volunteers (HV).** ***Methodology:*** **Oxidative stress was studied biochemically by markers of lipid peroxidation and biochemical assessment of anti-oxidant status and various cytokines were studied by ELISA.** ***Results:*** **Patients with NAFLD had significantly higher levels of malondialdehyde (MDA) ($p = 0.000$) and conjugated dienes (CD) ($p = 0.000$) in comparison to HVs. Patients with NAFLD also had significantly higher MDA levels ($p = 0.000$) in comparison to CVH patients. Patients with NAFLD had significantly lower GSH levels ($p = 0.004$) in comparison to HVs. Patients with NAFLD had higher GPx activity ($p = 0.028$) in comparison to HVs. Catalase activity was significantly decreased in both NAFLD ($p = 0.001$) and CVH patients ($p = 0.000$) in comparison to HVs. Patients with NAFLD had significantly higher SOD activity ($p = 0.000$) in comparison to CVH patients. There was no difference in serum levels of IL-1β and TNF-α amongst three groups. Patients with CVH were found to have higher IL-8 serum levels ($p = 0.039$) in comparison to HVs. CVH patients also had higher TGF-β levels ($p = 0.002$) in comparison to both NAFLD patients and HVs.** ***Conclusion:*** **Differences in the markers of oxidative stress and anti-oxidant status between NAFLD, CVH and healthy volunteers suggest presence of higher oxidative stress in patients with NAFLD. (J Clin Exp Hepatol 2013;3:12–18)**

NAFLD

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Address for correspondence: Ajay Duseja, Department of Hepatology, Post Graduate Institute of Medical Education and Research, Sector 12, Chandigarh 160012, India. Tel.: +91 172 2756336; fax: 91 172 2744401
E-mail: ajayduseja@yahoo.co.in

Abbreviations: NAFLD: nonalcoholic fatty liver disease; CVH: chronic viral hepatitis; HV: healthy volunteers; MDA: malondialdehyde; CD: conjugated dienes; NASH: nonalcoholic steatohepatitis; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; HCV: hepatitis C virus; AST: aspartate transaminase; ALT: alanine transaminase; ULN: upper limit of normal; ANA: anti nuclear anti bodies; ASMA: anti smooth muscle antibody; anti LKM: anti liver kidney microsomal antibody; AMA: antimitochondrial antibody; BMI: body mass index; WHR: waist hip ratio; HDL: high density lipoprotein; LDL: low density lipoprotein; TG: triglycerides; HOMA-IR: homeostasis model of insulin resistance; CHB: chronic hepatitis B; CHC: chronic hepatitis C; ICMR: Indian Council of Medical Research; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; TGF-β: transforming growth factor; GSH: glutathione reduced; GR: glutathione reductase; GPx: glutathione peroxidase; SOD: superoxide dismutase; PBS: phosphate buffered saline; NBT: nitroblue tetrazolium; MS: metabolic syndrome; CI: confidence intervals; ELISA: enzyme- linked immunosorbent assay; EDTA: ethylene diammine tetraacetic acid; RBC: red blood corpuscles (or cells); WBC: white blood corpuscles (or cells); KF rings: Kayser–Fleischer rings; ANOVA: analysis of variance

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Nonalcoholic fatty liver disease (NAFLD) is a recently coined entity and includes patients with simple steatosis and nonalcoholic steatohepatitis (NASH). Under the broad umbrella of NAFLD, NASH is an intermediate stage of liver damage and has the maximum propensity of progressing into cirrhosis and hepatocellular carcinoma (HCC).[1,2] NAFLD has become one of the commonest liver disease both in the eastern and western world and is responsible for significant liver disease.[3–5]

Pathogenesis of the NAFLD/NASH is not completely understood. Oxidative stress has been shown to play an important role in the pathogenesis of NAFLD/NASH in animal and human studies.[6–9] In the absence of alcohol intake, patients who either have metabolic syndrome or any of its components with insulin resistance, develop hepatic steatosis due to increased lipolysis and increased delivery of fatty acids from adipose tissue to liver.[10] The increased load of fatty acids in the hepatocytes increases the mitochondrial β-oxidation and increase in cytochrome P450 4A and cytochrome P4502E1 levels, leading to increase in reactive oxygen species. The increased mitochondrial oxidative stress leads to the second hit from steatosis to steatohepatitis and fibrosis by three main mechanisms, namely lipid peroxidation, cytokine induction, and Fas ligand induction. Lipid peroxidation,

apoptosis and recruitment of various cytokines lead on to hepatic inflammation and or fibrosis in addition to steatosis and progression from a stage of simple steatosis to NASH, setting the stage for future complications like cirrhosis and HCC.[11,12]

Oxidative stress has also been shown to play an important role in the pathogenesis of other liver diseases including chronic viral hepatitis. Thus the aim of the study was to assess the oxidative stress and cytokines in patients with nonalcoholic fatty liver disease (NAFLD) and to compare the results with those of chronic viral hepatitis.

PATIENTS AND METHODS

In a prospective study, twenty-five patients with NAFLD (Liver biopsy proven – 19) attending the liver clinic of our Institute were included over a period of 2 years. Results were compared with 25 age, sex and BMI-matched patients with chronic viral hepatitis (CVH) related to hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and 25 normal healthy volunteers (HV) selected from the attendants accompanying the patients. An informed consent was taken from all the subjects and study had the approval of the Institute Ethics committee.

The Nonalcoholic Fatty Liver Disease Group

Inclusion Criteria

Inclusion criteria for NAFLD patients included raised serum liver enzymes i.e. aspartate transaminase (AST) and alanine transaminase (ALT) at least 1.5 times the upper limit of normal (ULN) for more than 3 months, no history of alcohol intake or intake <20 g/day, negative viral markers (HBsAg and anti-HCV), negative autoimmune markers [anti nuclear anti bodies (ANA), anti smooth muscle antibody (ASMA), anti liver kidney microsomal antibody (anti LKM), antimitochondrial antibody (AMA)], normal ceruloplasmin levels, negative KF rings, normal iron studies and where available liver biopsy showing features of NAFLD. Pregnant females and patients with history of drug intake likely to cause NAFLD (e.g. corticosteroids, methotrexate, tamoxifen, etc) were excluded. Similarly patients with history of anti-tumor necrosis factor-α (e.g. pentoxyphylline, metformin, etc.) or anti-oxidant drugs (e.g. vitamin E or vitamin C, etc.) in last 3 months were excluded.

Anthropometric, Imaging and Biochemical Evaluation

All patients underwent a detailed evaluation including history of diabetes, hypertension, hyperlipidemia, coronary artery disease and drug intake/alcohol intake. Family history of diabetes, hypertension, coronary artery disease, and stroke was recorded. In particular body mass index (BMI) = Weight (kg)/Height (m^2), waist (cm), hip (cm) and waist hip ratio (WHR) for central obesity and abdominal examination for any organomegaly was recorded. Patients were classified as having overweight, obesity and central obesity as per the Asian Pacific criteria[13] (underweight BMI <18 kg/m^2, normal BMI ≥18 but <23 kg/m^2, overweight BMI ≥23 but <25 kg/m^2, class I obesity BMI ≥25 but <30 kg/m^2 and class II obesity BMI ≥30 kg/m^2, central obesity waist ≥90 cm and ≥80 cm in males and females, respectively). After routine hematological, biochemical (including fasting plasma glucose, lipid profile) and serological investigations, all patients were subjected to an ultrasound examination of abdomen. All examinations were performed on ATL 3500 ultrasound machine, using 5 MHz transducer and hepatic steatosis was noted and graded according to Saverymuttu et al.[14] A fasting plasma glucose of more than 126 mg/dl on more than one occasion or a random plasma glucose of more than 200 mg/dl in a symptomatic patient or a 2-h postprandial glucose more than 200 mg/dl was defined as diabetes.[15] In patients with known diabetes for long duration, dose and duration of drugs was recorded. Lipid profile was done in all patients and serum cholesterol >200 mg/dl, high density lipoprotein (HDL) less than 40 mg/dl in males and <50 mg/dl in females, low density lipoprotein (LDL) more than 130 mg/dl and serum triglycerides (TG) more than 150 mg/dl was taken as abnormal.[16]

Metabolic Syndrome and Insulin Resistance

Using Modified ATP III criteria i.e. modified waist circumference (≥90 cm in males, ≥80 cm in females), increased triglycerides (>150 mg/dl), low HDL cholesterol (<40 mg/dl in males, <50 mg/dl in females), high blood pressure (≥130/85 mmHg; or on antihypertensive drugs), and high fasting blood glucose (>110 mg/dl; or a known diabetic), metabolic syndrome was defined by the presence of any three or more criteria.[17] Homeostasis model of insulin resistance [HOMA-IR = fasting plasma glucose (mmol/L) × fasting plasma insulin (μU/ml)/22.5] was used to calculate the insulin resistance. HOMA-IR > 1.64 was taken as abnormal.[18,19]

Histopathology

All those fulfilling the inclusion criteria and with normal hemogram and coagulogram were admitted to the respective wards for liver biopsy. Liver biopsy was done under local anesthesia by the Menghini's/Trucut liver biopsy needle and the tissue thus obtained was subjected to histological examination. Diagnosis of NAFLD on histology was made by the defined criteria and the grading and staging of the disease was done as defined by Matteoni et al[20] and Brunt et al.[21]

Chronic Viral Hepatitis

Twenty-five age, sex and BMI-matched patients with CVH [chronic hepatitis B (CHB) and chronic hepatitis C (CHC)] were included. Eighteen patients were anti-HCV and HCV-RNA positive (CHC) while 7 showed the positivity for HBsAg with HBeAg positive or negative status and HBV-

DNA levels more than 2000 IU/L (CHB). In addition all patients with CVH had elevated ALT levels. The blood samples for investigations were taken before starting the treatment for chronic hepatitis B and C.

Healthy Volunteers

This group included age, sex, BMI-matched normotensive healthy subjects without a family history of diabetes with normal abdominal ultrasound, normal AST and ALT levels and fasting plasma glucose. The healthy subjects were selected according to Indian Council of Medical Research (ICMR) guidelines. Since serum lipids were not estimated in HVs, they were not evaluated for the presence of metabolic syndrome.

Oxidative Stress and Cytokines

During the same hospital admission in NAFLD patients and on outdoor basis in both healthy volunteers and disease controls (chronic viral hepatitis), 10 ml of fasting blood sample was taken for cytokines [interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), transforming growth factor (TGF-β)], markers of oxidative stress [malondialdehyde (MDA), conjugated dienes (CD)] and anti-oxidant status including reduced glutathione (GSH), glutathione reductase (GR) and peroxidase (GPx), catalase and superoxide dismutase (SOD).

Serum (from 5 mL blood in plain vial) and plasma (from 5 mL blood in EDTA vial) were separated from the sample within 30 min of collection and was stored in pyrogen free polypropylene cryotubes at −80°C until analysis. Remaining RBCs after separating plasma were washed three times with PBS (phosphate buffered saline) and WBCs were removed. The washed RBCs were also stored in pyrogen free polypropylene cryotubes at −80°C.

Oxidative Stress

Oxidative stress was studied by markers of lipid peroxidation.

Lipid Peroxidation

Markers of lipid peroxidation included determining plasma levels of malondialdehyde (MDA) and conjugated dienes (CD). MDA was assayed in plasma by the method of Buege and Aust.[22] MDA concentration was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and levels were expressed as μmol/L. Conjugated dienes were also assayed by the method of Buege and Aust.[22] Amount of hydroperoxide was calculated using a molar extinction coefficient of 2.52×10^4 M^{-1} cm^{-1} and conjugated diene levels were expressed as μmol/L.

Anti-oxidant Status

Anti-oxidant status was studied by various parameters including, reduced glutathione (GSH) levels, glutathione reductase (GR), glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD). GSH was assayed by the method of Beutler et al[23] and GSH levels were expressed as μmol/g Hb. GR was assayed by the method of Williams and Arscott.[24] Specific activity of GR was expressed as units/g Hb. One unit of GR activity was defined as the amount of enzyme required to reduce 1 μmol GSSG/min. GPx was assayed by the method of Flohe and Gunzler.[25] Specific activity of GPx was expressed as unit/g Hb. One unit of GPx activity was defined as the amount of enzyme required to produce 1 μmol GSSG/min. The method described by Luck[26] was used for estimating catalase activity in the hemolysate. Catalase activity was calculated using an extinction coefficient of 0.036 μM^{-1} cm^{-1}. Specific activity of catalase was expressed as μmol H_2O_2 oxidized/min/gHb. SOD was assayed by the method of Nishikimi et al.[27] The enzyme activity was expressed as units/mL where one unit of SOD activity was defined as the amount of the enzyme required to inhibit the rate of reduction of NBT (nitroblue tetrazolium) by 50%.

Cytokine Analyses

Serum cytokines including IL-1β, IL-6, IL-8, TNF-α and TGF-β in all the subjects were studied in a single batch using the commercially available kits (ELISA) as per the manufacturers' instructions. Serum ELISA kits for the evaluation of IL-1β, IL-6, IL-8, and TNF-α were purchased from Immunotech (A Beckman Coulter Company), France and TGF-β ELISA kit was procured from Raybiotech Inc., USA.

Statistical Analyses

Data is given as mean and 95% confidence intervals (CI). Comparison between different groups was performed using ANOVA and post-hoc analysis. Continuous variables were summarized as mean and 95% confidence intervals (CI). Student's *t*-test for unpaired data was used to compare groups when variables were normally distributed; otherwise, the Mann–Whitney test was used. A χ^2 test was used to compare differences in categorical variables. All analysis was performed using SPSS software version 13 (SPSS Inc., Chicago, IL). Differences were considered statistically significant if $p < 0.05$.

RESULTS

Demographic and Anthropometric Profile

The demographic and anthropometric profiles of all groups matched for age, gender and BMI are shown in Table 1. There was statistically no difference between age, gender and BMI among three groups. A higher waist circumference ($p = 0.010$) and a higher waist to hip ratio was observed in NAFLD patients ($p = 0.018$) as compared to HVs. Amongst NAFLD patients 9 (36%) patients were

Table 1 Demographic and biochemical characteristics of subjects with NAFLD, chronic viral hepatitis and healthy volunteers.

Parameter	Group-1	Group-2	Group-3	*p*-Value (ANOVA)
Mean (95% CI)	NAFLD, *n* = 25	Chronic viral hepatitis, *n* = 25	Healthy volunteers, *n* = 25	
Age (yrs)	38.60[a] (35–42.20)	35.56[b] (31.50–39.62)	37.04[c] (32.35–41.73)	0.566
Sex (M:F)	15:10	20:05	16:09	0.276
Height (cm)	163.56[d] (158.81–168.31)	168.55[e] (165.13–171.96)	163.64[f] (160.41–166.87)	0.104
Weight (kg)	71.39[g] (66.92–75.87)	69.60[h] (64.61–74.59)	64.38[i] (60.74–68.01)	0.060
BMI (kg/m^2)	26.71[j] (25.36–28.05)	24.51[k] (22.85–26.16)	24.17[l] (22.57–25.78)	0.039
Waist (cm)	91.20[m] (88.45–93.95)	86.44[n] (81.71–91.17)	82.62[o] (78.07–87.17)	0.012
Hip (cm)	92.42[p] (89.90–94.94)	89.62[q] (86.36–92.88)	89.08[r] (85.18–92.98)	0.284
Waist: Hip	0.99[s] (0.97–1.01)	0.96[t] (0.93–0.99)	0.93[u] (0.89–0.96)	0.022
Bilirubin (mg/dl)	0.96[v] (0.53–1.39)	0.64[w] (0.57–0.70)	0.56[x] (0.49–0.62)	0.058
AST (IU/L)	74.16[y] (58.33–90.00)	76.77[z] (63.48–90.05)	26.57[aa] (23.46–29.68)	0.000
ALT (IU/L)	93.36[bb] (76.02–110.69)	100.18[cc] (82.13–118.23)	28.97[dd] (26.05–31.88)	0.000

[ab]$p = 0.863$, [ac]$p = 1.000$, [bc]$p = 1.000$, [de]$p = 0.190$,[df]$p = 1.000$, [ef]$p = 0.202$, [gh]$p = 1.000$, [gi]$p = 0.068$, [hi]$p = 0.263$ [jk]$p = 0.123$, [jl]$p = 0.057$, [kl]$p = 1.000$, [mn]$p = 0.285$, [mo]$p = 0.010$, [no]$p = 0.537$, [pq]$p = 0.647$, [pr]$p = 0.422$, [qr]$p = 1.000$, [st]$p = 0.696$, [su]$p = 0.018$, [tu]$p = 0.330$, [vw]$p = 0.209$, [vx]$p = 0.074$, [wx]$p = 1.000$, [yz]$p = 1.000$, [yaa]$p = 0.000$, [zaa]$p = 0.000$, [bbcc]$p = 1.000$, [bbdd]$p = 0.000$, [ccdd]$p = 0.000$.

overweight, 9 (36%) were class I obese, 5 (20%) were class II obese and 2 (8%) patients were lean or had normal BMI. Central obesity was present in 20 of the 25 (80%) patients with NAFLD with females having more central obesity (90%) than males (73.30%). Amongst chronic viral hepatitis patients, 6 (24%) patients were overweight, 7 (28%) were class I obese, 4 (16%) were class II obese and 8 (32%) were lean or had normal BMI. Central obesity was present in 10 of the 25 (40%) patients of CVH with females having more central obesity (80%) than males (30%). Patients with NAFLD and CVH had higher AST (p = 0.000) and ALT (p = 0.000) levels in comparison to healthy volunteers with no difference between NAFLD and CVH patients.

Insulin Resistance

No differences were seen in serum insulin and HOMA-IR levels in patients with NAFLD compared to patients with chronic viral hepatitis and healthy volunteers. Presence of insulin resistance (HOMA-IR > 1.64) was present in 19 (76%), 14(56%), and 10(40%) patients respectively amongst NAFLD, CVH and HV groups with significant difference between NAFLD and HVs (p = 0.009).

Metabolic Syndrome

Thirteen patients (52%) with NAFLD had full-blown metabolic syndrome (≥3 components of MS) in comparison to 3 (12%) patients in the CVH group (p = 0.006). Conversely 10 (40%) patients in the CVH group had none of the components of metabolic syndrome in comparison to only 1 (4%) in the NAFLD group (p = 0.006). Two components of MS were present in 6 (24%) and one component of MS was present in 5 (20%) patients with NAFLD. Since serum lipids were not estimated in HVs, they were not evaluated for the presence of metabolic syndrome.

Histopathology

Liver biopsy was performed in 19 patients of NAFLD. Patients were classified as per Matteoni et al[20] and grading of necroinflammation and staging of fibrosis was performed according to Brunt et al.[21] One patient (5.26%) had class I NAFLD, 11 (57.89%) patients had Class II, 6 (31.58%) had Class III and 1 (5.26%) patient had Class IV NAFLD. Hence 7 out of 19 (36.84%) patients who underwent biopsy had histological NASH (i.e. Class III and IV). Inflammatory activity was grade 1 in 15 (78.94%) patients, grade 2 in 2 (10.53%) patients, and grade 3 in 2 (10.53%) patient. Twelve patients (63.16%) had no fibrosis, 4 patients (21.05%) had stage I fibrosis, 2 (10.53%) patients had stage II fibrosis and one (5.26%) patient had stage III fibrosis. None of the patients had stage IV (cirrhosis) on liver biopsy.

Oxidative Stress

Details of lipid peroxidation in three different groups have been shown in Table 2. Patients with NAFLD had significantly higher levels of malondialdehyde (MDA) (p = 0.000) and conjugated dienes (CD) (p = 0.000) in comparison to HVs. Patients with CVH also had higher MDA levels (p = 0.026) and CDs (p = 0.000) in comparison to HVs. Patients with NAFLD also had significantly higher MDA levels (p = 0.000) in comparison to CVH patients. However there was no difference in CDs levels between NAFLD and CVH patients.

Table 2 Markers of lipid peroxidation in subjects with NAFLD, chronic viral hepatitis and healthy volunteers.

Parameter	Group-1	Group-2	Group-3	*p*-Value (ANOVA)
Mean (95% CI)	NAFLD, (*n* = 25)	Chronic viral hepatitis (*n* = 25)	Healthy volunteers (*n* = 25)	
MDA (μmol/L)	2.41[a] (2.19–2.63)	1.78[b] (1.64–1.91)	1.45[c] (1.27–1.62)	0.000
CD (μmol/L)	24.66[d] (21.43–27.89)	23.01[e] (21.96–24.06)	16.45[f] (14.70–18.20)	0.000

[ab]p = 0.000, [ac]p = 0.000, [bc]p = 0.026, [de]p = 0.836, [df]p = 0.000, [ef]p = 0.000.

Anti-oxidant Status

Details of the anti-oxidant status have been shown in Table 3. Patients with NAFLD had significantly lower GSH levels (p = 0.004) in comparison to HVs. There was no significant difference in GR activity amongst three groups. Patients with NAFLD had higher GPx activity (p = 0.028) in comparison to HVs. CVH patients also had higher GPx activity (p = 0.006) in comparison to HVs. Catalase activity was significantly decreased in both NAFLD (p = 0.001) and CVH patients (p = 0.000) in comparison to HVs. Patients with NAFLD had significantly higher SOD activity (p = 0.000) in comparison to CVH patients.

Cytokine Analyses

Cytokines levels were analyzed in different groups and the results are shown in Table 4. There was no difference in serum levels of IL-1β and TNF-α amongst three groups. Patients with CVH were found to have higher IL-8 serum levels (p = 0.039) in comparison to HVs. CVH patients also had higher TGF-β levels (p = 0.002) in comparison to both NAFLD patients and HVs.

Cytokines and Oxidative Stress in Patients with and Without Histological Nonalcoholic steatohepatitis

Nineteen patients with NAFLD who underwent a liver biopsy were classified into those having histological NASH (class 3 and 4, n = 7) and NAFLD without NASH (class 1and2, n = 12). There was no difference in mean age, anthropometry, biochemical profile, insulin resistance and metabolic syndrome amongst these two groups. Markers of lipid peroxidation (MDA and CDs) and anti-oxidant markers (GSH, GR, GPx, catalase, SOD) were not different amongst patients with and without histological NASH. There was no difference in the serum levels of IL-6, TNF-α and TGF-β amongst patients with and without histological NASH (Data not shown).

DISCUSSION

Nonalcoholic fatty Liver Disease (NAFLD) presents a wide spectrum of hepatic injury due to longstanding fatty infiltration of the liver that ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (HCC).[28] Interplay of oxidative stress and cytokines has been documented as a second hit in the manifestation of NASH from simple steatosis, in the most conceivable 'two hit' hypothesis till date wherein the first step being the hepatic accumulation of fat in the form of triglycerides under the effect of insulin resistance.[29] The present study was conducted to assess the role of oxidative stress and cytokines in patients with nonalcoholic fatty liver disease.

Excessive fatty oxidation by the mitochondrial peroxisomes under the over expression of CYP2E1 enzymes, in the steatotic livers lay the foundation of oxidative stress.[30] We estimated in our patients the levels of conjugated dienes (CD) and malondialdehyde (MDA) as the early and end products of lipid peroxidation respectively. Both the markers were found to be increased in NAFLD and chronic viral hepatitis patients as compared to healthy volunteers. Similar results have been shown earlier with both CD and MDA and other markers of lipid peroxidation in patients

Table 3 Anti-oxidant status parameters in subjects with NAFLD, chronic viral hepatitis and healthy volunteers.

Parameter	Group-1	Group-2	Group-3	*p*-Value (ANOVA)
Mean (95% CI)	NAFLD (*n* = 25)	Chronic viral hepatitis (*n* = 25)	Healthy volunteers (*n* = 25)	
GSH (μmol/gHb)	2084.53[a] (1508.47–2660.59)	2995.20[b] (2195.92–3794.48)	3794.48[c] (2975.51–4613.45)	0.005
GR (units/gHb)	7.86[d] (5.85–9.87)	6.02[e] (5.04–7.01)	6.88[f] (5.27–8.49)	0.250
GPx (units/gHb)	25.74[g] (20.55–30.93)	27.25[h] (24.54–29.96)	18.17[i] (14.08–22.26)	0.004
Catalase (μmol H_2O_2 oxidized/min/gHb)	1667.88[j] (1475.45–1860.32)	1397.23[k] (1204.65–1589.81)	2162.00[l] (1963.43–2360.57)	0.000
SOD (units/mL)	64.90[m] (60.25–69.55)	44.11[n] (36.88–51.33)	54.28[o] (45.28–63.27)	0.000

[ab]p = 0.230, [ac]p = 0.004, [bc]p = 0.358, [de]p = 0.291, [df]p = 1.000, [ef]p = 1.000, [gh]p = 1.000, [gi]p = 0.028, [hi]p = 0.006, [jk]p = 0.138, [jl]p = 0.001, [kl]p = 0.000, [mn]p = 0.000, [mo]p = 0.102, [no]p = 0.127.

Table 4 Serum cytokines levels in subjects with NAFLD, chronic viral hepatitis and healthy volunteers.

Parameter	Group-1	Group-2	Group-3	*p*-Value (ANOVA)
Mean (95% CI)	NAFLD (n = 25)	Chronic viral hepatitis (n = 25)	Healthy volunteers (n = 25)	
IL-1β (pg/mL)	33.77[a] (30.56–36.99)	36.00[b] (30.37–41.63)	37.72[c] (33.84–41.59)	0.421
IL-6 (pg/mL)	17.55[d] (2.37–32.72)	72.64[e] (7.63–137.66)	9.69[f] (7.11–12.28)	0.040
IL-8 (pg/mL)	282.26[g] (141.22–423.29)	293.83[h] (81.42–506.25)	37.32[i] (32.14–42.51)	0.020
TNF-α (pg/mL)	50.90[j] (33.40–68.39)	57.25[k] (24.37–90.14)	48.36[l] (34.69–62.04)	0.844
TGF-β (ng/mL)	4.27[m] (3.8–4.75)	6.78[n] (5.21–8.36)	4.27[o] (3.68–4.87)	0.000

[ab]p = 1.000, [ac]p = 0.574, [bc]p = 1.000, [de]p = 0.122, [df]p = 1.000, [ef]p = 0.060, [gh]p = 1.000, [gi]p = 0.053, [hi]p = 0.039, [jk]p = 1.000, [jl]p = 1.000, [kl]p = 1.000, [mn]p = 0.002, [mo]p = 1.000, [no]p = 0.002.

with NAFLD and CVH.[8,31,32] In addition we found that patients with NAFLD had significantly higher MDA levels in comparison to patients with CVH. Even though the difference was not significant statistically, patients with NAFLD also had higher CD levels in comparison to patients with CVH. Patients with NAFLD also had significantly higher SOD activity in comparison to CVH patients. Our results thus suggest higher oxidative stress in patients with NAFLD in comparison to patients with chronic viral hepatitis and may indirectly also suggest that even though the oxidative stress is initiated at a same pace in both NAFLD and CVH patients, over a period it may get lower in patients with CVH.

Our results on anti-oxidant defense system yielded that the reduced glutathione (GSH) levels were significantly decreased in NAFLD patients in comparison to healthy volunteers even though the results were not significantly different from patients with CVH. Imbalance between pro-oxidants and anti-oxidants leads on to oxidative stress, when the balance is more toward pro-oxidants. GSH plays an integral role in the coordination of cellular anti-oxidant defense processes and its levels are shown to vary inversely with susceptibility to oxidative stress.[33] Decreased GSH levels have been shown earlier in patients with NAFLD as well as in experimental models of NAFLD.[34–36] Though not significant, GR activity was increased in patients with NAFLD in comparison to healthy volunteers. GR is thought to preserve the GSH levels and lower levels of GSH in spite of higher GR levels in our patients suggest the inability of the GR to maintain the GSH levels. We also found higher GPx activity in patients with NAFLD and CVH in comparison to healthy volunteers. GPx acts by reducing the lipid hydroperoxides using GSH as sole reductive source. Our results thus explain the lower GSH and higher CD and MDA levels in patients with NAFLD. Our data showed that patients with NAFLD had higher SOD activity in comparison patients with CVH. SOD dismutes the O_2^- to O_2 and H_2O_2. Increase in the activity further substantiates the overproduction of free radicals that sustains oxidative stress in patients with NAFLD. Same findings have been documented by Das et al[32] and are explained as an adaptive response toward oxidative stress.[37] Finally we found that catalase activity was decreased in patients with NAFLD and CVH in comparison to healthy volunteers. Catalase is associated with the decomposition of hydroperoxides especially H_2O_2. The decreased activity in our patients may be due to the structural and functional alterations induced in the enzyme due to excessive production of free radicals.[38,39]

In this study, we found that serum cytokine levels varied considerably between three groups with NAFLD, CVH and healthy volunteers. Among the pro-inflammatory cytokine profile, no significant difference in IL-1β, IL-6 and TNF-α level were observed in three different groups. Even though the liver biopsy was available only in 19 patients with NAFLD, majority of patients had mild inflammatory activity which could explain the lower levels of inflammatory cytokines. In contrast, IL-8 levels showed a significant increase in patients with CVH in comparison to HV. Patients with CVH also had higher TGF-β levels in comparison to both NAFLD and HV groups. Interleukin-8 is a cytokine synthesized by hepatocytes, Kupffer cells and macrophages and shows its effects by activating neutrophils and its higher levels in patients with CVH in comparison to HV may be related to hepatic inflammation. Even though the liver histology was not available in patients with CVH, higher TGF-β levels in CVH patients may be due to the higher stage of hepatic fibrosis in comparison to patients with NAFLD.

Even though our study is limited by small number of patients, the differences in the markers of oxidative stress and anti-oxidant status between NAFLD, CVH and healthy volunteers suggest presence of higher oxidative stress in patients with NAFLD in comparison to patients with CVH. Higher serum TGF-β levels in patients with CVH in comparison to NAFLD suggest higher hepatic fibrosis in patients with CVH. A study with larger number of patients is required to confirm the findings.

CONFLICTS OF INTEREST

All authors have none to declare.

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