

# Oxidative Toxic Stress and DNA Damage as a Promising Strategy for Identifying Patients with Nonalcoholic Fatty Liver Disease

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## Abstract

Non-alcoholic fatty liver disease (NAFLD) is considered as one of the common causes of chronic hepatitis and cirrhosis. The present study was performed to determine paraoxonase 1 (PON 1) and arylesterase (ARE) activities, biomarkers of oxidative toxic stress and DNA damage in patients with NAFLD and their suitability for identifying for NAFLD. In this case-control study, 40 NAFLD patients and 40 normal subjects were studied. Abdominal ultrasonography, serum PON 1 and ARE activities, level of lipid peroxidation (LPO), total thiol groups (TTG), total antioxidant capacity (TAC) and DNA damage biomarker were measured in both NAFLD patients and control group. There was a significant increase in salt stimulated PON 1 activity and LPO level in NAFLD patients compared to the control group ( $P < 0.05$ ). In contrast, TAC decreased in NAFLD patients compared to the control group. No significant difference was observed in biomarkers of DNA damage and ARE activity between the groups ( $P > 0.05$ ). PON 1 activity and LPO level can be considered as biomarkers for NAFLD diagnosis. Along with these biomarkers, total antioxidant capacity can be used for identifying patients with NAFLD.

**Keywords:** paraoxonase 1, oxidative stress, DNA damage, nonalcoholic fatty liver disease (NAFLD)

## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive lipid accumulation, inflammation and an imbalanced redox homeostasis. (Damba et al. 2020). NAFLD encompasses a wide spectrum of liver diseases, from simple steatosis to non-alcoholic steatohepatitis (NASH) (Chalasanani et al. 2018). The reported prevalence of NAFLD is 25–30% in Western countries (Ferro et al. 2020). Serum Paraoxonase (PON) and arylesterase (ARE) are esterase enzymes with lipophilic antioxidant characteristics. Serum PON and ARE act as a single enzyme (Ates et al. 2009). Paraoxonase 1 (PON1), a calcium-dependent esterase, is associated with high-density lipoprotein (HDL) cholesterol. It has been established that antioxidant property of PON is conferred by diminishing the storage of the products of lipid peroxidation (LPO) (Aslan et al. 2007). The liver plays an important role in the synthesis of PON 1 which is able to hydrolyse a number of substrates, including phenyl acetate, paraoxon, lipid peroxides, cholesterol esters and hydroperoxides (Shokri et al. 2020). Imbalance between the production of reactive oxygen species (ROS) and inadequate antioxidant defense systems can lead to oxidative toxic stress (OTS) and cell damage

not only directly but also indirectly by changing signaling pathways (Ghadermazi et al. 2018). ROS production results from endogenous factors such as elevation of mitochondrial dysfunction and oxidative enzymes in infections and inflammations and exogenous factors such as air pollution, radiation and drug exposures (Ranjbar et al. 2018). In a previous study, it has been shown that NAFLD was related to an increase in oxidative stress serological parameters (Damba et al. 2020). Since histological changes in liver biopsy for NAFLD diagnosis is invasive, finding non-invasive methods for NAFLD diagnosis is of great importance. The present study was performed to evaluate the presence of oxidative stress, DNA damage and activities of PON 1 and ARE in a sample of patients with NAFLD to find out their valuable potential as future biomarkers for NAFLD diagnosis.

## 2. Materials and methods

### 2.1. Chemicals

Reagents and Chemicals used in this study were tetraethoxypropane, 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), 5,5-Dithiobis-2-nitrobenzoic acid (DTNB), n-butanol, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), Tris base, Propofol, Ketamine, ethylene-di-amine tetra-acetic acid

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(EDTA), 2,4,6-tripyridyl-S-triazine (TPTZ) purchased from Sigma-Aldrich (St. Louis, USA). Paraoxonase and DNA damage kits were obtained from Cayman Chemical Co. (Ann Arbor, MI).

## 2.2. Study subjects

This case-control study was performed in Beheshti teaching hospital, Hamadan city, Iran, in 2016. Forty NAFLD patients who underwent a liver biopsy and 40 normal subjects were studied. All participants signed a formal consent before the commencement of the study. In addition, the protocol of the study was approved by the ethics committee of the Hamadan University of Medical Sciences (No: 940201454).

Histological diagnosis of nonalcoholic Steatohepatitis (NASH) and level of fibrosis were approved by an expert hepatopathologist. Standard (clinical and histological) criteria were used for the diagnosis of NAFLD. Patients were assigned into three distinct groups subjected to liver histology. Group 1, simple steatosis. Group 2, NASH in the absence of advanced fibrosis (NASH is described as steatosis along with portal and/or lobular inflammation and fibrosis stage 0-2). Group 3, NASH in the presence of advanced fibrosis (described as steatosis along with portal and/or lobular inflammation and fibrosis stage 3-4). Patients in the NAFLD group had not the history of alcohol drinking, autoimmune liver diseases, hemochromatosis and viral hepatitis.

Fasting blood samples were taken from the NAFLD patients in the morning of their programmed liver biopsy and control subjects. Samples were withdrawn from a cubital vein and then transferred into blood tubes and immediately stored at +4°C. Separation of cells was performed by centrifugation of samples at 3000 g for 10 min and kept in plastic vials at -80 °C until analysis.

## 2.3. Biochemical analysis

### 2.3.1. Paraoxonase 1 (PON1) and Arylesterase (ARE) activities measurement

Basal and salt-stimulated, with presence of NaCl, paraoxonase 1 activities were measured based on previously established findings. Generally, paraoxon (substrate) hydrolysis rate (di-ethyl p-nitro-phenyl-phosphate) was deliberated using an increase in absorbance level at 405 nm wave length at 37 °C. The analysis was carried out originally based upon the formation of p-nitrophenol using the molar absorptivity coefficient of 18050 M<sup>-1</sup>cm<sup>-1</sup> at pH = 8.5. The enzyme activity was expressed as U/L. Molar extinction coefficient of the resultant phenol (1310 M<sup>-1</sup>cm<sup>-1</sup>) was used to determine the activity of ARE, and phenyl acetate was utilized as substrate. The reaction was uninterruptedly monitored at 270 nm and 37°C. Under this condition, one unit of ARE activity was considered as the quantity of phenol in mol generated per min and expressed as U/L serum (Hashemi et al. 2011).

### 2.3.2. Measurement of Total Thiol groups (TTG)

Hu method was utilized to determine total sulfhydryl content in plasma. Briefly, 0.6 ml of the Tris-EDTA buffer (Tris base 0.25 M, EDTA 20 mM, pH = 8.2) was added to an aliquot of 0.2 ml plasma in a test tube. Then, 40 µl of 10 mM DTNB in methanol were added. The volume of the reaction mixture was made up to 4.0 ml by adding 3.16 ml

of methanol. The test tubes were capped and the color was developed for 15–20 min. Then, they centrifuged at 3000 g for 10 min at room temperature. The supernatant was collected, and its absorbance at 412 nm was measured (Hu and Dillard 1994).

### 2.3.3. Measurement of lipid peroxidation (LPO)

The level of LPO in blood was assessed by TBA reagent, and the LPO is expressed as the extent of aldehyde production, during acid heating reaction. Briefly, 1.5 ml of TCA (20% w/v) was added to 250 µl of each sample and then centrifuged at 3000 g for 10 min. The resultant precipitation was dissolved in sulfuric acid and then 1.5 ml of the mixture was added to 1.5 ml of TBA (0.2% w/v). After incubation in a boiling water bath for one hour, 2 ml of n-butanol was added to the mixture, and then it was centrifuged. After cooling at room temperature, the absorption of the supernatants was measured at 532 nm. Standard solutions of tetraethoxypropane were prepared to obtain calibration curve and to calculate the concentrations of TBA+MDA adducts in samples (Moore and Roberts 1998).

### 2.3.4. Measurement of Plasma total antioxidant capacity (TAC)

The ability of plasma in reducing Fe<sup>3+</sup> to Fe<sup>2+</sup> was used to assess the antioxidant capacity of plasma. The resultant complex of reaction between Fe<sup>2+</sup> and TPTZ provides a blue color with absorbance at 593 nm (Benzie and Strain 1999).

### 2.3.5. Measurement of DNA damage

The 8-OHdG content in the extracted DNA solutions was determined using the ELISA method (Highly Sensitive 8-OHdG ELISA kit, Japan). These meticulous assay kits were chosen because they require small amount of sample and provide high sensitivity and specificity, and inter- and intra-assay precision.

## 2.4. Statistical analysis

Data were analysed using version 16.0 of SPSS version 16 (SPSS, Chicago, IL). Mean and standard error values were ascertained for all the parameters, and the results were stated as Mean ± SEM. Differences between variables of case and control groups were analyzed by student t-test. A P-value less than 0.05 was considered statistically significant.

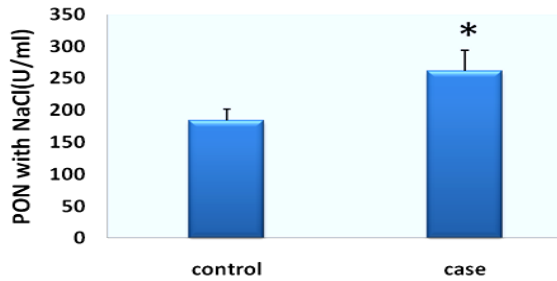
## 3. Results

None of the participants (40 NAFLD patients and 40 controls) declined to participate in the study. Three patients were excluded because of the lack of cold chain for their blood samples. Appropriateness of randomization was confirmed by comparison of general characteristics in both groups as shown in Table 1.

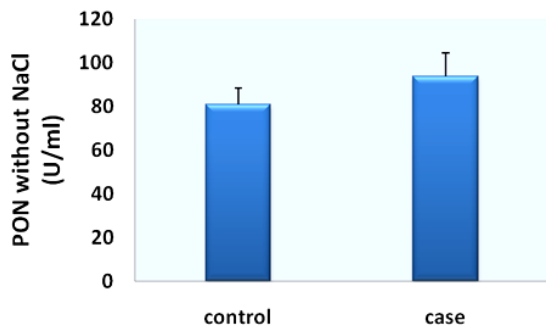
**Table 1.** Demographic characteristics of the studied subjects

Variables	Groups	
	Patients (n=40)	Control (n=40)
Age (years)	42.83±9.84	32.42±10.12
Weight (kg)	81.36±14.63	78.57±9.63
BMI (kg/m <sup>2</sup> )	29.31±3.79	24.50±2.13
Height (m)	166.33±11.20	176.17±6.74
Sex (%)	97.5% Male, 2.5% Female	52.8% Male, 47.2% Female

Figures 1 to 7 showed the biomarkers of oxidative stress in both groups. The salt-stimulated PON 1 activity in the NAFLD patients was significantly higher than that of control group ( $P = 0.04$ , Fig 1). The basal PON1 activity (without NaCl) in the NAFLD patients was higher than the control group; however, the difference was not statistically significant ( $P = 0.33$ , Fig 2).

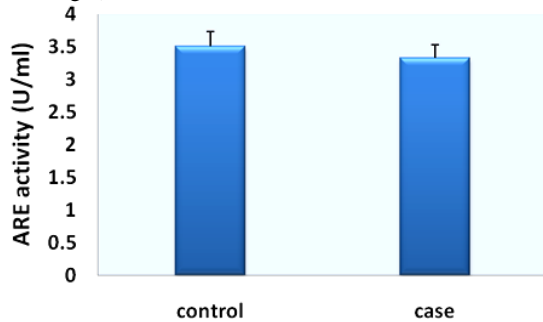


**Figure 1.** Salt stimulated activity of paraoxonase 1 in serum of patient and control groups. The difference between mean values was assessed by student-t test ( $P=0.04$ ) and is shown by (\*).



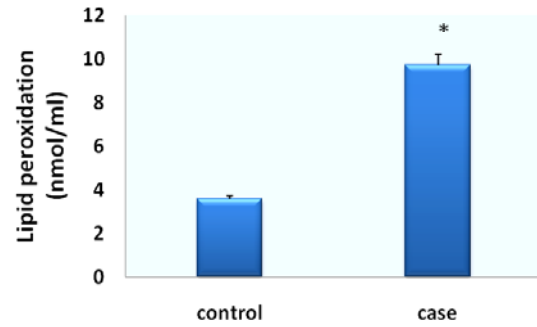
**Figure 2.** Basal paraoxonase 1 activity in serum of patient and control groups. The difference between mean values was assessed by student-t test ( $P=0.33$ ), and it was not significant

No significant difference of ARE activity was seen between the NAFLD patients and control groups ( $P = 0.60$ , Fig 3).

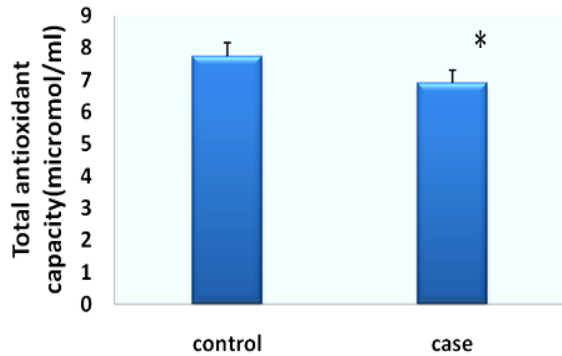


**Figure 3.** Arylesterase activity in serum of patient and control groups. The difference between mean values was assessed by student-t test ( $P=0.60$ ), and it was not significant.

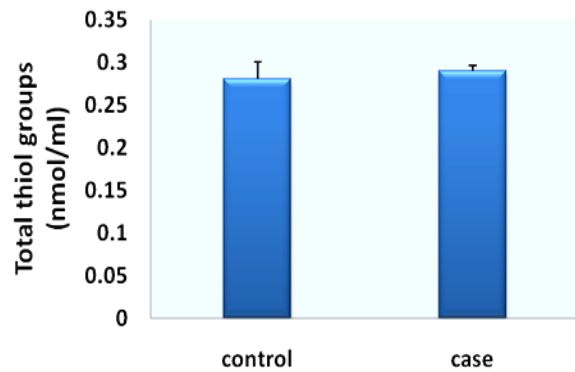
The LPO level of the NAFLD patients was significantly higher than that of the control group ( $P=0.001$ , Fig 4). In contrast, the TAC was significantly lower in the NAFLD patients than that of the control group ( $P=0.04$ , Fig 5). At the same time, total thiol molecules of the NAFLD patients were not significantly lower than that of the control group ( $P=0.66$ , Fig 6). No significant difference was also observed in the 8-OHdG content of the NAFLD patients and control groups ( $P= 0.6$ , Fig 7).



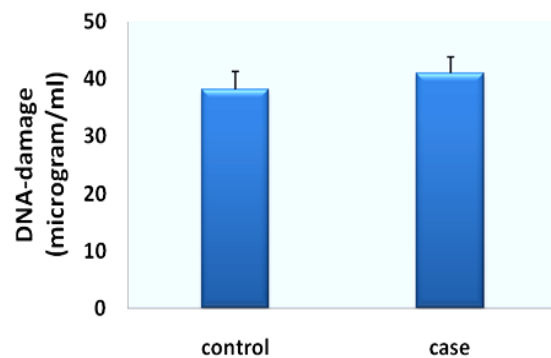
**Figure 4.** Lipid peroxidation level in serum of patient and control groups. The difference between mean values was assessed by student-t test ( $P=0.001$ ) and is shown by (\*).



**Figure 5.** Total antioxidant capacity in serum of patient and control groups. The difference between mean values was assessed by student-t test ( $P=0.04$ ) and is shown by (\*).



**Figure 6.** Total thiol groups in serum of patient and control groups. The difference between mean values was assessed by student-t test ( $P=0.66$ ), and it was not significant.



**Figure 7:** DNA damage level in serum of patient and control groups. The difference between mean values was assessed by student-t test ( $P=0.60$ ), and it was not significant.

#### 4. Discussion

In the present study, we revealed that NAFLD is accompanied by elevations in serum PON 1 activity and oxidative stress biomarkers such as LPO. Previous studies showed that LPO is greatly elevated in Nonalcoholic steatohepatitis (NASH) (Morita et al. 2012). Excess ROS is detoxified by several enzymatic mechanisms. PON 1, a liver-produced peroxidase, is famous for its antioxidant role in the circulation (Ramadan et al. 2012, Zaki et al. 2014). Therefore, our findings provide evidence in support of the theory that PON 1 may act as an antioxidant. This theory was first expressed by Marsillach et al. (2007) when they observed a wide-range of paraoxonases in chronic alcoholics (Marsillach et al. 2007). Previously, Hashemi et al. (2011) studied the effect of PON 1 deficit in a mouse model of NAFLD induced by a high-fat and high-cholesterol diet. They found that PON 1 deficit can induce higher levels of oxidative stress which confirm an antioxidant role for PON 1 in the liver (Hashemi et al. 2011). As mentioned before, liver plays a critical role in PON 1 synthesis. Chronic liver disorders can lead to increased levels of oxidative stress and inflammation (Shokri et al. 2020). PON 1 protects the liver against inflammation, liver disease and fibrosis (Aharoni, Aviram and Fuhrman 2013, Loued et al. 2012). It has already been revealed that increased PON 1 activity could be correlated with the reduction of serum levels of oxidative biomarkers in patients with progressive liver cirrhosis (Desai et al. 2014). Likewise, considering the antioxidant characteristics of PON-1 enzyme in NAFLD patients, the results of the previous studies demonstrated that PON-1 is paradoxically maintained and may even be increased in NAFLD despite its inverse associations with metabolic disorders and low HDL cholesterol (van den Berg et al. 2019). Hepatotoxicity induced by oxidative stress may lead to loss of fundamental biomolecules functions and cell viability due to direct attack of RNS and ROS (Çekmez and Dündar 2013, Awad et al. 2016). Alternatively, indirect activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B), activator protein-1 (AP-1), and redox-sensitive transcription factors can be induced by ROS, which are initiating the production of cytotoxic, fibrogenic and/or proinflammatory mediators by Kupffer cells and other non-parenchymal cells (Awad et al. 2016, Marí et al. 2015, Wan et al. 2014). Several studies in animal models of NAFLD displayed a higher free radical activity via the following mechanisms: (i) elevation of mitochondrial superoxide radicals and H<sub>2</sub>O<sub>2</sub> generation, (ii) induction of microsomal cytochrome P450 isoforms such as CYP2E1 and CYP3A4, characterized by their high pro-oxidant activity, and (iii) LPO responses (Marí et al. 2015, Kumar et al. 2012, Schmilovitz-Weiss et al. 2013, Woolsey et al. 2013). We did not find a significant difference in the level of DNA damage biomarker (8-OHdG) between the NAFLD patients and controls. In addition, no significant difference in the ARE activity was observed between the groups. As a consequence of ROS overproduction and the shortage of endogenous antioxidant molecules, higher oxidative stress is recognized as a well-established cause of liver injury due to extensive oxidative biomolecular damage, and subsequently alterations in PON and ARE activities may lead to progressive fibrosis (Ferro et al. 2020). Furthermore, in previous studies, interference of OTS in NAFLD patients was proved and antioxidants usage for protection against these pathways was suggested,

but in this study patients with NAFLD had the induction of OTS and PON 1 activity which can be used as valuable future biomarkers for NAFLD diagnosis.

## 5. Conclusions

Patients with NAFLD had the induction of oxidative toxic stress and PON 1 activity which can be used as valuable future biomarkers for NAFLD diagnosis. Further studies are recommended to assess the mechanism of oxidative stress and the beneficial role of antioxidant treatment in patients with NAFLD.

## 6. Conflict of interest

None.

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