Hadinia A, et al. Iran J Immunol. 2019; 16(4):327-333.
https://doi.org/10.22034/iji.2019.80284.

Iranian Journal of Immunology (IJI)
iji@sums.ac.ir

SHORT PAPER

Circulating Levels of Pro-inflammatory Cytokines in Patients with Nonalcoholic Fatty Liver Disease and Non-Alcoholic Steatohepatitis

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ABSTRACT

Background: Pro-inflammatory cytokines are associated with systemic inflammatory responses. **Objective:** To investigate the levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in patients with non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) compared to healthy individuals. **Methods:** This case-control study was conducted on 30 patients with NAFL, 30 patients with NASH, and 30 healthy volunteers. The plasma level of IL-1β, IL-6, and TNF-α were determined by ELISA, and biochemical parameters were measured using colorimetric methods. **Results:** IL-1β and IL-6 levels were significantly higher in patients with NASH compared with NAFL and control group. However, TNF-α levels had no significant variations in NAFL and NASH patients compared to the control group (p=0.903 and p=0.960, respectively). **Conclusion:** Results showed that the levels of ALT activity and pro-inflammatory cytokines were higher in patients with NASH compared to control and NAFL subjects; Therefore, steatosis and inflammation develop as a result of excessive pro-inflammatory factors in NASH.

Received: 2019-05-09, Revised: 2019-10-21, Accepted: 2019-11-08.
Citation: Hadinia A, Doustimotlagh AH, Goodarzi HR, Arya A, Jafarinia M. Circulating Levels of Pro-inflammatory Cytokines in Patients with Nonalcoholic Fatty Liver Disease and Non-Alcoholic Steatohepatitis. *Iran J Immunol.* 2019; 16(4):327-333. doi: 10.22034/iji.2019.80284.

Keywords: Interleukin-1β, Interleukin-6, Non-Alcoholic Fatty Liver, Non-Alcoholic Steatohepatitis, Tumor Necrosis Factor-α

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) refers to the fat accumulation within hepatocytes, with no history of alcohol abuse or other causes of secondary hepatic steatosis. This accumulation is strongly associated with obesity and metabolic syndrome (1). There are two types of non-alcoholic fatty liver disease (NAFLD), namely non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) (2). NAFL is non-inflammatory liver steatosis while NASH is hepatic steatosis associated with an inflammation not histologically distinct from alcohol steatohepatitis (3). NASH is the critical stage of NAFLD, and data have shown that 15%-20% of NASH patients develop liver cirrhosis in 10 to 20 years (4). The pathogenesis of NAFL and NASH has not been fully elucidated. Recent investigations have shown that the immune system plays an essential role in the initiation, maintenance, and progression of these diseases (5). Various immune parameters, such as cytokine level, have been studied for a better understanding of the pathophysiology of these diseases and as biomarkers for severity assessment and outcome prediction. General inflammatory biomarkers such as pro-inflammatory cytokines have been associated with the occurrence and prognosis of NAFLD (6). The interactions between cytokines and oxidative stress and lipid peroxidation play a vital role in the induction of alcoholic and nonalcoholic steatohepatitis (7). Cytokines have a critical part as mediators of injury, inflammation, fibrosis, and cirrhosis in NASH (8,9).The balance between pro-inflammatory and anti-inflammatory cytokines also seems to play a significant role in systemic, local metabolic, and inflammatory processes involved in the development of NAFL and NASH (1,10). Because cytokines play an etiologic role in liver diseases and are characterized by contradictory results, liver biopsy remains the standard gold test for the diagnosis of NASH; however, since it is invasive and not readily accepted by patients, non-invasive diagnosis by serum biomarkers are eagerly needed (11). The objective of this study was to evaluate the levels of plasma pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in NAFL and NASH compared to healthy individuals.

MATERIALS AND METHODS

Study Population. This case-control study was performed on 30 patients with NAFL, 30 patients with NASH and 30 healthy subjects, all recruited at the same time. The subjects of both case and control groups were matched with age, sex, and other demographic variables while none of the controls had a history of liver disease. All patients underwent liver sonography, and clinical examinations to ensure a definitive diagnosis of the disease. The inclusion criteria were no smoking or alcohol consumption of higher than 20 g/day; male patients aged 30 to 60 years, no history of any hepatic diseases such as hepatitis infections (B and C), liver cirrhosis and liver cancer, and no use of statins, anticonvulsants, isoniazid, rifampin and glucocorticoids, known to promote fatty liver disease. The body mass index (BMI) was calculated through dividing the weight by height squared (kg/m^2). Five ml of antecubital vein blood was collected from each subject after overnight fasting. Blood samples were centrifuged at 3000 rpm for 10 min, and plasma aliquots were stored at -20°C for detection.

Biochemical Assays. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), triglyceride (TG), and direct bilirubin (DB) were measured using relevant diagnostic kits (Pars Azmoon Inc., Tehran, Iran).

Cytokines Analysis. The plasma samples were employed for IL-1β, IL-6, and TNF-α measurements using a commercial ELISA kit (Karmania Pars Gene, Kerman, Iran) based on the manufacturer's instructions. The lower detection limit of the IL-1β and TNF-α assay was 2 pg/ml, and that of IL-6 was 3 pg/ml. Absorption was measured via an ELISA reader (BioTek; Winooski, Vermont, USA) at 450 nm.

Statistical Analysis. One-way analysis of variance was used to compare data expressed as the mean ± SEM. Associations between variables were calculated by Spearman correlation. p-value of 0.05 or less was considered as statistically significant.

RESULTS and DISCUSSION

All patients and healthy subjects were male. The mean ages of NAFL, NASH, and control subjects were 44 ± 1.84, 44 ± 2.34, and 38 ± 2.04 years, respectively. BMI levels showed significant differences between NASH and control group (p=0.001); however, NASH and NAFL cases were not significantly different in terms of BMI levels (p=0.181). The patients (NAFL and NASH) and the control group had differences regarding clinical and biochemical parameters. Nevertheless, significant differences existed between NASH and NAFL concerning ALT and AST levels (for ALT: 46.22 ± 5.50 in NASH Vs 28.84 ± 2.30 pg/ml in NAFL: p=0.004, for AST: 27.33 ± 1.62 in NASH Vs 21.15 ± 0.86 pg/ml in NAFL: p=0.015) and between NASH and healthy subjects regarding the level of ALT (46.22 ± 5.50 in NASH Vs 29.89 ± 2.27 pg/ml in control group: p=0.007), (Table 1).

Table 1. Plasma levels of biochemical parameters and demographic variables in patients and control groups.

Parameters	Control	NAFL	NASH
ALT (U/L)	29.89 ± 2.27	28.84 ± 2.30	46.22 ± 5.50[a,b]
AST (U/L)	24.73 ± 1.83	21.15 ± 0.86	27.33 ± 1.62[b]
ALP (U/L)	209.94 ± 19.93	207.63 ± 17.66	229.94 ± 22.59
DB (mg/dl)	0.31 ± 0.03	0.30 ± 0.03	0.33 ± 0.03
TG (mg/dl)	145.21 ± 16.59	147.57 ± 13.19	156.00 ± 15.11
BMI (kg/m^2)	24.59 ± 0.69	26.79 ± 0.65	28.81 ± 0.99[a]
Age (years)	38.66 ± 2.04	44.00 ± 1.84	44.00 ± 2.34

ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; DB: direct bilirubin; TG: triglyceride. Each value represents the mean ± SEM. NAFL: non-alcoholic fatty liver; NASH: non-alcoholic steatohepatitis. [a]Significantly different from control group, p-value ≤ 0.05. [b]Significantly different from NAFL group, p-value ≤ 0.05.

After analyzing the plasma cytokine levels in patients and controls, we found that IL-1β was significantly higher in NASH patients (5.01 ± 0.40 pg/ml) compared with NAFL (4.00 ± 0.16 pg/ml, p=0.004) and controls (3.73 ± 0.15 pg/ml, p=0.026). Furthermore, IL-6 level was significantly higher in NASH patients (4.64 ± 0.16 pg/ml) as compared to NAFL (4.19 ± 0.07 pg/ml, p=0.004) and control group (4.24 ± 0.05 pg/ml, p=0.013).

On the contrary, TNF-α levels had no significant variations in NAFL (6.69 ± 0.92 pg/ml) and NASH patients (6.04 ± 0.95 pg/ml) in comparison with the control group (5.63 ± 1.23 pg/ml) (p=0.903 and p=0.960). However, there was no significant difference between NAFL and control group regarding IL-1β, IL-6, and TNF-α levels (Table 2).

Table 2. Plasma levels of pro-inflammatory cytokines in patients and control groups.

Cytokines	Control	NAFL	NASH
IL-1β (pg/ml)	3.73 ± 0.15	4.00 ± 0.16	5.01 ± 0.40[a,b]
IL-6 (pg/ml)	4.24 ± 0.05	4.19 ± 0.07	4.64 ± 0.16[a,b]
TNF-α (pg/ml)	6.04 ± 0.95	6.69 ± 0.92	5.63 ± 1.23

IL-1β: Interleukin-1β; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α. Each value represents the mean ± SEM. NAFL: non-alcoholic fatty liver; NASH: non-alcoholic steatohepatitis. [a]Significantly different from control group, p-value ≤ 0.05. [b]Significantly different from NAFL group, p-value ≤ 0.05.

Further observed were statistically significant correlations between plasma TG and ALT activity, plasma TG, and BMI, as well as ALT activity and BMI. Moreover, positive correlations were observed between the plasma levels of inflammatory cytokines (IL-6, TNF-α, and IL-1β). However, our findings did not show significant correlations between the plasma levels of inflammatory cytokines (IL-6, TNF-α, and IL-1β) and ALT activity or TG plasma levels (Table 3).

Table 3. Statistical significant correlations between studied parameters.

Correlation Between	Correlation coefficient	p-value
TG plasma levels and ALT activity	0.276	**0.048**
TG plasma levels and BMI	0.377	**0.006**
TG and IL-6 plasma levels	-0.011	**NS***
TG and TNF-α plasma levels	-0.148	**NS***
TG and IL-1β plasma levels	-0.247	**NS***
BMI and ALT activity	0.562	**0.000**
ALT activity and IL-6 plasma level	0.131	**NS***
ALT activity and TNF-α plasma level	-0.222	**NS***
ALT activity and IL-1β plasma level	-0.151	**NS***
IL-6 and TNF-α plasma levels	0.356	**0.010**
IL-6 and IL-1β plasma levels	0.384	**0.040**
TNF-α and IL-1β plasma levels	0.495	**0.000**

*NS; Not significant

The pathogenesis of NAFL and NASH is highly convoluted (12). Pro-inflammatory cytokines play a pathophysiological role in the progression of NAFL and NASH through the induction of liver inflammation, apoptosis and fibrosis (13). Our findings showed that the levels of pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β

were not significantly different between NAFL and control subjects; however, IL-6 and IL-1β significantly increased in NASH patients as compared to the controls and NAFL subjects. According to the present results, NAFL is a condition without inflammation while NASH is a disorder with inflammation. The role of IL-6 in fatty liver disease is also very complex, and its involvement in the progression of NAFLD is yet to be clarified (13). Numerous studies have demonstrated that the levels of IL-6 are higher in patients and animal models with NAFLD as compared to normal subjects (7,14,15). Mas *et al.* showed that diet-induced NASH was reduced in IL-6 knockout mice compared with the controls (16). In humans with NASH, a positive correlation was observed between IL-6 expression in hepatocytes and the severity of NAFLD (12,13). Yamaguchi *et al.* illustrated the paradoxical role of IL-6 in NAFLD (17). Therefore, we cannot exclude the possibility that IL-6 might also play an indirect deleterious role in NAFLD and NASH pathogenesis; however, its exact role in the pathogenesis of NAFLD and NASH remains to be specified. Several studies have demonstrated that IL-1β, in many ways, contributes to the development of NAFLD (18-20). Kumar *et al.* reported that IL-1 plasma levels were significantly higher among NAFLD patients compared to other chronic liver diseases, with significantly high levels in the advanced stage of fibrosis (21). In a diet-induced model of steatosis/steatohepatitis, an increase was observed in the hepatic expression of IL-1α /β (12). TNF-α is a main factor in the progression of NAFL and NASH. Hotamisligil *et al.* reported a correlation between the expressions of TNF-α and insulin resistance in NASH subjects (22). Although the inhibition of TNF-α in NAFLD animal model is a therapeutic approach, the role of this cytokine in humans remains controversial. Moreover, a positive correlation was reported between TNF-α level and the degree of liver fibrosis in NASH patients (23). Zahran *et al.* showed that TNF-α was increased in NAFLD patients compared to the control group (24). In a cross-sectional study by Hui *et al.*, TNF-α levels were found to be significantly higher in NAFL patients compared to the controls; however, there existed no significant difference in terms of TNF-α levels between NAFL and NASH as diagnosed by liver biopsy (25). However, the role of TNF-α in NAFLD and insulin resistance is questionable. Lucero *et al.* did not observe any difference in the serum levels of TNF-α between NAFLD patients and healthy subjects (26). In another study, Musso *et al.* reported no significant differences concerning TNF-α serum levels among obese, nondiabetic NASH patients, and matched controls (27). Among all pro-inflammatory cytokines involved in the pathogenesis of NAFLD, TNF-α is the most commonly characterized by conflicting results. This is probably ascribed to the heterogeneity in study populations or various factors that might affect the plasma levels of TNF-α, sample sizes and factors that possibly interfere with plasma TNF-α level detection (1,28). The role of the TNF-α in NAFLD needs to be corroborated in future studies. In conclusion, pro-inflammatory cytokines did not differ between NAFL and control subjects, suggesting that the clinical data pertaining to the early stage of NAFLD (NAFL) are similar to healthy subjects; moreover, steatosis and inflammation develop in response to excessive pro-inflammatory factors in NASH patients. Furthermore, high plasma levels of IL-1β and IL-6 imply a systemic inflammation. Consequently, pro-inflammatory cytokines may induce an essential correlation between metabolic and liver disorders in fat accumulation and liver fibrosis.

ACKNOWLEDGEMENTS

The study was financially supported by Deputy of Research and Technology of Yasuj University of Medical Sciences (Grant No: 960161), Yasuj, Iran.

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