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Effects of Non-Alcoholic Fatty Liver Disease on Visfatin and IL-6 Levels in Mice: An Immunohistochemical Study

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Abstract

Non-alcoholic Fatty Liver Disease (NAFLD) includes a wide range of diseases from simple fatty liver to steatohepatitis, fibrosis and cirrhosis. Alcoholic fatty liver disease and its more serious form, NAFLD, are related mostly to obesity and insulin resistance. Adipokines, secreted form fatty tissues, increases with weight gain and body-mass index (BMI). Previous studies showed that most adipokines increased with liver steatosis but changes of visfatin and IL-6 levels are still controversial. We applied a high dose fructose diet to mice via adding 30% w/v fructose to the drinking water for 4-6 weeks. High levels of liver damage and NAFLD were observed within the experiment group. Visfatin and IL-6 levels were also evaluated using immunohistochemistry. We found high levels of liver damage in fructose group along with increased levels of biochemical liver damage markers. Both IL-6 and visfatin staining were observed higher in the fructose group.

Our study suggests that the IL-6 and visfatin levels were increased with obesity and liver damage. We aimed that this study will create an opportunity for new research with IL-6 and visfatin.

Keywords: Fructose; Interleukin-6; Visfatin; Non-alcoholic fatty liver disease; Immunohistochemistry

Abbreviations

AST: Aspartate Amino Transferase; BMI: Body-Mass Index; CRP: C-reactive Protein; DAB: Diaminobenzedine; DM: Diabetes Mellitus; ER: Endoplasmic Reticulum; FIAF: Fasting Induced Adipose Factor; FFA: Free Faty Acid; HDL: High Density Lipoprotein; H-E: Hemotoxylin-Eozin; HNE: Hydroxynonenal; ICAM-1: Intracellular Cell Adhesion Molecule 1; IL-6: Interleukin-6; INF-γ: Interferon gamma; LDL: Light Density Lipoprotein; MDA: Malondialdehyde; NAFLD: Nonalcoholic Fatty Liver Disease; NASH: Nonalcoholic Steatohepatit; NAMPT: Nicotinamide Phosphotransferase; PBS: Phosphate Buffer Solution; PNL: Polymorph Leukocyte Nuclei.

Introduction

Alcoholic (ASH) and non-alcoholic steatohepatitis (NASH) are chronic liver diseases that are significantly increasing day by day [1]. ASH affects millions of patients worldwide and it is one of the major causes of death in developed countries. The progression of ASH is characterized by steatosis (fatty infiltration), inflammation, necrosis, and finally fibrosis and cirrhosis; when severe hepatitis occurs, death is a common outcome [1,2]. NAFLD is not necessarily a disease, since it may be reversed by physical exercise, food restriction and body weight reduction. NAFLD is the hepatic manifestation of the metabolic syndrome and is closely associated with visceral obesity and insulin resistance [3,4]. Obesity is currently regarded as a systemic, low-grade inflammation, in which adipose tissue and its hormones have a central role [5,6].

Fructose is a monosaccharide which is commonly used as a sweetener, e.g., Industrially, it is frequently found in soft drinks and pre-packaged foods. A correlation is observed between dietary fructose intake and the prevalence of metabolic syndrome and fatty liver [7]. Studies have suggested a link between the consumption of sugar-sweetened beverages and dental caries, weight gain, type 2 diabetes, dyslipidemia, and nonalcoholic fatty liver disease [8,9]. There is a growing body of evidence linking a Western diet (high in saturated fat, trans-saturated fatty acids (trans-fat) and table sugar) with the increasing incidence of NASH [10].

Hypertrophic adipocytes release chemokines, which recruit macrophages and a vicious cycle commences, as macrophages release inflammatory cytokines, which stimulate inflammatory and suppress anti-inflammatory adipokines [7,11].

Fatty liver diseases increased in countries with increased fast food consumption. NAFLD were related mostly to diabetes, dyslipidemia and insulin resistance [2]. Insulin resistance was recently described as a metabolic syndrome by World Health Organization, as a mixture of five different risk factors including central obesity, high blood pressure, hypertriglyceridemia, low HDL and hyperglycemia. These risk factors are easily evaluated in clinical practice and used in epidemiological studies [10,12].

Visfatin also known as the main enzyme of nicotinamide adenine dinucleotide, nicotinamide phosphotransferase (NAMPT) or pre-B cell colony enhancing factor (PBEF), was first found within inflammatory cells [13]. Visfatin is secreted mostly from vascular adipose tissues and

acts like insulin on insulin sensitive organs such as liver, muscle and adipose tissues [14,15]. Visfatin has a protective effect against insulin resistance and is very helpful in treatment of diabetes. Visfatin binds insulin receptor thereby decreases glucose secretion from hepatocytes and promotes glucose consumption from peripheral tissues [14,16].

IL-6, secreted from vascular adipose tissues and endothelial cells, has more complex structures than other adipokines. IL-6 levels increase with obesity and are related to coronary artery diseases and atherosclerosis [17]. IL-6 increases insulin resistance and endothelial adhesion molecules and also regulates triglycerides (TG) secretion and procoagulant substances. IL-6 protects fatty liver via preventing mitochondrial function disorders and suppressing oxidative stress [16]. Other roles of IL-6 such as apoptosis induction are in contrast with the liver protective roles of IL-6. Long term treatment of IL-6 slowed liver regeneration despite short-term beneficial effects being observed in an animal study. These data indicate that the roles of visfatin and IL-6 are not clarified on the NAFLD [18]. Hence, the main aim of the current study is to evaluate the changes of these two adipokines with an animal model by creating NAFLD with high doses of fructose consumption [19,20].

Materials and Methods

Animals

Twenty-eight male Swiss albino mice weighting between 30-35 g were used for the study. This study has been approved by Süleyman Demirel University Animal Experiments and Local Ethical Committee on January 2010 (2010-1/029). All animals were kept in 12 h light/12 h dark cycles and fed with limitless food but had limited access to water.

Experimental procedure

Mice were randomly divided into two groups, one of as a healthy control group consisting of seven mice and remaining 21 animals were experiment groups and experiment groups divided into three other groups (4,5,6 week)

Group I (Control, n=7) fed with normal tap water.

Group II (4-week, n=7) fed with tap water containing 30% (w/v) fructose, (Fluka, Neu-Ulm, Germany).

Group III (5-week, n=7) fed with tap water containing 30% (w/v) fructose.

Group IV (6-week, n=7) fed with tap water containing 30% (w/v) fructose.

The rats were provided unlimited access (ad libitum) to water and food (Animal Food Institution Standard Rat Chow). Group I fed with normal tap water, but group II, III, IV fed with tap water containing 30% (w/v) fructose [20]. Normal tap water without fructose was given to the animals three times a week for half an hour in order to help them handle over-thirstiness. Experiment group animals were sacrificed at the end of the fourth, fifth and sixth weeks as seven animals in each week under anesthesia with ketamine-xylazine (10% Alphamine and 2% Alfazyne, Alfasan IBV, Woerden, Netherlands). Blood, fat (abdominal) and liver tissues were taken for biochemical and histological analyses.

Histological procedure

Liver and fat tissues fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) in phosphate buffered saline (Sigma-Aldrich, Munchen, Germany) were washed for 48 h, dehydrated and cleared in xylenes. Tissues were embedded in paraffin and were cut in 4-5 μm sections. Consecutive four of every twenty sections were taken from paraffin blocks. One of the sections was stained with hematoxylin-eosin (H-E) (Merck) and the other three were stained immunohistochemically.

Immunohistochemical analysis was performed using the streptavidin-peroxidase method. Slides, which are reserved for immunohistochemistry were rehydrated, quenched in 3% H_2O_2 (Thermo Scientific, Fremont, CA) and heat activated antigen retrieval was performed in Tris- ethylenediaminetetraacetic acid (EDTA) buffer (Merck) at pH 9.0. Non-specific binding was eliminated via Ultra V Block (Thermo). First of the sections was stained with mouse monoclonal visfatin antibody (Santa Cruz Biotechnology, Santa Cruz, CA.), second section stained with IL-6 antibody (Abcam, Cambridge, MA.) and last section stained with secondary antibody as control. Tissues were incubated with primary antibodies at +4°C overnight in a humidified chamber. anti-polyvalent biotinylated antibody (Thermo Scientific, Fremont, CA) was used as secondary antibody. 3.3'-Diaminobenzidine (DAB) staining was performed with Ultra Vision Plus Large Volume Detection System (Thermo Scientific, Fremont, CA) and slides counterstained with hematoxylin. Slides were examined under Olympus BX51 light microscope (Olympus Optical Co., Ltd., Tokyo, Japan). H-E stained liver sections scored 1 (none) to 5 (maximal) according to the method of Abdel-Wahhab for apoptotic changes, edema, cellular disorganization, vacuolization and vascular congestion and hemorragia. Immunohistochemical slides analyzed semi-quantitatively and staining density was scored 1 to 3 in visfatin and IL-6 stained sections [21].

Biochemical analyses

Rat blood samples were collected in biochemistry tubes without preservatives, and they were centrifuged at 4000 rpm for 10 min (Hettich, Germany). The levels aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (T.COL) and glucose were measured photometrically in a biochemistry autoanalyser (Mindray BS 800 M, China).

Statistical analysis

SPSS 15.0 software (SPSS, Chicago, IL) for Windows was used for statistical analysis of the data. The data were presented as means ± standard error of mean (SEM). Kruskal-Wallis test used for semi-quantitative evaluations and Mann-Whitney U tests were performed for group comparisons. P values of less than 0.05 were considered as statistically significant.

Results

Animal weight and behavior

Animals consumed about 500 ml water per day per seven animals in all groups. After second week, experiment animals started to refuse fructose containing water. In order to handle increased thirstiness, normal tap water was given to the animals for 30 minutes for twice a week. Food consumption was also decreased along with a decrease of

movements within the cage although they were not measured. Animals were weighted weekly and weight gain evaluated. Surprisingly, animals in the experiment groups didn't gain weight and some animals even lost weight in spite of the high glucose diet.

Histological evaluation

Normal histological structures were observed in the liver tissues of control group animals. After fourth week, increased amounts of macro and micro vesicular steatosis, hemorrhage, mononuclear cell infiltrations, necrotic and apoptotic cell residues with picnotic nuclei and granular degenerations were observed within the liver tissues of experiment group animals ($P<0.05$). There was a slight increase of hepatocellular damage among second, third and fourth groups ($P<0.05$) although there was significant difference between experimental groups and control group tissues (Figure 1).

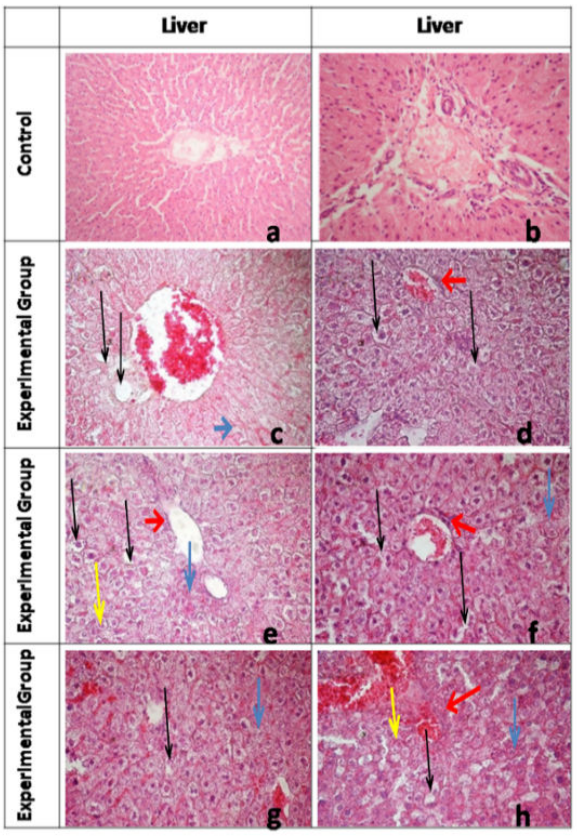

Figure 1: Effect of fructose (30% w/v) on liver tissue. Histopathological sections from control group showed near normal liver histology (a-b, H-E, × 10 and × 20). Whereas, sections from the experimental groups (4, 5, 6 weeks, respectively) showed extensive macro and micro vesicular steatosis (black arrow) and hepatic injury, such as inflammatory cell infiltration (red arrow), pycnotic nucleus (yellow arrow), granular degeneration (blue arrow) (c-d, H-E, × 10 and × 20).

Immunohistochemical results

Immunohistochemical results show significant difference between experiment and control tissues (Figure 2). A semi-quantitative evaluation revealed that visfatin and IL - 6 staining in the adipose tissues was seen dense (a-b), liver tissue staining of the control rats, was either very slight or nonexistent (c-d). However, the staining in the liver tissues of experimental groups was dense (e, f, g, h).

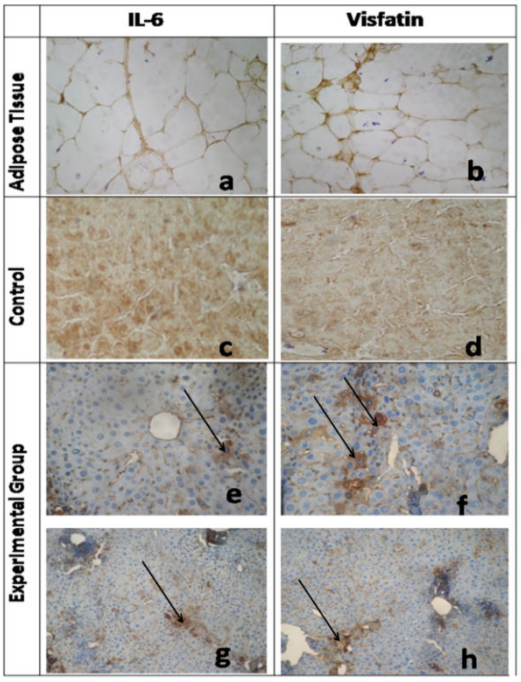

Figure 2: Effect of fructose (30% w/v) on liver tissue. Immunohistological sections from control group showed less staining intensity (c, IL-6 immunstaining and d, visfatin immunstaining, × 10-40). Conversely, sections from the experimental group showed intense staining (black arrow) (e-g IL-6 immunstaining and f-h visfatin immunstaining, × 10).

Biochemical findings

The analyses of the biochemical parameter revealed an elevation of the serum in control and experimental groups, indicating significant increases especially in T.COL and glucose levels respectively (125.14 ± 18.62, 278.71 ± 50.52 mg/dL, Table 1).

High-fructose supplemented in drinking water clearly increased the serum AST, ALT and ALP levels increased in the experimental group compared to the control group respectively (82.28 ± 37.31 U/L, 45.00 ± 47.51 U/L, 51.42 ± 10.50 U/L, Table 1).

	Control	4 Weeks	5 Weeks	6 Weeks
Glucose (mg/dL)	187.6 ± 40.04	278.71 ± 50.82[a]	319.14 ± 61.70[a]	271.42 ± 49.23[a]
AST (U/L)	72.25 ± 3.37	68.14 ± 9.58[a]	82.28 ± 37.31[a]	88.28 ± 40.75[a]
ALT (µ/L)	31.75 ± 8.59	20.71 ± 1.70	45.00 ± 7.51[a]	31.14 ± 4.50[a]
ALP (U/L)	33.62 ± 13.52	51.42 ± 10.50[a]	63.28 ± 11.84[a]	68.28 ± 13.88[a]
T.COL (mg/dL)	91.62 ± 7.55	125.14 ± 18.62[a]	107.85 ± 20.79[a]	113.85 ± 15.39[a]

Table 1: Biochemical results and P values between groups based on Mann-Whitney U Test. Values are expressed as mean ± standard deviation (SD) ($P<0.05$), [a]$p<0.05$.

Discussion

Non-alcoholic fatty liver disease is a disease group which is intensely characterized with macro vesicular steatosis in the liver regardless of the routine alcohol use [1,22]. Non-alcoholic fatty liver disease (NAFLD) is the most common factor associated with liver damage [20]. Following that a relation between fatty liver and alcoholic cirrhosis became apparent, data on alcohol and fatty liver started to be collected but it was considered until very lately that there was no significant pathology except for alcoholic cases. Then, it was reported that this pathology could develop in a group of cases which don't use alcohol [23-25].

Previous studies have shown that chronic fructose consumption induced leptin resistance before body weight, adiposity, serum leptin, insulin and glucose increase High-fructose corn syrup-induced NAFLD and hepatic steatosis is linked with insulin resistance [9]. The effect of fructose is different from other carbohydrates because fructose does not stimulate insulin secretion from pancreatic β-cells. Studies using fructose or high-fructose corn syrup have demonstrated that high fructose consumption causes inflammation, leptin resistance, steatosis and decreased catabolism of fatty acids [10,26].

Visfatin is an adipokine which is particularly synthesized from vascular adipose tissue and becomes active by cleaving to the insulin receptor. It is known that visfatin shows insulin like impact in insulin sensitive tissues and has a protective characteristic against insulin resistance [27,28].

Visfatin is still considered as an adipokine in some sources. Visfatin increases in the human blood values in accordance with the body mass increase and it is known that it doesn't directly increase with the visceral adipose tissue increase [18]. It is also stated that visfatin increasing in specifically the patients with metabolic syndromes has a role in inflammation according to many sources [29].

Plasma visfatin values of human and experimental animal models are closely related to weight gain. Visfatin release increases in fat animal models and it also increases in humans with plasma concentrations in people with abdominal obesity or type II DM [30]. Visfatin connecting to the insulin receptor distant from the insulin decreases the release of glucose from hepatocytes and creates a hypoglycemic impact by promoting the use of glucose in nearby tissues [27,31]. Therefore, this molecule is very beneficial in treatment of diabetes patients. Comparing the control groups and experiment groups (fat mice groups) in the studies carried out, it was observed that the plasma visfatin value is higher in fat mice. Although the main reason underlying the increase in plasma visfatin value isn't certainly known, it is attributed to the endothelial functional disorder in type II DM patients [28,32].

Some studies showed no meaningful relationship between the serum levels of visfatin and liver fibrosis severity [19]. However, in a same study designed for measuring visfatin levels in the adipose tissue of NAFLD patients, visfatin levels were high in the non-NAFLD group and reduced in the mild steatosis, moderate steatosis, and non-alcoholic steatohepatitis groups, respectively. Reduction in mild steatosis demonstrates a reverse relationship between visfatin levels and the incidence of mild steatosis in non-alcoholic fatty liver. But in another studies, it was found that visfatin plasma levels can predict the presence of portal inflammation in NAFLD [19]. In our study we found correlation between visfatin, IL-6 and NAFLD with consumption fructose and also our results were supported by other studies [2,4,22,28].

Interleukin-6 has a protective effect in fatty liver; it performs this preservative impact by means of preventing the disorder of mitochondria and suppressing the oxidative stress [17]. At the same time, IL-6 protects the liver in ischemia conditions. This condition is generally critical for survival of mice the livers of which were removed. Besides the protective impact of interleukin-6, it has impacts such as triggering the liver damage and apoptosis related cell death [20]. This contradictory situation shows that the increase in the amount of IL-6 in the chronic liver damage increases the inflammation and creates an inflammation inhibiting response in acute liver damage. In other words, IL-6 has contradictory functions based on its long and short-term impact mechanism [9,12,33-35]. Although IL-6 levels have increased in some studies, in some studies it was not found definite result [10]. But in our study IL-6 levels increased in NAFLD which was formed with consumption of fructose.

We did plan in our study to assess the impacts of visfatin and IL-6 released from the adipose tissue histochemically and immunohistochemically in consideration to the above-mentioned conditions by means of creating a non-alcoholic fatty liver by using dietary fructose.

In our study, simple steatosis was observed in mice fed with fructose since the fourth week, steatosis even slightly increased in time and it caused liver damage in some groups. Although histologically liver steatosis was observed in the mice of our experiment group, there was no significant increase with regards to weights, while some mice gained weight others lost weight to the end of the experiment. We considered the cause of it as the difference between the amount of water the mice drank, and also thought that the consumption of fructose included water could result in loss of appetite in mice.

However, in previous studies suggested that the amount of adipokines might fluctuate during the course of NAFLD [19].

Based on the immunohistochemistry study, with regards to the evaluations made according to the staining differences, there were staining differences between control and experiment groups for visfatin and IL-6 and immunohistochemical studies [19,20]. Some other study on non-alcoholic fatty liver disease; rat groups with simple steatosis, control group, obese mice and experiment groups with non-alcoholic steatohepatitis were compared with regards to their adipokine amounts. Serum visfatin values were higher in obese mice compared to the control group, however it was interesting that lower rates of visfatin were observed in the non-alcoholic steatohepatitis group as a result of the comparison between non-alcoholic steatohepatitis group, mice with simple steatohepatitis and control group. Comparing the interleukin-6 value, more IL-6 was confirmed in obese mice compared to the control group and the non-alcoholic steatohepatitis group. It was considered that the visfatin value changes in different groups and lower levels of it in non-alcoholic steatohepatitis mice were due to the changing IL-6 values and visfatin takes a role in the occurrence of non-alcoholic fatty liver disease. Because, it is known that there are positive and negative feedback cycles between IL-6 and visfatin, while visfatin promotes the IL-6 production in human CD14 monocytes, IL-6, on the other hand, regulates the visfatin gene expression in 3T3-L1 adipocytes in a negative way [20,27,35].

According to the results of our study, significant differences were found between the control and experiment group as regards to AST, ALT, ALP, T cholesterol and glucose amounts based on the biochemical analyses ($P<0.05$), because of fatty liver. Our results were supported by some studies [10,12,34].

It was preferred in our experiment to use the mice instead of rats in order to create a non-alcoholic liver steatosis because the other studies, which were carried out, showed that the steatosis took longer in rats [36]. The reason we made immunohistochemical studies on liver tissue instead of plasma while monitoring the visfatin and IL-6 levels is because we didn't have the chance to take enough blood from the mice. Therefore, we formed non-alcoholic fatty liver disease on the mouse and examined the visfatin and IL-6 rates. We were able to develop non-alcoholic fatty liver on mice within 6 weeks during our experiment. As a result of our immunohistochemical studies, it was observed that visfatin and IL-6 increased in the experiment group compared to the control group.

It is known that adipokines take a role in occurrence of non-alcoholic fatty liver disease and the adipokine amount increases in line with the steatosis increase [16,37]. Though this parallel increase is known in many adipokines, the increase in visfatin and IL-6 adipokines hasn't become definite yet. In many of the studies carried out, the adipokine amounts in the plasma were examined [17]. We observed in this study the change in the adipokine amount as immunohistochemical in the liver tissues and found that adipokine amounts increase in connection with the NAFLD. In this regard, we believe that the experiment will shed light on the next studies.

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