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RESEARCH ARTICLE

Association between Obesity Measures, Inflammatory, Oxidative Stress and Neuronal Injury Markers in Obese Women with Non-Alcoholic Fatty Liver

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Abstract

The present study aimed to investigate the association of Nuclear Factor (NF)-κB, oxidized –low density lipoprotein (o-LDL), total antioxidant capacity (TAC), neuron-specific enolase (NSE) and CD4 in obese women with non-alcoholic fatty liver (NAFLD). A total of 36 NAFLD obese women were enrolled in this study and 36 female control healthy women (aged 30-39) without cognitive impairment were examined. NAFLD patients were diagnosed by magnetic resonance elastography. Anthropometric parameters including body height, weight, waist circumference (WC) and body fat % were measured. Serum NF-Kb, TAC, CD4, o- LDL and NSE were measured by ELISA. Also, liver function tests, lipid profile were done. The present results showed marked elevation in serum NF-κB and o-LDL, while significant reduction in TAC and CD4 in obese NFALD than normal control subjects. In conclusion results provide evidence of association between obesity, high levels of oxidative stress, inflammatory markers and brain biomarker in women with NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a significant health problem and the most common form of chronic liver disease worldwide [1]. The prevalence of NAFLD parallels the steady increases in the rates of obesity, and consumption of saturated fat is positively associated with the risk of NAFLD.

Clinico-pathologically, NAFLD comprises a wide spectrum of liver damage ranging from bland steatosis (NAFL) to non-alcoholic steatohepatitis(NASH), fibrosis and ultimately cirrhosis [2]. Bland steatosis is benign whereas NASH is characterized by hepatocyte injury, TNF α-mediated inflammation, and a high risk of liver-related morbidity and mortality [3]. The pathogenesis of NAFLD is not fully understood, and the factors that contribute to disease progression from bland steatosis to NASH remain enigmatic.

Epidemiological and human observational studies do provide indications that progressive NAFLD is strongly associated with white adipose tissue (WAT) inflammation, insulin resistance and elevated circulating levels of inflammatory mediators including certain adipokines and lipids [4].

Furthermore, longitudinal rodent studies demonstrated that high-fat diet(HFD)-induced expression of inflammatory genes in WAT precedes the development of NASH in disease models with obesity, suggesting a potential role of inflamed WAT in NAFLD progression [5]. Also, the severity of the

NAFLD pathology appears to be closely linked to WAT dysfunction, that is, hypertrophy of adipocytes combined with macrophage infiltration, formation of crown-like structures (CLS) and enhanced expression of inflammatory genes [6].

Hence it has been postulated that obesity-induced inflammation in WAT is critical for the development of NAFLD, but experimental evidence for an involvement of WAT is still lacking. WAT is a complex endocrine organ that is composed of different depots, among which are the intra-abdominal (e.g., epididymal and mesenteric) and subcutaneous (e.g., inguinal) WAT depots [4].

These depots are thought to have different roles in energy storage and inflammation [7] and may thus have different contributions to the pathogenesis of NAFLD. The temporal development of inflammation (i.e., CLS formation) in WAT depots has not been systematically investigated and it is not known whether a particular depot is more prone to become inflamed during HFD-induced obesity and associated NAFLD.

Adipose tissue is a highly metabolic active organ. Hence, it might be reasonable to assume that abdominal fat is closely involved in the production of oxidative stress, which in turn responsible for LDL oxidation which is thought to play a crucial role in the generation of atherosclerotic lesions [8]. The progression of obese /and or diabetic atherosclerosis entails complex interactions between the modified low-density lipoproteins (LDL) and the cells of the arterial wall. LDL and intermediate density lipoprotein (IDL) induced the mRNA expression of MCP-1 in cultured human endothelial cells, possibly through the activation of NFκB pathway.

The transcription factor NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells) promotes immunity by on trolling the expression of genes involved in inflammation. Cytokines and pathogen-associated molecular patterns (PAMPs) stimulate cell surface receptors including toll-like receptors (TLRs) to initiate a signaling cascade resulting in the activation of NF-κB. NF-κB drives expression of target genes that mediate cell proliferation and release of antimicrobial molecules and cytokines to activate the immune response[9]. Indeed, a number of recent reports have demonstrated a key role for the NF-κB signal pathway in the liver, adipose tissue, and central nervous system in the development of inflammation-associated metabolic diseases. In addition to their role in insulin resistance in both liver and adipose tissue, proinflammatory pathways regulated by NF-κB also contribute to vascular disease associated with metabolic excess. Oxidized lipoproteins trigger secretion of chemokines MIP-1α and MCP-1 by vascular endothelia, recruiting leukocytes to the site of inflammation [10].

The detection of NF-κB in nuclei of macrophages in atherosclerotic lesions [11] suggests that NF-κB activation is associated with atherogenesis. Additionally T-lymphocytes may collaborate with monocytic leukocytes to regulate insulin responsiveness during HFD-induced obesity. Lymphocytes regulate macrophage differentiation and activation by producing IL-6 and TNF-α. Obese adipose tissue is enriched in CD8 cytotoxic T lymphocytes and TH1-type helper CD4 T-cells, and depleted in CD4+CD25+ regulatory T-cells[12].

IFNγ-producing TH1 CD4 helper T-cells infiltrate adipose tissue of mice on a high-fat diet, suggesting that T cells mediate inflammation resulting in obesity and metabolic disease. In NAFLD and atherosclerotic heart, total Antioxidant Capacity which considers the cumulative action of all the antioxidants present in plasma and body fluids [13]. Previous studies have shown that obesity and central fat accumulation are associated with chronic inflammatory state and increased oxidative stress (OS). HFD induced obesity has been discussed as a risk factor for neurodegenerative disorders, such as Alzheimer's disease, a disorder characterized by learning and memory deficits [14].

Neuro imaging studies have identified regional cerebral atrophy paralleling high body mass index (BMI) using computed tomography7 and magnetic resonance imaging (MRI). However, the mechanisms underlying this apparent alteration in brain structure of obese subjects are yet unclear. In order to clarify whether obesity-associated brain atrophy could be explained by a putative brain injury, we include measurements of neuron-specific enolase

(NSE) serum levels. NSE is primarily localized in the cytoplasm of neurons and not secreted. Thus, this enzyme can be used as reliable marker for structural neuronal damage and increased serum NSE concentrations have been observed in patients suffering from traumatic brain injury and neurodegenerative disease. In the present study, w ehypothesize a higher BMI to be associated with increased NSE serum levels) [15]. The present study, we aimed to study ;obesity -evoked changes in circulating ox-LDL, TAC, CD4, NF-κB and NSE in NFLD obese women compared to normal control one.

Subjects and Methods

Diagnosis of Patients with Nonalcoholic Fatty Liver

A total of 36 NAFLD obese women were enrolled in the study and 36 control normal weight healthy women in matched age (aged 30-39) without cognitive impairment. This study was conducted between February 2017 and March 2018 in the National Research Centre, Egypt; Medical Research Centre of Excellence. The study was approved by the Ethical Committee form of National Research Centre, Egypt (number = 16361), in accordance with the World Medical Association's Declaration of Helsinki

Methods

Because nonalcoholic fatty liver disease causes no symptoms in most cases, so we take medical attention when tests done for other reasons point to a liver problem. This can happen if the patient's liver looks unusual on ultrasound or if it has an abnormal liver enzyme test. Tests done to pinpoint the diagnosis and determine disease severity include: Blood tests included, complete blood count, liver enzyme and liver function tests, tests for chronic viral hepatitis (hepatitis A, hepatitis C and others), celiac disease screening test, fasting blood sugar, hemoglobin A1 c, and lipid profile.

Demographic data: age, gender; Biochemical data: Fasting glucose (mg/dl), total cholesterol (mg/dl), triglycerides(mg/dl), High density lipoprotein (HDL-cholesterol) (mg/dl), Low density lipoprotein (LDL-cholesterol) (mg/dl), and anthropometric data: weight (kg), Body Mass Index (BMI) (kg/m2), weight gain (kg) and weight loss (kg).

Data were obtained from structured clinical interviews and questionnaires. Weight was measured without shoes and outdoor clothes to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm, again without shoes and with participants standing upright with their back to the stadiometer. Participants were excluded if, they were receiving hormone replacement therapy or had received it within the last year before the start of the study or had drugs for cancer, cardiovascular disease, diabetes mellitus and other endocrinal disorders, bronchial asthma, acute or chronic inflammatory diseases, autoimmune diseases and rheumatic diseases (n=17).

All participants signed informed written consent after the explanation of the study procedure. All procedures in this study were conducted in accordance with the guidelines of NRC. Body mass index (BMI) was calculated as weight divided by height squared (kg/m2); overweight was defined as BMI between 25 and 30, and obesity asBMI≥30 (19). Blood pressure was calculated. Participants were seated for 5 minutes before the measurements were made, and blood pressure readings were made at 1-minute intervals (Table 1).

Imaging Procedures

Imaging procedures used to diagnose patients with nonalcoholic fatty liver disease include: Transient elastography, an enhanced form of ultrasound that measures the stiffness of patient's liver. Liver stiffness indicates fibrosis or scarring and Magnetic resonance elastography, which combines magnetic resonance imaging with patterns formed by sound waves bouncing off the liver to create a visual map showing gradients of stiffness throughout the liver reflecting fibrosis or scarring. Medical records were used to gather the following information:

Laboratory Measurements

Venous blood was obtained in the morning after the subjects had fasted for 12 h and was kept frozen at 40 °C until assayed. The concentration of ox-LDL in serum a was measured with a sandwich enzyme-linked immunosorbent assay procedure by using the murine monoclonal antibody mAb-4E6 as capture antibody (bound to micro titration wells) and a peroxidase conjugated

antiapolipoprotein B antibody recognizing oxidized LDL ox-LDL bound to the solid phase (Sigma chemical company, USA).

Anthropometric measurements were obtained according to standardized equipment and following the recommendations of the International Biological Program [16]. Body fat % was assessed by Tanita Body Composition Analyzer (SC-330). Antiapolipoprotein B antibody recognizing ox-LDL bound to the solid phase (Sigma chemical company, USA). For TAC, 5 mL of blood sample were taken from the vein, in fasting state. Blood samples were centrifuged at 4oC for 10 min at 3000 rpm to obtain serum.

The serum samples were stored at -80oC until analysis. The samples were thawed at room temperature only once at the time of assay. Serum total antioxidant capacity was measured by quantitative colorimetric assay, kit (Biodiagnostic, Cairo, Egypt). By this method Cu2+ is reduced by antioxidant to Cu+. The resulting Cu+ specifically forms a colored complex with a dye reagent. The color intensity at 570 nm is proportional to TAC in the sample. Serum total antioxidant capacity is expressed in mMTrolox equivalents.

Referral TAC values using this kit is within the linear range of 1.5-1000 mMTrolox equivalents [17] . Serum NSE levels were determined by monoclonal 2-site immunoassay assays[15]. ELISA for active NF-kB : nuclear extracts were prepared from 5x105monocytes, pretreated and not with DHMEQ (10µg/mL) for 2 h and after which stimulated with 100 ng/ml LPS for the desired period of time, using a Nuclear Extract Kit (Sigma Chemical Co, USA) according to the manufacturer's protocol. Levels of nuclear p65 concentrations were determined by a sensitive ELISA assay (Sigma Chemical Co, USA) [18]. Determination of CD4 marker was performed in serum using ELISA technique [19].

Statistical Analysis

All data are presented as mean ± SD. Significance of differences of continuous variables between obese and controls were analyzed using Student's t-test. Results were considered statistically significant at P≤0.05.

Results

Data in Table 1 shows comparison of clinical and biochemical characteristics between obese women with NAFLD and controls. Significant increase of obesity measures, fasting glucose, serum lipid and liver enzyme levels (AST and ALT) as compared to controls. Table 2 shows significant increase in the serum NF-κB, O-LDL and neuromarker NSE compared to control subjects. However, CD4 and TAC showed marked reduction in NAFLD obese subjects compared to normal one.

Table 1: Characteristics of obese women with NAFLD and controls

Characteristics	NAFLD	Controls
Age (year)	32.4 ± 4.7	31.4 ± 4.5
BMI(Kg/m2)	35. 25 ± 4.80**	22.21 ± 3.2
WC (cm)	93.89±10.00*	81.7 ± 4.2
Body fat %	39.7 ± 5.87*	26.5 ± 3.4
Fasting glucose (mg/dl)	100.90 ± 6.10*	81.5 ± 10.6
Total Cholesterol (mg/dl)	190.0 ± 10.0*	118.9 ± 21.1
Triglycerides (mg/dl)	157.0 ± 8.0*	104.2 ± 12.8
HDL-cholesterol (mg/dl)	35.10 ± 3.9*	49.84 ±13.7
LDL-cholesterol (mg/dl)	109.2 ± 9.43*	114 ± 9.7
AST (U/L)	56.90 ± 4.87**	25.5 ± 7.2
ALT(U/L)	70.00 ± 4.98**	23.4 ± 6.9

*p <0.05, ** p<.001 vs. controls; Data are presented as mean ±SD; BMI, body mass index; WC, waist circumference.

Table 2: Different biomarkers level in serum of NAFLD and controls

Markers	NAFLD	Controls
NF-κB (ng/ml)	76.00±4.00**	31.00±1.98
O-LDL (mg/dl)	17.98±1.90**	4.3±0.87
NSE ug/ l	15.63± 0.22**	5.51± 0.01
CD4 U/ ml	16.9±0.54*	28.80± 0.55
TAC mmol/L	265.0±16.03**	383.63±12.11

TAC: Total antioxidant capacity; NSE: neuron-specific enolase; O-LDL: oxidized LDL

Discussion

The present study showed significant increase of serum NF-κB levels in NAFLD compared to control subjects. This may be discusses based on the transcription factor NF-κB promotes immunity by controlling the expression of genes involved in inflammation. Cytokines and pathogen-associated molecular patterns (PAMPs) stimulate cell surface receptors including toll-like receptors (TLRs) to initiate a signaling cascade resulting in the activation of NF-κB. NF-κB drives expression of target genes that mediate cell proliferation and release of antimicrobial molecules and cytokines to activate the immune response [10].

Although NF-κB was first characterized in cells of the hematopoietic system, subsequent research has revealed NF-κB activation can occur in most cell types. Indeed, a number of recent high profile reports have demonstrated a key role for the NF-κB signaling pathway in the liver, adipose tissue, and central nervous system in the development of inflammation-associated metabolic diseases.

The potential mechanism, the possible ligation of Toll-like receptors on adipocytes or macrophages by dietary lipids could exploit the canonical inflammatory signaling pathway to activate NF-κB and produce inflammatory mediators**.**

The non-canonical IKK kinase IKKε was shown to be required for high fat diet [20]. IKKε is not expressed in most resting cells, but is transcriptionally induced by NF-κB downstream of inflammatory stimuli. IKKε contributes to the late phase of the NF-κB transcriptional activity but primarily plays an important role in interferon signaling, and is therefore required to combat certain viral infections [21].

IKKε expression is dramatically upregulated in response to nutrient excess, up to 40-fold in adipocytes and fat-infiltrating macrophages[20] . IKKε deficiency uncoupled obesity from high fat diet by increasing energy usage, oxygen respiration, and thermogenesis. NF-κB activation by over nutrition also results from detection of extracellular inflammatory triggers by classical inflammatory pathways. TLR4 deficiency uncouples lipid excess and high fat diet from inflammatory signaling in adipocytes, insulin resistance in the muscle and glucose intolerance [22].

A loss of function mutation in TLR4 protects against insulin resistance in adipose tissue as well as diet-induced obesity [23]. NAFLD obese cases showed also, high serum levels of O-LDL which is discussed as an independent risk factor for atherosclerosis because of its associations with oxidative stress and inflammation[24]. The marker ox-LDL was strongly associated with metabolic syndrome (MS) and its components independently of central obesity and insulin resistance.

The variation in ox-LDL, may presumably cause by other factors than central obesity, which is its association with changes in metabolic parameters. Thus, we suggest that ox-LDL could be a useful early predictive marker of cardiometabolic abnormalities before the appearance of insulin resistance. In particular, the accumulation of abdominal fat, which can be indirectly measured through waist circumference (WC), is an important coronary artery disease (CAD) risk factor.

This is due to its association with a series of metabolic disorders such as diabetes mellitus, hypertension, and dyslipidemia[24].

Oxidation of LDL is a hallmark of atherosclerosis development[25]. Patients with manifest CAD have elevated plasma concentration of circulating oxidized LDL (ox-LDL) ,which are associated with the severity of symptoms and degree of CAD[26]. Circulating ox-LDL has been shown to be a prognostic marker of CAD in cardiac transplant patients. The few studies that used circulating ox-LDL to investigate oxidative stress induced by obesity have shown decreased circulating ox-LDL concentrations in morbidly obese patients after weight loss following surgery. However, the results concerning the correlations between BMI and ox-LDL are controversial [27].

It has been shown that obesity increases oxidative stress [28]. Adipose tissue is a highly metabolic active organ. Hence, it might be reasonable to assume that abdominal fat is closely involved in the production of oxidative stress. However, data on the association of abdominal fat and oxidative stress are scarce. The hypothesis of the present study was that abdominal fat, measured indirectly through WC, is independently related tocirculating ox-LDL and inflammatory markers as well as in the Coronary Artery Risk Development in young adults study (CARDIA), a higher ox-LDL was associated with an increased incidence of MS [29].

We investigated in the present study serum levels of neuron specific enolase (NSE), a marker for neuronal injury, in a set of NAFLD –overweight/obese subjects and controls. There is growing evidence that obesity represents a risk for enhanced gray matter (GM) density changes comparable to those demonstrated for mild cognitive impairment in the elderly. However, it is not clear what mechanisms underlie this apparent alteration in brain structure of overweight subjects and to what extent these changes can already occur in the adolescent human brain serum level NSE is primarily localized in the cytoplasm of neurons and not secreted.

Thus, this enzyme can be used as reliable marker for structural neuronal damage and increased serum NSE concentrations have been observed in patients suffering from traumatic brain injury and neurodegenerative disease [30].

In the present study, we hypothesize a higher BMI in NAFLD patients to be associated with increased NSE serum levels. Previous studies declared decreased hippocampal Gray Matter (GM) correlating with elevated NSE levels in obese subjects which is considered the first evidence in young adults to link obesity with a specific indicator for neuronal injury in a brain region of particular sensitivity to neuro degeneration and cognitive decline. It was found that , aerobic exercise is a significant beneficial factor for maintenance of cognitive function and integrity of brain structure vulnerable to obesity-related decline in older adults[31].

Further, the dramatic decrease in CD4 in serum of NAFLD obese patients may be, as a result of the decreased production rate of circulating lymphocytes resulting from the depletion in peripheral T cells , apoptosis enhancement, and no new T cell production [24]. Other study [32] agreed that immune system restoration is quantitatively and qualitatively partial despite having shown an increase in these cell populations together with a reduction in apoptosis after treatment. Previous study demonstrated that ,the greatest averages weight gain was observed among patients with lower CD4 [33].

Additionally, IFNγ-producing TH1 CD4 helper T-cells infiltrate adipose tissue of mice on a high-fat diet [34], suggesting that T cells mediate inflammation resulting in obesity and metabolic disease. The main result of this study was that the TAC in NAFLD obese subjects were lower than controls[35], have also demonstrated that TAC was reduced in obese children especially with metabolic syndrome and they have commented that obese children with metabolic syndrome are prone to oxidative stress [36]. However, other studies [37] have reported that the BMI, standard deviation score of BMI and total body fat were inversely associated with dietary TAC in obese subjects . Many studies report the ability of the dietary intake to modulate antioxidant status after the acute consumption of antioxidant rich foods [38] it may be possible that antioxidant metabolism or absorption may be altered.

In this study we demonstrated that TAC was reduced in NAFLD- obese patients. Therefore it must be recommended to obese subjects to consume some products containing more antioxidants.

The presence of oxidative stress has been evaluated previously in obese children[39].On the other hand other studies have reported that oxidized LDL is associated with insulin resistance, independent of body fatness, and they suggested that oxidative stress may be independently related to the development of insulin resistance early in life [40] . Oxidative stress has a correlation with insulin resistance. It has been supposed that oxidative stress (OS) which was induced by obesity may have a role in pathogenesis of these obesity related diseases. Several mechanisms are involved in generating OS in obesity.

Obesity may induce systemic OS and, in turn, OS is associated with an irregular production of adipokines, which contributes to the development of the numerous diseases [41]. Our results are also consistent with the results from [42] who found that plasma TAC levels were significantly lower in obese women compared to healthy women group.

This suggests that increased adiposity leads to increased oxidative stress which in turn lowers TAC levels counteracting increased radical production. Several studies have also shown that antioxidant defense markers are lower according to the amount of body fat and central obesity [43]. Possible reasons for our findings might be attributable to the fact that visceral fat mass is more metabolically active compared to subcutaneous fat mass. Adipose tissue secretes various adipokines acting in autocrine, paracrine and endocrine manner. Excessive storage of adipose tissue, specifically in the abdomen, leads to disturbances in adipokines secretion.

This promotes endothelial dysfunction and chronic low grade pro-inflammatory state leading to numerous diseases. In physiological and, pathological conditions, adipokines induce the production of ROS, thus generating oxidative stress. However, the hypothesis that visceral fat mass alone contributes to decrease in total antioxidant capacity is not valid , but rather the total fat mass and body mass index as measures of overall adiposity. It has been shown that excessive adipose tissue is a source of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1ß, and IL-6. Upon activation, many immune cells generate free radicals and, in the same way, the synthesis of ROS promotes an inflammatory status [44]. Obese persons may have an antioxidant deficit as a result of poor antioxidant intake or activities of the major antioxidant enzymes may be inadequate [35].

Conclusion

It could be concluded that, NF-κB activation by obesity in NAFLD subjects in which results from extracellular inflammatory triggers by classical inflammatory pathways**.** Marked reduction in CD4 cells in NAFLD obese patients suggests that T cells mediate inflammation resulting in obesity and metabolic disease**.** Our findings can thus be interpreted as a confirmation of the neuronal injury associated with elevated NSE marker linked with NAFLD obese women which could contribute to the heightened vulnerability brain regions, that host cognitive function, exhibit to neuro-degeneration, O-LDL exhibited high level in NAFLD obese subjects, supposed that oxidative stress which was induced by obesity may have a role in pathogenesis of these obesity related diseases. Balance between oxidant and antioxidant system is disrupted due to the reduced TAC in NAFLD obese subjects. Further studies should also be performed to evaluate the beneficial effects of dietary intake of antioxidants in these cases.

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