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Original article

EVALUATION OF THE RELATIONSHIP BETWEEN SERUM ADIPONECTIN LEVELS AND OXIDATIVE STRESS, PROINFLAMMATORY CYTOKINES IN HYPERLIPIDEMIA

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Abstract

In this study, adiponectin, protein carbonylation, TAC, TNF-α and IL-6 levels were measured in patients with hyperlipidemia and compared with healthy volunteers. Adiponectin, , TNF-α and IL-6 levels were analysed by ELISA method using kit. TAC and protein carbonylation levels were spectrophotometrically measured. In total patient group, a significant differences was found in adiponectin and TAC levels compared with controls ($p<0.05$, $p<0.01$). Total patients were divided into three groups: hyperlipidemia, hyperlipidemia with coronary artery stenosis and coronary artery stenosis. Adiponectin levels were increased in hyperlipidemia with coronary artery stenosis group compared with control group ($p<0.01$). Protein carbonylation in hyperlipidemic group were higher than controls ($p<0.01$). TAC levels were decreased in both hyperlipidemic group and hyperlipidemia with coronary artery stenosis group compared with control group ($p<0.05$, $p<0.01$). In hyperlipidemic patients, protein carbonylation was higher than hyperlipidemia with coronary artery stenosis patients ($p<0.01$). In conclusion, our study shows that oxidative stress is increased in hyperlipidemia. High adiponectin levels in hyperlipidemia with coronary artery stenosis may be due to protecting heart from oxidative damage.

Key words: *Adiponectin, Hyperlipidemia, Oxidative stress, Coronary artery stenosis, Protein carbonylation, Antioxidant.*

Hiperlipidemide Serum Adiponektin Düzeyleri ile Oksidatif Stres, Proinflamatuvar Sitokinler Arasındaki İlişkinin Değerlendirilmesi

Bu çalışmada hiperlipidemik hastalarda adiponektin, protein karbonilasyonu, TAK, TNF-α ve IL-6 düzeyleri ölçülerek sağlıklı grup ile karşılaştırılmıştır. Adiponektin, TNF-α and IL-6 düzeyleri ELISA metodu ile analiz edilmiştir. TAK ve protein karbonilasyonu, düzeyleri spektrofotometrik olarak ölçülmüştür. Sağlıklı kontroller ile karşılaştırıldığında total hasta grubunda adiponektin ve TAK düzeylerinde anlamlı farklılıklar bulunmuştur ($p<0.05$, $p<0.01$). Total hastalar üç gruba ayrılmıştır: hiperlipidemik, hiperlipidemik ve koroner arter stenozlu ve koroner arter stenozlu. Adiponektin düzeyleri kontrol grubu ile karşılaştırıldığında hiperlipidemili ve koroner arter stenozu olan grupta yüksekti ($p<0.01$). Protein karbonilasyonu hiperlipidemili grupta kontrol grubundan daha yüksekti ($p<0.01$). TAK düzeyleri kontrol grubu ile karşılaştırıldığında hem hiperlipidemili grupta hem de hiperlipidemili ve koroner arter stenozu olan grupta azalmıştı ($p<0.05$, $p<0.01$). Protein karbonilasyonu hiperlipidemili hastalarda hiperlipidemili ve koroner arter stenozu olan hastalardan daha yüksekti ($p<0.01$). Sonuç olarak, çalışmamız hiperlipidemide oksidatif stresin arttığını göstermektedir. Hiperlipidemi ile birlikte koroner arter stenozunda adiponektin düzeylerinin kalbi oksidatif hasardan korumak için artmış olabileceğini söyleyebiliriz.

Anahtar kelimeler: *Adiponektin, Hiperlipidemi, Oksidatif stres, Koroner arter stenozu, Protein karbonilasyonu, Antioksidan.*

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INTRODUCTION

Hyperlipidemia is associated with several manifestations of endothelial dysfunctions, and it is one of the major risk factors for atherosclerosis (1). Besides its role in energy storage, adipose tissue is considered an important endocrine organ that produces numerous factors affecting metabolism of lipids and carbohydrates and numerous other processes in human body (2). Adiponectin is a circulating hormone secreted by adipose tissue that plays a key role in glucose homeostasis and fatty acid metabolism (3). It may have antiatherogenic properties and may play a role in the pathogenesis of cardiovascular disease (4). Previous studies reported that circulating levels of adiponectin are decreased in disorders associated with obesity, dyslipidemia, insulin resistance and inflammation (5–7). On the other hand, chronic heart failure patients had poor prognosis with a high adiponectin levels (8,9).

Atherosclerosis is a chronic inflammatory condition associated with an overproduction of reactive oxygen species (ROS), endothelial cell activation, and the accumulation of leukocytes in the walls of arteries (10). Several studies have demonstrated that plasma markers of oxidative stress are increased while plasma markers of antioxidant defence are decreased in cardiovascular disease (11-13). Oxidative modification of proteins by ROS can cause loss of catalytic activity of the proteins and marks the protein for subsequent proteolytic degradation. Such damaged protein can seriously compromise cellular integrity (14,15). The biological oxidative effects of free radicals on cell components are controlled by a spectrum of antioxidants. Antioxidants may inhibit atherogenesis and improve vascular function by different mechanisms (16). Clinical studies have demonstrated that patients with cardiovascular disease have markers suggestive of decreased antioxidant activity when compared with normal subjects (17,18).

Adipose tissue expresses a variety of adipocytokines, which participate in the immune and inflammation system. Proinflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-6 have been regarded as a key mediators in the development of atherosclerosis (19). Damaged endothelial cells release cytokines and growth factors and then macrophages and adhesion molecules accumulate into the subendothelial space of the injured region, promoting the atherogenic change (20). Numerous studies show enhanced inflammation in adult patients with hypercholesterolemia or cardiovascular disease(21-23).

The aim of this study was to examine in serum adiponectin, protein carbonylation, TAC, TNF-α and IL-6 levels in patients with hyperlipidemia and evaluate relationship between adiponectin and oxidative stress in this disease.

MATERIALS AND METHODS

Subjects

This study was approved by the Clinical Research Ethics Committee of Gazi University. Written consent was obtained from the patients or their relatives. We studied 87 patients with newly diagnosed hyperlipidemia and/or coronary artery stenosis and 28 age-matched control subjects at Türkiye Yüksek İhtisas Education and Research Hospital, Ankara, Turkey. All patients included in the study had never been treated for hyperlipidemia and/or coronary artery stenosis. Patients were divided into three group; Thirty-two patients with hyperlipidemia, forty-one patients with hyperlipidemia+coronary artery stenosis and fourteen patients with coronary artery stenosis. Eligible patients including men and women at least 18 years old with hypercholesterolemia defined as LDL ≥130 mg/dl and triglyceride ≤200 mg/dl who were not on lipid lowering medication. CAD patients were revealed to have ≥50% reduction in at least 1 main coronary artery. Exclusion criteria for patients were: diabetes mellitus, renal deficiency, hypothyroidism, , hepatic disease, arthritis, cancer and treatment with anti-inflammatory drugs.

Venous blood samples were collected by venipuncture after a 12-h overnight fast. Blood samples were drawn, following overnight fasting, into tubes and serum was immediately separated by centrifugation. Biochemical measurements were carried out according to validated methods.

Determination of serum Adiponectin levels

Adiponectin levels (Human Adiponectin Enzyme-Linked Immunosorbent Assay Kit, Biovendor, BRNO, Czech Republic) were measured by enzyme immunoassay. 100 µL of standard/sample are incubated in microplate wells pre-coated with polyclonal anti-human adiponectin antibody. After 60 minutes incubation and washing, polyclonal anti-human adiponectin antibody, 100 µL of conjugated with horseradish peroxidase (HRP) is added to the wells and incubated for 60 minutes with captured adiponectin. Following another washing step, the remaining HRP conjugate is allowed to react with 100 µL of the substrate solution (TMB). The reaction is stopped by addition of 100 µL of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. A standard curve is constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve. The inter-assay coefficient of variation of adiponectin is 3.1%.

Determination of serum total antioxidant capacity (TAC) levels

Serum TAC levels were determined according to the ABTS radical cation ($ABTS^{\cdot+}$) decolorization assay described by Re et al. (24). In this method, when the aliquot of serum is added to the $ABTS^{\cdot+}$ solution, decolorization as a result of the presence of serum antioxidants which reverse the formation of $ABTS^{\cdot+}$ is observed. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate (final concentration) in a ratio of 1:0.5 and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. $ABTS^{\cdot+}$ solution was diluted with PBS, pH 7.4, to give an absorbance of 0.700 (± 0.020) at 734 nm and 30°C. After addition of 1.0 mL of diluted $ABTS^{\cdot+}$ solution ($A_{734\ nm}$ = 0.700 ± 0.020) to 10 µL serum or Trolox standard in PBS the absorbance reading was taken at 30°C exactly 6 min after initial mixing. Percentage inhibition values of samples and standards were calculated and TAC levels were calculated from a calibration curve. The inter-assay coefficient of variation of TAC is 2.1%.

Determination of serum Protein carbonylation levels

Oxidative damage to proteins was determined in the serum based on the formation of protein-hydrazone as a result of the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls using the Cayman's Protein Carbonyl Assay kit. Absorbance of the samples was measured at 370 nm using the Versemax Microplate Reader. Carbonyl content was determined using the extinction coefficient of DNPH (0.022 $\mu M^{-1}cm^{-1}$). The total serum protein was then measured at 280 nm on spectrophotometer. Bovine Serum Albumin (BSA) was used to construct a protein standard curve. Protein carbonyl content in the serum was then expressed as nanomoles per milligram protein. The inter-assay coefficient of variation of protein carbonylation is 4.4%.

Determination of TNF-α levels

TNF-α levels was determined using a human TNF-α ELISA kit (eBioscience, San Diego, California). Each sample was measured in dublicate. Briefly, 100 µL of distilled water were added to all wells. Subsequently, serum/standard samples (50 µL) were added to wells of a microplate and incubated at room temperature for 3 hours on a microplate shaker at 200 rpm. The microwell contained antihuman TNF-α monoclonal coating antibodies, a lyophilized detection antibody, streptavidin-horseradish peroxidase (HRP), and sample diluents. After incubation, the samples were washed, 100 µL of tetramethylbenzidine HRP substrate solution

was added to the wells, and the samples were incubated at room temperature for 10 minutes; the reaction was stopped with 100 µL stop solution. The results were read using a microplate spectrophotometer at 450 nm. A standard curve was used to calculate the TNF-α levels of the serum samples. The interassay coefficient of variation is 4.1%.

Determination of IL-6 levels

Serum IL-6 levels was determined using a human IL-6 ELISA kit (eBioscience, San Diego, California). The assay was performed according to the manufacturer's recommendations. 100 µL of distilled water were added to all wells. Serum/standard samples (50 µL) were added to wells of a microplate and incubated at room temperature for 3 hours on a microplate shaker at 200 rpm. 100 µL of TMB substrate solution was added to all wells, including the blank wells. The microwell strips were incubated at room temperature for about 10 minutes. The reaction was stopped with 100 µL stop solution. The results were read using a microplate spectrophotometer at 450 nm. The inter-assay coefficient of variation of IL-6 is 3.4%.

Determination of other blood parameters

Serum total cholesterol, HDL-cholesterol and triglyceride levels were measured spectrophotometrically on the Roche-Hytachi P-800 autoanalyzer by using a commercial kit (Roche, Mannheim, Germany). Concentration of serum LDL-cholesterol was calculated by the standard Friedewald formula.

Statistical analysis

All results are expressed as means ±SE. Statistical analyses were performed by using SPSS packed programme (version 18 software, SPSS Inc. Chicago, Illinois, USA). Analysis of the distribution of constant variance for normality was assessed using the Shapiro-Wilk test. The mean difference between groups was evaluated with Student's t-test when the number of independent groups was two and with one-way analysis of variance when the number of independent groups was more than two. Because some of data set were not normally distributed, statistical comparisons between two groups were performed using Mann-Whitney U test. P value <0.05 was considered statistically significant.

RESULTS

Clinical characteristics and laboratory data from total patients and control group were shown on Table 1. The levels of total cholesterol and LDL-cholesterol in control group were statistically lower than those of patient group ($p<0.01$). Similarly, serum triglycerides in healthy controls were lower than those of total patient group ($p<0.05$). No significant differences were determined in the comparison of HDL-cholesterol levels between patients and control group ($p>0.05$).

Table 1. Clinical characteristics and laboratory data from patients and controls (X±S.E.).

	Total patients (n=87)	Controls (n=28)
Age (years)	54.09±1.04	51.54±2.01
Sex (m/f)	70/17	21/7
Quetelet index (kg/m^2)	27.42±0.54	28.31±0.60
Total cholesterol (mg/dL)	212.64±3.99**	171.14±3.23
HDL-cholesterol (mg/dL)	41.69±1.02	41.54±1.39
LDL-cholesterol (mg/dL)	137.59±3.23**	105.36±3.35
Triglycerides (mg/dL)	171.35± 10.79*	121.43±7.09

* significant difference from control group ($p<0.05$).
** significant difference from control group ($p<0.01$).

Our data showed a significant increase of adiponectin levels in total patients (11.37±0.85) as compared with controls (7.63±0.95) ($p<0.05$) (Table 2). But, serum TAC levels in total patient group (2.04±0.01) were found lower than those of controls (2.15±0.20) ($p<0.01$). Protein carbonylation in total patients (21.21±0.71) and healthy controls (19.01±0.84) were similar ($p>0.05$). No significant difference were determined in the comparison of TNF-α levels between patient (20.12±1.16) and (18.29±1.06) control groups ($p>0.05$). IL-6 levels in total patients (11.95±0.80) were not different from healthy controls (13.68±0.71) ($p>0.05$)(Table 2).

Table 2. Comparison of measured parameters between patient and control groups (X±S.E.)

	Total patients (n=87)	Controls (n=28)
Adiponectin (µg/mL)	11.37±0.85*	7.63±0.95
TAC (mmol/L)	2.04±0.01**	2.15±0.20
Protein carbonylation (nmol/mg)	21.21±0.71	19.01±0.84
TNF-α (pg/mL)	20.12±1.16	18.29±1.06
IL-6 (pg/mL)	11.95±0.80	13.68±0.71

* significant difference from control group ($p<0.05$).
** significant difference from control group ($p<0.01$).

When patient group were divided into three group as hyperlipidemia, hyperlipidemia with coronary artery stenosis, and coronary artery stenosis. Adiponectin levels in patients with hyperlipidemia with coronary artery stenosis (13.78±1.54) were increased compared with controls (7.63±0.94) ($p<0.01$)(Table 3). But, TAC levels in patients with hyperlipidemia (2.03±0.03) and hyperlipidemia with coronary artery stenosis (2.01±0.01) were diminished compared with controls (2.15±0.02)($p<0.05$, $p<0.01$, respectively). In patients with

hyperlipidemia with coronary artery stenosis, TAC levels were decreased compared with coronary artery stenosis group (2.11±0.02) ($p<0.01$). A significant increase of protein carbonylation levels were observed in hyperlipidemic group (27.20±0.95) when compared to hyperlipidemia with coronary artery stenosis group (18.65±0.69), coronary artery stenosis group (15.05±0.92) and control group (19.01±0.84) ($p<0.01$, $p<0.01$, $p<0.01$, respectively). Protein carbonylation levels in hyperlipidemia with coronary artery stenosis group were higher than coronary artery stenosis group ($p<0.05$). In coronary artery stenosis group, protein carbonylation levels were lower than control group ($p<0.05$). No statistical difference was determined in the comparison of TNF-α and IL-6 levels between patient goups and controls ($p>0.05$) (Table 3). IL-6 levels were higher in hyperlipidemic patients compared to coronary artery stenosis group ($p<0.05$).

Negative correlations were found between TAC levels and protein carbonylation levels of the total patients ($r= -0.294$, $p=0.006$) and also between TAC levels and protein carbonylation levels of the hyperlipidemic group ($r= -0.514$, $p=0.003$) (Table 4).

Table 3. Comparison of adiponectin, TAC, protein carbonylation, TNF-α and IL-6 levels between patient groups and controls (X±S.E.).

	Adiponectin (μg/mL)	TAC (mmol/L)	Protein carbonylation (nmol/mg)	TNF-α (pg/mL)	IL-6 (pg/mL)
Hyperlipidemic group (N=32)	9.65±0.75	2.03±0.03[a]	27.20±0.95[b]	18.13±1.71	16.26±1.55
Hyperlipidemia+coronary artery stenosis group (N=41)	13.78±1.54[b]	2.01±0.01[b,e]	18.65±0.69[c,f]	18.33±1.14	12.66±0.72
Coronary artery stenosis group (N=14)	8.27±1.70	2.11±0.02	15.05±0.92[a,c]	18.51±4.43	10.78±0.97[d]
Controls (N=28)	7.63±0.94	2.15±0.02	19.01±0.84	20.12±1.16	11.95±0.80

[a] significant difference from control group ($p<0.05$).
[b] significant difference from control group ($p<0.01$).
[c] significant difference from hyperlipidemic group ($p<0.01$).
[d] significant difference from hyperlipidemic group ($p<0.05$).
[e] significant difference from coronary artery stenosis group ($p<0.01$).
[f] significant difference from coronary artery stenosis group ($p<0.05$).

Table 4. Pearson correlation coefficients between measured parameters in patients.

		Protein carbonylation
Total patients (N=87)	TAC	r=-0.294 p=0.006
Hyperlipidemic group (N=32)	TAC	r=-0.514 p=0.003

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DISCUSSION

In this study, we show that patients with both hyperlipidemia and coronary artery stenosis have a higher level of adiponectin, but lower level of antioxidant activity compared to healthy controls. We have also found increased protein oxidation and reduced antioxidant activity in hyperlipidemic patients.

Adiponectin, a 244 amino-acid fat-derived peptide, produced and secreted exclusively by adipose tissues, has been reported to be linked with visceral adiposity, insulin resistance and cardiovascular risk (25,26). Experimental data suggest that adiponectin is involved in prevention of foam cell formation, down regulation of adhesion molecules, inhibition of endothelial dysfunction, and smooth muscle cell proliferation and migration (27,28). Therefore, adiponectin is supposed to be protective against cardiovascular disease. Zhang et al. (29) report that higher adiponectin concentrations were associated with a lower prevalence of diabetes, lower body mass index, fasting insulin, fasting glucose, LDL cholesterol, and triglycerides, and higher HDL-cholesterol levels in 899 outpatients with stable coronary artery disease. In earlier study (30), coronary artery disease subjects had lower adiponectin concentrations than subjects without coronary artery disease. Arca et al. show that patients with familial combined hyperlipidemia have a reduced level of adiponectin compared to controls (31). However its role seems paradoxical in different conditions. The study results by Haugen et al. (32) have shown significantly increased adiponectin level in chronic heart failure as compared with control group for those over 70 years old. They think that increased adiponectin level in these patients is most probably compensatory as an indicator of disease severity rather than as a pathophysiological mediator. Similar results were also obtained in another study (8). Higher adiponectin concentrations were obtained in chronic obstructive pulmonary disease patients compared to control subjects who had normal pulmonary function by Tomoda et al. (33). Guebre Egziabher et al. has been reported to be increased plasma adiponectin levels in chronic kidney disease (34). Consistent with reports by Haugen et al., George et al., Tomoda et al. and Guebre Egziabher et al., our study found hyperlipidemia with coronary artery stenosis patients had higher serum concentrations of adiponectin than healthy controls. This means that the increased adiponectin concentrations seems to be caused by hyperlipidemia plus coronary artery stenosis in the same patients. This also shows the functional adiponectin resistance, as suggested by Kintscher (35). We did not observe any significant differences between patient groups of hyperlipidemia or coronary artery stenosis and healthy controls, in this study.

Oxidative stress is also specifically implicated in the pathogenesis of atherosclerosis or atherosclerosis-related conditions including cardiovascular disease (36). ROS production overwhelming the capacity of antioxidant defense leads to the establishment of oxidative stress. Vasconcelos et al. (37) found significantly higher levels of ROS in hyperlipidemic patients compared with healthy controls. These data provide evidence that ROS production by circulating monocytes from hyperlipidemic subjects may contribute to the systemic oxidative stress and possibly to atherogenesis. ROS attack and modify subcellular components, nucleic acid, lipids and proteins (38). Many different types of protein oxidative modifications can be induced by free radicals. However, protein carbonyl formation has been accepted as a common phenomenon of protein oxidation (39). When comparing hypercholesterolemic patients with normocholesterolemic controls, Özdemirler et al. found increased levels of protein carbonyls as a representative indicator of oxidative stress in the former group (40). Mutlu Türkoğlu et al. (41) have reported that advanced oxidation protein products increased in the plasma in patients with coronary artery disease and there was no correlation between the plasma levels of protein carbonyls among patients grouped into affected coronary artery vessel. The results of the

present study suggest that oxidation of proteins occurs in the serum of patients with hyperlipidemia. Similar to our results, Serdar et al. (42) demonstrated that the levels of protein oxidation products were high in the plasma of CAD patients. Nonenzymatic detoxification against ROS is provided by antioxidants. Clinical studies have demonstrated that both patients with hyperlipidemia and CAD have markers suggestive of decreased antioxidant activity when compared with normal subjects (37,41,43). In this study, TAC levels in patients with hyperlipidemia and hyperlipidemia with coronary artery stenosis were diminished compared with controls. Moreover, we found negative correlation with TAC and protein carbonyls in hyperlipidemic patients. Our findings consist with the results of other studies (13,37) that reported decreased total antioxidant status as an indicator of oxidative stress in hyperlipidemia.

Atherosclerosis is recognized as a chronic inflammatory disease of the arterial wall in which an inflammatory response is a key event that leads to the formation of atheromatous lesions (44). The inflammatory cytokine TNF-α has been regarded a mediator in the development of atherosclerosis due to its involvement in several stages in this process. Narverud et al. reported an elevated TNF-α levels in children with familial hypercholesterolemia compared to control children (45). But, El Messal et al. found no significant difference between hypercholesterolemic patients and controls (46). The present data showing unchanged TNF-α levels in hyperlipidemic patients is consistent with the earlier report by El Messal et al. Among pro-inflammatory cytokines, IL-6 is one of the most important factors (47). It is hypothesized that inflammatory mediator CRP, via IL-6, may exacerbate vascular dysfunction (48). In previous study, high IL-6 concentration has been shown in patients with coronary artery stenosis (49). But, in another study, similar IL-6 concentrations in coronary artery patients and healthy controls have been reported (50). In this study, no statistical difference was determined in the comparison of IL-6 levels between patient goups and controls. Only, increased IL-6 levels were obtained between patients group of hyperlipidemia and coronary artery stenosis.

In summary, the results of the present study corroborate earlier studies showing increased oxidative stress in patients with hyperlipidemia. High adiponectin levels in hyperlipidemia with coronary artery stenosis may be due to the protecting effect of adiponectin of heart from oxidative damage.

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