

## OXIDIZED LOW-DENSITY PROTEIN, INTERLEUKIN-6 AND TUMOR NECROSIS FACTOR ALPHA LEVELS AND PARAOXONASE ACTIVITIES IN HYPERTENSIVE PATIENTS

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### Abstract

*Oxidative stress is thought to play a critical role in the pathogenesis of essential hypertension. Oxidized low-density lipoprotein (ox-LDL) has an important role during the atherosclerosis process and paraoxonase can significantly inhibit lipid peroxidation. We aimed to examine serum oxidized LDL, TNF- $\alpha$ , IL-6 levels and paraoxonase (PON1) activity as a biomarkers of oxidative stress in hypertensive patients. Thirty-eight hypertensive patients and twenty-two healthy control subjects were included in the study. Clinical assessment and blood pressure measurement were performed in patients. Serum Ox-LDL, TNF- $\alpha$  and IL-6 levels were measured by Enzyme-linked immunosorbent assay (ELISA) method. PON1 activities were measured by spectrophotometric method. Ox-LDL and IL-6 levels were significantly higher in hypertensive patients compared to controls ( $p<0.01$ ,  $p<0.01$ , respectively). PON1 activities were found at low level in patients with hypertension compared to control group ( $p<0.01$ ). Hypertensive patients were divided into two groups: dipper and non-dipper. Decreased Ox-LDL and TNF- $\alpha$  levels were found in dipper patients compared to non-dipper patients ( $p<0.01$ ,  $p<0.01$ , respectively). PON1 activities were found higher in dipper patients than those of non-dipper patients ( $p<0.05$ ). Increased Ox-LDL, IL-6 levels and decreased PON1 activities in hypertensive patients might indicate increased oxidative stress, which plays an important role in the development of cardiovascular diseases. Dipper patients had mild signs of oxidative stress compared with non-dipper patients.*

**Key words:** Oxidized LDL, Paraoxonase, Inflammatory cytokines, Hypertension, Oxidative stress.

### Hipertansiyon Hastalarında Okside Düşük Dansiteli Lipoprotein, İnterlökin-6 ve Tümör Nekroz Faktör- $\alpha$ Düzeyleri ve Paraoksonaz Aktiviteleri

*Oksidatif stresin esansiyel hipertansiyonun patojenezinde kritik bir rol oynadığı düşünülmektedir. Okside düşük dansiteli lipoprotein (ox-LDL) ateroskleroz sürecinde önemli bir role sahiptir ve paraoksonaz belirgin olarak lipid peroksidasyonunu inhibe edebilir. Biz hipertansiyon hastalarında oksidatif stresin markerleri olarak serum okside LDL, TNF- $\alpha$ , IL-6 düzeyleri ve paraoksonaz (PON1) aktivitesini incelemeyi amaçladık. Otuz sekiz hipertansif hasta ve yirmi iki sağlıklı kontrol çalışmaya dahil edildi. Hastaların klinik değerlendirmesi ve kan basıncı ölçümleri yapıldı. Serum Ox-LDL, TNF- $\alpha$  and IL-6 düzeyleri ELISA metoduyla ölçüldü. PON1 aktiviteleri spektrofotometrik methodla ölçüldü. Hipertansif hastalarda sağlıklı kontrollerle karşılaştırıldığında daha yüksek Ox-LDL and IL-6 düzeyleri bulundu (sırasıyla,  $p<0.01$ ,  $p<0.01$ ). PON1 aktiviteleri sağlıklı kontrollerle karşılaştırıldığında hipertansiyonlu hastalarda daha düşüktü ( $p<0.01$ ). Hipertansif hastalar iki gruba ayrıldı: dipper ve dipper olmayan. Dipper olmayan hastalarla karşılaştırıldığında dipper hastalarda azalmış Ox-LDL and TNF- $\alpha$  düzeyleri bulundu (sırasıyla,  $p<0.01$ ,  $p<0.01$ ). Dipper hastalardaki PON1 aktiviteleri dipper olmayan hastaların aktivitelerinden daha yüksek bulundu ( $p<0.05$ ). Artan Ox-LDL, IL-6 düzeyleri ve azalan PON1 aktivitesi hipertansif hastalarda kardiyovasküler hastalıkların gelişiminde önemli bir rol oynayan artmış oksidatif stresi gösterebilir. Non-dipper hastalarla karşılaştırıldığında dipper hastalarda oksidatif stresin daha hafif belirtileri vardı.*

**Anahtar kelimeler:** Okside LDL, Paraoksonaz, İnflamatuvar sitokinler, Hipertansiyon, Oksidatif stres.

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## INTRODUCTION

Essential hypertension, or hypertension of unknown cause, accounts for more than 90% of cases of hypertension (1,2). It is one of the most important risk factors for cardiovascular diseases and clinical outcomes (3). In addition, hypertension is associated to target-organ damage such as left ventricular hypertrophy (4), microalbuminuria (5,6), or subclinical vascular impairment as endothelial dysfunction (7,8), an early marker of atherosclerosis.

Numerous mechanisms or causes of hypertension have been well characterized over the years. Several vasoconstrictive mechanisms, the sympathetic nervous system, the endothelin system, the vasopressin system and more recently the reactive oxygen species have all been implicated in the development of experimental or human hypertension. Increased vascular oxidative stress could be involved in the pathogenesis of hypertension (9, 10), a major risk factor for cardiovascular disease mortality. Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species and the antioxidant defense systems so that the latter become overwhelmed (11, 12). One of the most important oxidative processes is oxidation of lipids and lipoproteins such as oxidized low-density lipoprotein (Ox-LDL). Ox-LDL is a major cause of vessel wall injury and atherosclerosis. Ox-LDL has a prominent role in the pathogenesis of atherosclerosis (13), and the elevation of ox-LDL levels in atherosclerotic plaques is an important event in the development of atherosclerosis (14). It induces foam cell formation from macrophages that plays a key role in early atherogenesis. Oxidation of LDL occurs primarily in the vessels wall, thus activating many inflammatory and atherogenic reactions. Moreover, elevated ox-LDL contributes to plaque instability and vulnerability (15).

Paraoxonase (PON1) is an enzyme with three activities, which are paraoxonase, arylesterase and diazoxonase. PON1 is a calcium-dependent esterase consisted of 354 amino acids with a molecular mass of approximately 45 kDa, and it is found exclusively associated with high density lipoprotein (HDL) in serum. It protects LDL from oxidative stress by destroying biologically active phospholipids (16). Human serum PON1 activity was shown to be inversely related to the risk of cardiovascular diseases (17), and low PON1 activities were observed in atherosclerotic, hypercholesterolemic and hypertensive patients (18,19).

Endothelial injury is the first and crucial step in the pathogenesis of atherosclerosis. It triggers inflammation with increased adhesiveness and activation of monocytes and platelets, which is accompanied by the production of cytokines including tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6), chemokines, vasoactive molecules and growth factors. Several studies have revealed an association between biochemical markers of systemic inflammation and cardiovascular disease such as atherosclerosis and hypertension (20–22).

In this study, we aimed to examine serum ox-LDL, TNF- $\alpha$ , IL-6 levels and PON1 activities as a biomarkers of oxidative stress in hypertensive patients and compare these parameters between dipper and non-dipper patients.

## MATERIALS AND METHODS

### *Subjects*

Newly diagnosed thirty-eight essential hypertensive patients (19 men and 19 women, mean age 52.92 $\pm$ 1.76 years) were recruited from the department of cardiology, then compared to twenty-two non-hypertensive healthy volunteers (10 men and 12 women, mean age 50.09 $\pm$ 2.17 years). No person in the study group was administrated any vitamin supplementation or any drugs. Hypertension was considered to be present if the systolic pressure was >140 mm Hg and/or diastolic pressure was >90 mmHg. Blood pressure was measured using a mechanical sphygmomanometer in hospital setting. Systolic blood pressure and diastolic blood pressure were taken as the first and fifth phases of Korotkoff sounds. Exclusion criteria for entry in the

patient group were clinical evidence of cardiovascular or any other atherosclerotic disease, presence of diabetes mellitus, neoplastic disease, antihypertensive drug use, antioxidant supplement use, chronic renal failure. Hypertensive patients were divided into two groups: 18 dipper patients and 20 non-dipper patients. Patients with blood pressure decrease of 10% or more during nighttime were accepted as dipper status, whereas patients with blood pressure decreases less than 10% were accepted as non-dipper hypertensive cases. Healthy control subjects with blood pressure < 140/90 mmHg in multiple measurements and with same age range with hypertensive patients, were also enrolled in the study. The Institution's Ethics Committee for studies on human subjects approved the study and each patient and healthy subject consented to participate in the research.

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 ox-LDL levels*

The measurement of serum ox-LDL was performed using a sandwich ELISA method (Immundiagnostic AG, Bensheim, Germany). Standards, controls and samples containing ox-LDL were added to wells of microplates coated with high affinity antibodies. During the first incubation period, antibodies immobilized on the wall of the microtiter wells capture the antigen in the patient samples. After washing of the unbound components from samples, a peroxidase-conjugated antibody was added to each microtiter well. Tetramethylbenzidine (TMB) was used as a substrate for peroxidase. Finally, an acidic stop solution was added to terminate the reaction. The intensity of the yellow color was directly proportional to the ox-LDL concentration in the sample. A curve of absorbance unit vs. concentration was generated using the values obtained from standards. Ox-LDL present in the sample was directly determined. The inter-assay coefficient of variation of the ox-LDL was 4.4%.

#### *Determination of paraoxonase activities*

Serum paraoxonase activity were measured using commercially available kit (Rel Assay Diagnostics, Gaziantep, Turkey). Fully automated paraoxonase activity measurement method consists of two different sequential reagents. The first reagent is an appropriate Tris buffer and it also contains calcium ion, which is a cofactor of paraoxonase enzyme. The second reagent is a new developed stable substrate solution. The sample is mixed with the Reagent 1 and the substrate solution is added. Linear increase of the absorbance of *p*-nitrophenol, produced from paraoxon, is followed at kinetic measurement mode. Nonenzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. The molar absorptivity of *p*-nitrophenol is  $18.29 \text{ M}^{-1} \text{ cm}^{-1}$  and one unit of paraoxonase activity is equal to 1 mol of paraoxon hydrolyzed per liter per minute at 37 °C. Paraoxonase activity was expressed as U/L serum. The inter-assay CV is 5.1 %.

#### *Determination of TNF- $\alpha$ levels*

TNF- $\alpha$  levels was determined using a human TNF- $\alpha$  ELISA kit (eBioscience, San Diego, California). Each sample was measured in duplicate. Briefly, 100  $\mu\text{L}$  of distilled water were added to all wells. Subsequently, serum/standard samples (50  $\mu\text{L}$ ) 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- $\alpha$  monoclonal coating antibodies, a lyophilized detection antibody, streptavidin-horseradish peroxidase (HRP), and sample diluents. After incubation, the samples were washed, 100  $\mu\text{L}$  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  $\mu\text{L}$  stop solution. The results were read using a microplate

spectrophotometer at 450 nm. A standard curve was used to calculate the TNF- $\alpha$  levels of the serum samples. The interassay coefficient of variation is 2.8%.

#### *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  $\mu$ L of distilled water were added to all wells. Serum/standard samples (50  $\mu$ L) were added to wells of a microplate and incubated at room temperature for 3 hours on a microplate shaker at 200 rpm. 100  $\mu$ L 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  $\mu$ L stop solution. The results were read using a microplate spectrophotometer at 450 nm. The inter-assay coefficient of variation of IL-6 is 3.1%.

#### *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  $\pm$ SE. Statistical analyses were performed by using SPSS packed programme (version 10 software, SPSS Inc. Chicago, Illinois, USA). Data were statistically analysed by using the non-parametric Mann-Whitney U test. P value  $<0.05$  was considered statistically significant.

## **RESULTS**

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

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

	Hypertensive patients (n=38)	Controls (n=22)
Age (years)	52.92±1.76	50.09±2.17
Sex (m/f)	19/19	10/12
Quetelet index (kg/m <sup>2</sup> )	26.21±0.53	27.02±0.76
Systolic pressure (mm Hg)	141.21±1.25*	114.77±1.17
Diastolic pressure (mm Hg)	87.53±0.68*	72.41±0.96
Total cholesterol (mg/dL)	216.79±8.30	201.91±6.02
HDL-cholesterol (mg/dL)	40.89±1.29**	46.14±1.81
LDL-cholesterol (mg/dL)	131.88± 6.78	124.98±6.08
Triglycerides (mg/dL)	159.92± 13.69	129.77±12.54

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

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

Our data showed a significant increase of Ox-LDL levels in hypertensives (30.31±1.58) as compared with controls (22.68±1.63) (p<0.01) (Table 2). Serum paraoxonase activities in hypertensive group (98.78±5.22) were found lower than those of controls (130.85±8.75) (p<0.01). No significant differences were determined in the comparison of TNF-α levels between patient (35.25±1.80) and (31.15±1.88) control groups (p>0.05). IL-6 levels in patients with hypertension (78.10±6.65) were higher than healthy controls (51.19±5.95) (p<0.01) (Table 2).

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

	Hypertensive patients (n=38)	Controls (n=22)
Ox-LDL (ng/mL)	30.31±1.58*	22.68±1.63
Paraoxonase (U/L)	98.78±5.22*	130.85±8.75
TNF-α (μmol/L)	35.25±1.80	31.15±1.88
IL-6 (ng/mL)	78.10±6.65*	51.19±5.95

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

When patient group were divided into two group as dipper and non-dipper, Ox-LDL levels in dipper patients (26.30±2.37) were decreased compared with non-dipper patients (33.93±1.82) (p<0.01) (Table 3). But, serum paraoxonase activities in dipper patients (112.01±7.95) were elevated compared with non-dipper patients (86.87±5.85) (p<0.05). No statistical difference was determined in the comparison of IL-6 levels between dippers (80.57±11.07) and nondippers (75.88±8.01) (p>0.05). A significant decrease of TNF-α levels

were observed in dipper patients ( $30.07\pm 2.57$ ) when compared to non-dipper patients ( $39.91\pm 2.06$ ) ( $p<0.01$ ) (Table 3).

**Table 3.** Comparison of measured parameters between patient groups ( $\bar{X}\pm S.E.$ ).

	Dipper patients (n=18)	Non-dipper patients (n=20)
Ox-LDL (ng/mL)	$26.30\pm 2.37^*$	$33.93\pm 1.82$
Paraoxonase (U/L)	$112.01\pm 7.95^{**}$	$86.87\pm 5.85$
TNF- $\alpha$ ( $\mu\text{mol/L}$ )	$30.07\pm 2.57^*$	$39.91\pm 2.06$
IL-6 (ng/mL)	$80.57\pm 11.07$	$75.88\pm 8.01$

\*significant difference from non-dipper group ( $p<0.01$ ).

\*\*significant difference from non-dipper group ( $p<0.05$ ).

## DISCUSSION

In the present study, we examined the oxidant and antioxidant status to evaluate the oxidative stress in hypertensive patients and compared with that of normotensives. With respect to ox-LDL and paraoxonase, our findings indicated that oxidative stress increased in patients with essential hypertension compared to normotensives. Dipper patients had mild signs of oxidative stress compared to non-dipper patients.

Hypertension is a common disease. It is an important cause of morbidity and mortality worldwide. Elevated systolic blood pressure is a major risk factor for cardiovascular diseases. Hypertension has unfavorable consequences on tissues, cells, and molecules, which in turn cause specific injuries; the most important of them is atherosclerosis. LDL is generally believed to be important in the development of atherosclerosis, and its atherogenicity is likely to depend on modification, especially by oxidation (23). Ox-LDL stimulates endothelial adhesiveness and promotes T-cell and monocyte activation. Ox-LDL is taken up by macrophages in the artery wall, which develop into foam cells, and is therefore generally believed to be atherogenic (24). Previous studies have suggested that hypertension may be related to increased Ox-LDL, by showing either increased in vitro oxidizability of LDL or elevated titers of autoantibodies against Ox-LDL in subjects with essential hypertension (25,26,27). Konukoglu et al. (27) found that Ox-LDL levels of the hypertensive patients were higher than the normotensives. Their data suggested that an imbalance between oxidant and antioxidant status in hypertension was related with elevation in Ox-LDL levels and reduction in PON1 activity. In another study conducted by Wu et al, decreased titers of autoantibodies against oxidized LDL were observed in subjects with borderline hypertension (28). This discrepancy might be explained by the possibility that decreased titers of autoantibodies against oxidized LDL may represent increased consumption of autoantibodies due to binding to early atherosclerotic lesions. Our finding that ox-LDL was significantly higher in patients with essential hypertension relative to the control subjects is in line with the findings of Chrysohoou et al.(25) and Konukoglu et al. (27). Since serum concentrations of ox-LDL have been suggested as a marker for in vivo oxidative stress, results reported here suggest that patients with essential hypertension may be under oxidative stress. Patients with essential hypertension are divided into two groups: dippers and non-dippers. Non-dipper hypertensives have greater vascular damage in the carotid arteries and higher carotid

intima media thickness as well (29). In previous studies, oxidative stress was shown to be increased in non-dipper hypertensive patients compared with dipper hypertensive patients (30,31). Yildiz et al. reported that non-dippers have higher lipid peroxidation than dippers (31). In this study, we found increased ox-LDL levels in non-dipper patients compared with dipper patients. Therefore, in the present study oxidative stress may be increased in hypertensive patients particularly in non-dipper group.

LDL oxidation and thus the development of atherosclerotic process has been shown to be inhibited by some protective properties of high-density lipoprotein (HDL). The proposed antioxidant properties of HDL are due to the effect of some molecules in the lipoprotein such as PON1. It has been shown that PON1 prevents LDL from oxidation by removing oxidised phospholipids from LDL (32).

In previous study, PON1 activity was found at low level in both sustained hypertension and white coat hypertension (18). Uzun et al. (18) suggested that reduced paraoxonase activity might be a basis of increased oxidative stress in patients with hypertension. Our results were in agreement with mentioned reports. In the present study, PON1 activity was decreased in essential hypertensive patients compared with healthy controls. Reduced PON1 activity could be related to the increased oxidative stress in serum from these patients. Yildiz et al. were found PON1 activity was decreased in non-dipper patients compared with dipper patients and healthy controls (31). In this study, similar results were obtained in terms of PON1 activity. We found lower activity of PON1 in non-dipper hypertensive patients than dipper hypertensive patients. Thus, in non-dipper hypertensives, more qualitative changes occur to LDL which render them more susceptible to oxidation; coupled to a reduction in the potential antioxidant activity of HDL. This is suggestive of non-dipper hypertensives as high-risk group for atherosclerosis compared to dipper hypertensives.

Proinflammatory cytokines are peptide mediators of endothelial cell activation and dysfunction. These cytokines enhance the formation of a number of endothelial cell substances (33). Thus, endothelial dysfunction associated with hypertension may be mediated by proinflammatory cytokines. The renin-angiotensin system and sympathetic nervous system interact with the proinflammatory cytokines. The sympathetic nervous system stimulates the release of proinflammatory cytokines or proinflammatory cytokines may activate the sympathetic nervous system (34). Angiotensin II enhances the synthesis of TNF- $\alpha$  and IL-6 (35). It also increases the production of reactive oxygen species that participate in the process of inflammation (36). Bogdanski et al. reported that circulating TNF- $\alpha$  and IL-6 are elevated in hypertensive patients compared to healthy controls (37). We found that IL-6 as an innate inflammatory marker is elevated in essential hypertension. This study suggests a causal role of IL-6 in the pathogenesis of hypertension. When we examined according to dipper or non-dipper status, we found higher TNF- $\alpha$  levels in non-dipper hypertensive patients than dipper hypertensive patients.

In conclusion, the present study revealed that hypertensive patients have increased ox-LDL levels, increased IL-6 levels and decreased paraoxonase activities compared to healthy controls. Dipper patients had mild signs of oxidative stress compared to non-dipper patients.

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