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The association of sialic acid levels with nitric oxide and C-reactive protein in patients with diabetic foot ulcer

Diyabetik ayak ülseri olan hastalarda sialik asit düzeylerinin nitrik oksit ve C-reaktif protein ile ilişkisi

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Abstract: Objective: The present study aimed to evaluate the association of serum sialic acid (SA) levels with nitric oxide (NO) and C-reactive protein (CRP) in patients with diabetic foot ulcer.

Methods: This study included total 56 type 2 diabetic patients (27 of them had diabetic foot ulcer and 29 without it) and 22 healthy volunteers. Serum SA, NO and CRP levels were measured with spectrophotometric and nephelometric methods respectively.

Results: Serum SA levels were higher in diabetic foot group than diabetes group ($p<0.05$). CRP and NO levels were found to be significantly higher in diabetic foot group compared to the diabetes ($p<0.001$ and $p=0.002$, respectively) and control groups ($p<0.001$, both). Although there was no correlation between SA and NO ($p>0.05$), serum SA levels were significantly correlated with CRP in diabetic foot group ($p<0.001$).

Conclusion: We suggest that SA could be related to acute phase response in patients with diabetic foot ulcer. Elevated serum SA and NO levels may be indicators of oxidative stress-induced vascular damage.

Keywords: Sialic acid, diabetic foot ulcer, C-reactive protein, nitric oxide

Özet: Amaç: Bu çalışma, diyabetik ayak ülseri olan hastalarda serum sialik asit (SA) düzeylerinin nitrik oksit (NO) ve C-reaktif protein (CRP) ile ilişkisini belirlemeyi amaçladı.

Metod: Bu çalışma, toplam 56 tip 2 diyabetik hasta (27 diyabetik ayak ülserli ve 29 ayak ülseri olmayan) ve 22 sağlıklı gönüllüyü kapsadı. Serum SA, NO ve CRP düzeyleri sırasıyla spektrofotometrik ve nefelometrik metodlarla ölçüldü.

Bulgular: Serum SA düzeyleri, diyabetik ayak grubunda diyabet grubundan daha yüksekti ($p<0.05$). CRP ve NO düzeyleri, diyabetik ayak grubunda diyabet ($p<0.001$ ve $p=0.002$, sırasıyla) ve kontrol grupları ($p<0.001$, her ikisinde) ile karşılaştırıldığında anlamlı olarak daha yüksek bulundu. Diyabetik ayak grubunda, SA ve NO arasında korelasyon olmamasına rağmen ($p>0.05$), serum SA düzeyleri CRP ile anlamlı olarak koreleydi ($p<0.001$).

Sonuç: Diyabetik ayak ülseri olan hastalarda, SA'nın akut faz cevabıyla ilişkili olduğunu ileri sürebiliriz. Artmış serum SA ve NO düzeyleri oksidatif stresin neden olduğu vasküler hasarın göstergeleri olabilir.

Anahtar Kelimeler: Sialik asit, diyabetik ayak ülseri, C-reaktif protein, nitrik oksit

1 Introduction

Diabetes is a huge and growing problem, and the costs to society are high and escalating. International Diabe-

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tes Federation (IDF)'s most recent estimates indicate that 8.3% of adults – 382 million people – have diabetes, and the number of people with the disease is set to rise beyond 592 million in less than 25 years. Yet, with 175 million of cases currently undiagnosed, a vast amount of people with diabetes are progressing towards complications unawares [1]. An estimated 15% of patients with diabetes will develop a lower extremity ulcer during the course of their disease and despite considerable international efforts, foot ulcers continue to be responsible for a high number of lower-limb amputations that are associated with a substantial decrease in quality of life [2,3].

Sialic acid (SA) is a N-acetylated derivative of neuraminic acid that is an abundant terminal monosaccharide of glycoconjugates [4]. Normal human serum SA is largely bound to glycoproteins or glycolipids, with small amounts of free SA [5]. Sialic acid-rich glycoprotein is found mainly in cell membranes, and elevated levels may indicate excessive cell membrane damage, but more specifically for cells of vascular tissue [6].

Additionally, SA forms the terminal component of many acute-phase proteins such as α 1-antichymotrypsin, α 1-antitrypsin, haptoglobin and orosomucoid, and these glycoproteins together explain 70% of the total serum SA concentration [7].

Hyperglycemia favors, through the activation of nuclear factor kappa B (NF- κ B), an increased expression of both NAD(P)H oxidase and inducible isoform of nitric oxide synthase (iNOS). Overexpression of iNOS is accompanied by increased generation of nitric oxide (NO). Superoxide overproduction induced by hyperglycemia, when accompanied by increased NO generation, favors the formation of the strong oxidant peroxynitrite which is cytotoxic, proinflammatory, and can contribute to further vascular damage [8,9].

The levels of SA are known to increase in type 2 diabetes [10,11]. However, there is not enough study concerning the SA levels in patients with diabetic foot ulcer. The present study aimed to evaluate serum SA levels in patients with diabetic foot ulcer and its relationships with NO and C-reactive protein (CRP).

2 Materials and Methods

2.1 Patients

This study was a case control study and the groups of patient and control were as follows: Control group: 22 healthy volunteers (12 males; 10 females)

Diabetes group: 29 patients (14 males; 15 females) with type 2 diabetes who never had foot ulcers

Diabetic foot group: 27 type 2 diabetic patients (15 males; 12 females) with diabetic foot ulcer

The study was conducted in the Department of Endocrinology, Faculty of Medicine, Erciyes University and according to the Declaration of Helsinki. This study was approved by local ethic committee. Informed consents were obtained from all participants. The elimination criteria in healthy volunteers were systemic disease and medication intake. The American Diabetes Association (ADA) criteria were used for the diagnosis of diabetes mellitus. The ulcers in patients with diabetic foot were classified according to Wagner [12] Classification by taking into consideration the examination findings, culture and radiology results. The patients with type 2 diabetes mellitus were evaluated according to their history of myocardial infarction or electrocardiographic findings. The patients with acute infections in diabetes group were excluded from the study by CRP levels. Also recent and current myocardial infarction, end-stage renal failure, a history of thyroid or liver disease or any malignancy were excluded.

2.2 Laboratory analysis

Fasting blood samples were collected into plain tubes and anticoagulated tubes containing EDTA. Anticoagulated whole blood was used for hemoglobin A1c (HbA1c) measurement. After centrifuging the blood samples for 10 min at 2000 $\times$ g, serum glucose and CRP levels were measured on the same day. The separating serum aliquots for NO and SA assays were stored at -70°C. Serum glucose levels were analyzed on an automatic analyzer (Architect c8000, Abbott, USA). HbA1c value was detected with an HPLC system (Agilent 1100, Germany). Serum CRP was measured by using immunonephelometry (Dade Behring, Marburg, Germany). Serum NO levels were measured by spectrophotometric method using Griess reaction (Cayman's nitrate/nitrite colorimetric assay kit, Michigan, USA). SA in serum samples was determined by using the method described by Svennerholm *et al.* [13]. In this method, SA was first dissociated with acid hydrolysis, then resorcinol and Cu²⁺ were used to obtain chromophore. Finally, chromophore was extracted with n-butanol and read at 580 nm. The intra- and interassay coefficients of variation (CV) were 3.6% and 5.5% for SA; 2.7% and 3.4% for NO respectively. The detection limit was 2.5 μ mol/L for NO. Serum CRP, NO and SA levels were given as mg/L, μ mol/L and mg/dL, respectively.

Table 1: Demographic and laboratory findings of patient and control groups.

Variables	Control (n=22)	Diabetes (n=29)	Diabetic foot (n=27)	p value
Age (years)	56.27±6.99	57.24±8.16	59.85±6.79	0.211
Male/Female n (%)	12 (54.5)/10 (45.5)	14 (51.7)/15 (48.3)	15 (55.6)/12 (44.4)	0.512
Diabetic age (years)		11.79±5.61	13.4±6.45	0.311
Glucose (mg/dL)	92.68±7.72	196.27±55.85 ^a	184.7±57.06 ^a	<0.001
HbA1c (%)	5.13±0.82	9.08±2.26 ^a	7.92±1.70 ^{a,b}	<0.001

Data were presented as mean±standard deviation or n (%). ^astatistically significant with respect to control group; ^bstatistically significant with respect to diabetes group.

2.3 Statistical analysis

All statistical analyses were performed using “IBM SPSS Statistics 20” statistical package software. The Shapiro-Wilk test was used to determine whether the distributions of continuous variables were normal. Study groups with a normal distribution were compared with one-way analysis of variance (ANOVA) and those without a normal distribution were compared with the Kruskal-Wallis analysis. Parametric and non-parametric Tukey tests were used for multiple comparisons. Summary statistics of categorical variables were compared with Chi-square exact method. The independent t-test was used to compare the two groups. The descriptive statistics of numerical variables with a normal distribution were presented as mean±standard deviation. The statistics of numerical variables without a normal distribution were presented as medians (25th–75th percentile). The Spearman test was used for correlation analysis. P<0.05 was considered statistically significant.

3 Results

The demographic and laboratory findings of patient and control groups were presented in Table 1. The gender distribution and age of the subjects are similar in all groups. There was no statistically significant difference in terms of diabetic age between the patient groups. Serum glucose and HbA1c levels were significantly higher in both patient groups than in the control group. Additionally, HbA1c levels were found to be significantly lower in diabetic foot group compared to diabetes group (Table 1).

As shown in Figure 1, CRP levels were found to be significantly ($p<0.001$) higher in diabetic foot group [(22.8 (7.14–63.7)] compared to control [3.41 (3.32–3.41)] and diabetes groups [3.34 (3.24–4.21)]. Also nitric oxide levels were significantly higher in diabetic foot group [20.87 (14.67–29.75)] than control [11.02 (9.11–13.45)] and diabetes groups [13.4 (11.57–16.24)] ($p<0.001$ and $p=0.002$, respectively).

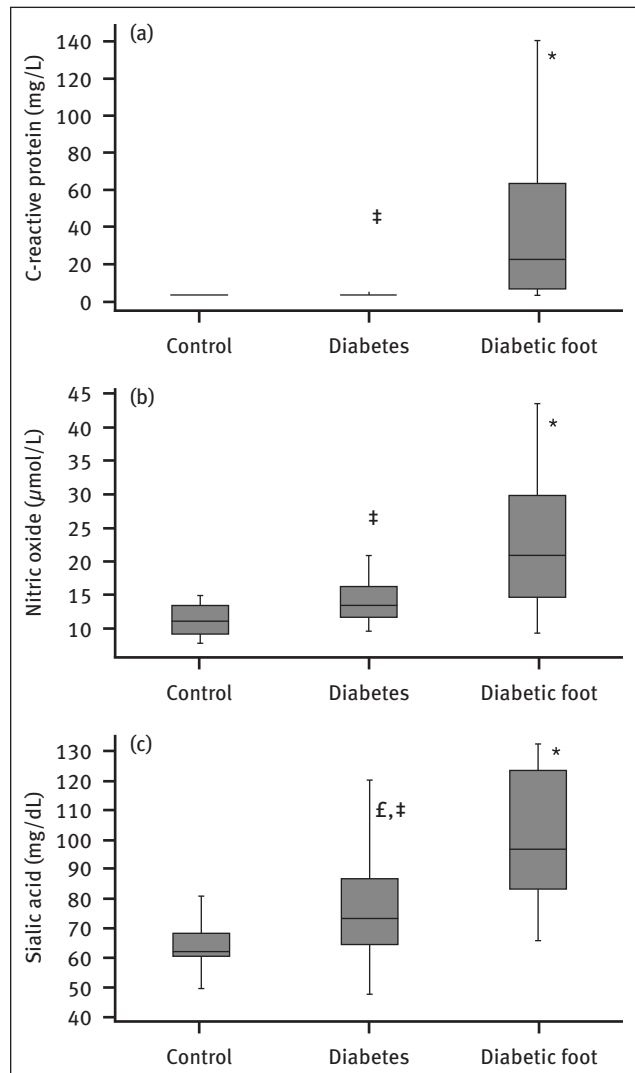


Figure 1: Comparison of serum C-reactive protein (a), nitric oxide (b) and sialic acid (c) levels of patient groups and control group. Data were presented as median (25th–75th percentile). Horizontal lines: median values; coloured: 25th–75th percentile; error bars: 95% confidence intervals. *Diabetic foot vs. control groups, (C-reactive protein $p<0.001$, nitric oxide $p<0.001$, sialic acid $p<0.001$). †Diabetes vs. control groups $p<0.05$ (C-reactive protein $p>0.05$, nitric oxide $p>0.05$, sialic acid $p<0.05$). ‡Diabetes vs. diabetic foot, $p<0.05$ (C-reactive protein $p<0.001$, nitric oxide $p=0.002$, sialic acid $p=0.001$).

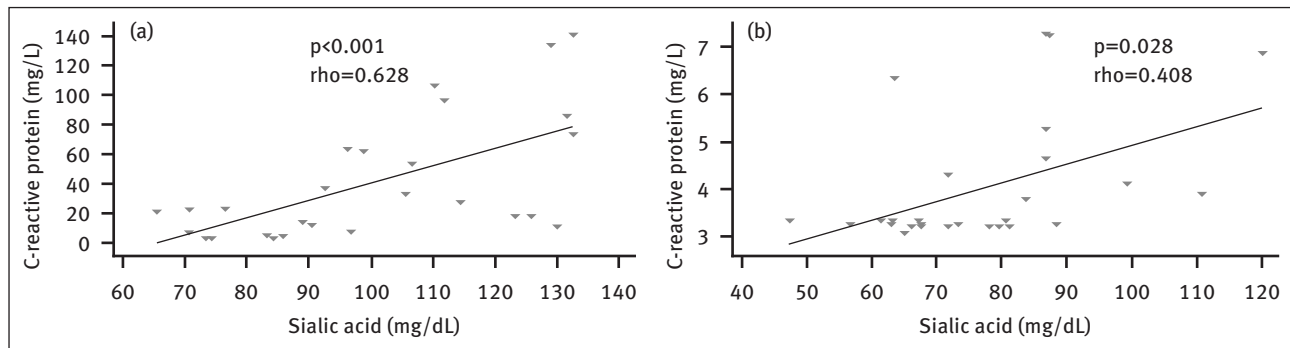


Figure 2: The correlation of sialic acid with C-reactive protein (a) in diabetic foot group and (b) in diabetes group, rho is correlation coefficient.

There was significant difference in SA levels among three groups ($p < 0.001$). Serum SA levels were significantly higher in diabetic foot than the diabetes group [96.77 (83.2–123) vs. 73.3 (64.2–86.9) respectively, $p = 0.001$] and the significant difference was also shown in diabetes group compared to control [73.3 (64.2–86.9) vs. 62.1 (60.2–68.4) respectively, $p < 0.05$]. Additionally, there was a significant difference in sialic acid levels between the patients with diabetic foot ulcer and control group [96.77 (83.2–123) vs. 62.1 (60.2–68.4) respectively, $p < 0.001$].

Although SA levels did not show significant correlations with NO ($p > 0.05$, $\rho = 0.188$) and HbA1c ($p > 0.05$, $\rho = -0.090$), SA was correlated with CRP in the diabetic foot group ($p < 0.001$, $\rho = 0.628$) (Figure 2). There was no correlation between SA and NO, HbA1c, CRP levels in control group ($p > 0.05$). Additionally, SA levels were correlated with CRP ($p < 0.028$, $\rho = 0.408$) (Figure 2), but SA levels did not show significant correlations with other parameters in diabetes group ($p > 0.05$).

4 Discussion

Diabetic foot ulcers may develop as a result of polyneuropathy, ischaemia or subclinical inflammation [14]. It does not occur spontaneously, usually follows some form of trauma, which may go unrealized by the patient. Infection of foot ulcers in diabetic patients is estimated to be the most common cause of diabetes-related admission to hospital [15]. The infected foot ulcer can be painless as a result of neuropathy and thus may lead to delay in requesting medical attention [16].

Serum CRP level represents a very useful non specific inflammatory biomarker and also plays an important role in monitoring the response to treatment and helps in detection of recurrent infection [17]. Ammal *et al.* [18] showed that CRP increases significantly in diabetic

patients with foot ulcers compared to those without foot ulcers. In addition, in diabetic patients with uninfected ulcers, circulating levels of CRP did not significantly differ from those found in diabetic patients [19]. Another study showed that the significant independent predictors for recurrence of diabetic foot ulcers were CRP > 5 mg/L [20].

Sialic acid acts as an integrated marker of a number of acute-phase proteins and is representative of the overall acute-phase response, whereas CRP is one of acute-phase proteins [7]. The association of type 2 diabetes with increased SA could possibly be due to an acute phase response which involves the release of acute phase glycoproteins with SA from the liver into general circulation [21,22]. It has been reported that plasma SA concentration has shown a strong correlation with CRP [23]. Browning *et al.* [24] suggested that SA is a more stable inflammatory marker and a single measurement of serum SA may be the most useful estimate of an individual's habitual inflammatory status. In large population-based cohort study followed for more than 40 years, it has been found that elevated SA, as a marker of systemic inflammation, was independently associated with risk of diabetes and diabetes-related hospitalizations [25].

In present study, we found significantly high CRP and SA levels in patients with diabetic foot ulcer than with diabetes. Although SA levels were higher in patients with diabetes than healthy volunteers, there was not difference in term of CRP. These findings may be related to the use of non high-sensitivity CRP for assay.

Hyperglycemia activates numerous metabolic pathways like polyol, protein kinase C (PKC), advanced glycation end products (AGE), and hexosamine pathway. All these pathways are known to integrate through hyperglycemia mediated mitochondrial ROS production. Oxidative stress and these classical pathways activate transcription factors, resulting in neuroinflammation and vascular impairment. Tissue injury caused by diabetic vascular complications stimulates local cytokine secretions from cells involved in

the complications such as macrophages and endothelium. This induces an acute phase response which involves the release of acute phase glycoproteins [26,27].

The vascular endothelium carries a high concentration of sialic acid and hence extensive microvascular damage associated with diabetes, could account for its shedding into the circulation leading to an increase in SA concentration [28]. Earlier studies have indicated that serum SA levels are elevated in type-2 diabetics with and without nephropathy [11,29]. Nayak *et al.* [27] have shown that serum SA may be used as an inflammatory marker and possible indicator of complications among the Caribbean Type 2 diabetics. In addition, it has been also indicated that SA is an independent risk factor for cardiovascular disease [30,31] and elevated serum SA levels are associated with increased coagulability and higher risk of thrombus formation in type 2 diabetic patients [32]. Some researchers reported that SA levels were elevated in obese subjects and its association with insulin resistance may enhance the cardiovascular risk in these subjects [33,34]. In the literature, to our knowledge there is only one study concerning SA levels in diabetic patients with ischemic disease of the lower extremities and it has been reported that SA reflects the progression of ischemic disease of the lower extremities in type 2 diabetes [35].

We found high levels of SA in patients with diabetic foot ulcer than with diabetes. We suggest that elevated serum SA levels could reflect acute inflammatory reaction due to the strong positive correlation between SA and CRP. Additionally, elevated serum SA levels may be related to oxidative stress-induced vascular damage in patients with diabetic foot ulcer.

Cytokines stimulate iNOS and this isoform produces much larger quantities of NO than the other isoforms. Endothelial nitric oxide synthase (eNOS)-derived NO may protect the vascular wall from atherosclerosis, while the NO arising from iNOS may promote the formation of atherosclerotic lesions [8,9]. Once released into the lumen of the vessel, the NO deriving from the endothelium is oxidized or participates in nitrosylation reactions. An increase in the levels of peroxynitrite have been observed when iNOS is expressed [36]. It has been shown that NO levels increase in diabetes and diabetic retinopathy [37] and associate with severity of diabetic retinopathy [38]. Veves *et al.* [39] reported that the immunostaining of skin biopsies for eNOS was reduced or absent in a higher percentage of patients with the neuropathic and ischemic-neuropathic foot ulcers when compared with the control subjects. Additionally, Jude *et al.* [40] found that plasma NO levels were significantly elevated in patients with active and healed foot ulcers and also reported that

NO within the wound was produced by the infiltrating macrophages and fibroblasts. In another study, it has been found significantly higher plasma nitrite levels in patients with peripheral arterial disease (PAD) than in the healthy subjects and, also there was a linear correlation between these levels and the clinical severity degree. Additionally, no correlation was found between hsCRP and nitrites levels [41].

In present study, it was found that NO levels were higher in patients with diabetic foot ulcer and there was no correlation between SA and NO levels. The increased SA and NO levels may reflect different stages of the inflammation process.

The main limitation of our study is the small number of patients with diabetic foot ulcer. Therefore, we could not classify diabetic foot ulcer according to the presence of infection and/or gangrene. Also we could not determine whether there was difference in SA and CRP levels between the diabetic patients with uninfected foot ulcers and those without foot ulcer in terms of subclinical inflammation. Additionally, as unexpected finding no correlation between SA and HbA1c was found in patient groups.

In conclusion, the results of this study suggest that the increased SA levels are strongly related to acute phase response in patients with diabetic foot ulcer. Additionally, elevated serum SA and NO levels may be indicators of oxidative stress-induced vascular damage. The use of sialic acid in the follow-up patients with type 2 diabetes mellitus may be beneficial in evaluating inflammatory status.

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Conflict of interest: None declared.

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