Serum NOX-2 concentrations and paraoxanase-1 activity in subclinical hypothyroidism: a pilot study

Subklinik hipotiroidizmli hastalarda serum NOX-2 konsantrasyonları ve paraoksonaz-1 aktivitesi: bir pilot çalışma

Abstract

Objective: Subclinical hypothyroidism (SH) is one of the most prevalent endocrine disorders. Although recent data suggest an imbalanced oxidative status in SH, the mechanisms of increased oxidative stress are poorly figured out. The primary goal of this research was to analyze potential sources of ROS and the relationship between serum NOX-2 levels and paraoxonase-1 (PON-1) activity in SH. Serum lipid changes in SH patients which had been determined were compared to healthy control group.

Materials and methods: Thirty-one patients diagnosed with SH and 30 healthy controls were included in the study. The quantitative sandwich ELISA was used for the detection of serum NOX-2 levels. Spectrophotometric method was used to determine serum PON-1 activity.

Results: Higher median serum NOX-2 levels were determined in patients than in the control group (p = 0.004). Lower median serum PON-1 activity was determined in patients as to aforementioned control group (p < 0.0001). As a consequence, no statistically remarkable correlation was identified between PON-1 activity and NOX-2 levels. Triglyceride (TG) concentrations were determined as superior in patients to control group (p < 0.0001).

Conclusion: Over-production of NOX-2 and decreased PON-1 activity contribute to the increased oxidative stress in SH patients. Larger prospective studies required to confirm these findings.

Keywords: Subclinical hypothyroidism; NADPH oxidase; Triglyceride; Paraoxonase; Oxidative stress.

Öz

Amaç: Subklinik hipotiroidi (SH) en yaygın endokrin hastalıklardan biridir. Her ne kadar yeni veriler SH’de dengesiz bir oksidatif durum gösterse de, oksidatif stresin arttığı mekanizmalar henüz iyi çözümlenmiş değildir. Bu araştırmaın temel amacı, reaktif oksidasyon ürünlerinin (ROS) potansiyel kaynaklarını ve SH’deki serum NOX-2 düzeyleri ve paraoksonaz-1 (PON-1) aktivitesi arasındaki ilişkiyi analiz etmektir. Yarı sira SH hastalıına serum lipit değişiklikleri sağlıklı kontrol grubu ile karşılaştırılmaktır.


Bulgarlar: Hastalarda ortanca serum NOX-2 düzeyleri kontrol grubuna göre daha yüksek bulundu (p = 0,004). Kontrol grubuna göre hastalarda düşük median serum PON-1 aktivitesi belirlendi (p < 0,0001). Sonuç olarak, PON-1 aktivitesi ile NOX-2 seviyeleri arasında istatistiksel olarak kayda değer bir korelasyon saptanmadı. Triglerisit (TG) konsantrasyonları kontrol grubundaki hastalardan yüksek bulundu (p < 0,0001).
Introduction

Free radicals are destructive oxygen-derived molecules [1]. There are two major classes of free radicals in biological systems, known as reactive oxygen (ROS) and reactive nitrogen species (RNS) [2]. Over-production of the mentioned substances is related to different endocrine disorders in the human body [3–5]. Subclinical hypothyroidism (SH) is the leading endocrine disorder, which is described as serum TSH greater than the upper limit of normal values in concur with normal circulating free thyroxine (FT4) and free tri-iodothyronine (FT3). SH has an increased cardiovascular disease risk because of dyslipidemia and accelerated endothelial dysfunction [6]. Researches have consistently indicated the presence of the increased oxidative stress in SH patients [7, 8] although there are limited data about the mechanism of increased oxidative stress in SH.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) are the primary sources of abnormal and non-physiological ROS production. Increased NOXs activation and elevated serum concentration are necessary for ROS production [9]. Different NOXs are expressed by the endothelium but NOX-2 is the most important NOX in the context of vascular pathology [10]. Although the importance of NOXs in the regulation of thyroid hormone biosynthesis and ROS production is well known, no studies have examined the relationship between NOX-2 and SH. Paraoxonase1 (PON-1) is a calcium-dependent high density lipoprotein (HDL) associated enzyme, which is primarily synthesized in the liver. PON-1 activity has antioxidant and anti-inflammatory effects including protection against low density lipoprotein (LDL) oxidation [11]. Different studies have evaluated the activity of PON-1 in SH patients [12, 13]. However, data on PON-1 activity are more conflicting. Furthermore, no previous study has examined the relationship between serum NOX-2 level and PON-1 activity. Thyroid hormones regulate the basal metabolic rate and lipid metabolism. An imbalance of thyroid hormone homeostasis has been related to dyslipidemia and some cardiovascular risk factors. Although a confident correlation has been clearly reported among TSH and serum lipids including triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and total amount of cholesterol (TC) [14], discordant findings have been reported on the serum lipid levels in SH patients [15–18].

The objectives of this research were to determine serum levels of NOX-2, PON-1 activity and serum lipids in patients with SH, and identify potential sources of ROS, beside the relationship between serum NOX-2 levels and PON-1 activity in SH. Serum lipid changes in SH patients were also identified and made an extensive comparison with a healthy control group.

Materials and methods

Study population

This study was conducted in Cumhuriyet University, School of Medicine Department of Endocrinology and Metabolism and Department of Biochemistry with the consent of the Local Ethics Committee (Approval number: 2017/10/01). The procedures which had been performed in study protocol including human subjects were conducted with the 1964 Helsinki Declaration in accordance with its ethical standards and its later amendments or measurable ethical standards.

Thirty-one newly-diagnosed SH patients were admitted to this study. Patients with diabetes mellitus, heart failure, cerebrovascular event, liver disease, renal disease, taking medicine including L-T4 or herbal drugs, malignancy, infectious and inflammatory diseases and other chronic or endocrinological diseases were discarded from the study. SH is used to describe an asymptomatic patient with an elevated serum TSH level over the reference range (0.270–4.20 μIU/mL) and a normal FT4 level (0.93–1.7 ng/dL). Borderline values for TSH and FT4 were determined according to kit inserts.

The control group was formed of 30 healthy individuals without known thyroid disease, and no drug use, or infection history (viral or bacterial) that may affect thyroid functions. All the subjects in the control group had normal serum thyroid function test and anti-TPO levels.

Morning blood sample after overnight fasting had been gathered from each participant into red top tubes (Becton Dickinson, UK) after diagnosis. Centrifugation was performed at the following condition, 4°C for 10 min at 4000 rpm and the sera were collected, then frozen within 30 min at −80°C.
Laboratory analyses

Serum concentrations of thyroid stimulating hormone (TSH), FT4, anti-thyroid peroxidase antibody (TPOAb), and insulin were analyzed using Roche Cobas e 601® (Mannheim, Germany). Glucose, TG, TC, LDL-C and HDL-C values were determined using Beckman Coulter DXC 800 UniCel® Autoanalyser (Fullerton, CA, USA). The quantitative sandwich ELISA commercial kit was employed for the determination of serum NOX-2 (LZ Biotech, Shanghai, China). The variation values for intra and inter-assay coefficient were 10 and 12%, respectively. The measurement of the arylerase activity of PON1 was performed using the method described by Eckerson et al. [19], with minor modifications. Paraoxonase activity of PON1 was determined spectrophotometrically using paraoxon (Sigma-Aldrich, St. Louis, MO, USA) as the substrate and measured by rises in the absorbance owing to the formation of 4-nitrophenol. The generation of 4-nitrophenol was monitored spectrophotometrically: 50 μL serum was dissolved in 1 mL Tris-HCl buffer (0.1 mol/L, pH: 7.6) involving 1 mmol/L CaCl₂ and 1 mmol/L paraoxon. Absorbance at 412 nm was measured using a Rayleigh UV 1601 spectrophotometer. Enzyme activity was determined using the molar absorptivity coefficient 17,100 L·mol⁻¹·cm⁻¹. One unit of PON1 activity was described as 1 nmol of 4-nitrophenol formed per minute (U/min) under the above-mentioned assay conditions. Samples of patient and control groups are randomized and studied at the same run in PON1 activity measurement. The inter and intra-assay CV values of PON-1 activity were 2 and 3.1%, respectively.

Statistical analysis

Data analyses were performed by using GraphPad Prism version 7.01 for Windows (GraphPad Software, La Jolla, CA, USA). Data normality was evaluated using q-q graphs, histogram and the D’Agostino and Pearson normality test. For group comparisons of NOX-2, PON-1 activity, TSH, FT4, TC, HDL-C, TG, TPOAb and fasting insulin Mann-Whitney U test was applied. The Student’s t-test with Welch’s correction was used to compare fasting blood glucose in group comparisons. Correlations between quantitative data were assessed with Spearman analysis. p < 0.05 was accepted as statistically significant. Figures were acquired by using BoxPlotR: a web-tool for the generation of box plots (http://shiny.chemgrid.org/boxplotr/).

Results

Statistically substantial differences were monitored in terms of median TG (p < 0.0001), TSH (p < 0.0001), FT4 (p < 0.0001) levels between patient and control groups. No statistically significant difference was observed between the groups in terms of median HDL-C, LDL-C and TC levels. The basic characteristics and concentrations of some laboratory parameters in our study subjects were presented in Table 1. The median NOX-2 levels were determined as 41.00 (37.00–66.00) in the patient group and 29.00 (26.00–76.50) ng/mL in the controls (p = 0.004). The comparisons of the serum NOX-2 levels between the patient and control groups are shown in Figure 1. Median PON-1 activity values were 15.03 (9.32–24.05) and 323.8 (254.70–405.90) U/L in the

Table 1: Basic characteristics and the concentrations of some laboratory parameters in study population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients</th>
<th>Controls</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>31</td>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>Male/Female (n)</td>
<td>2/29</td>
<td>3/27</td>
<td>–</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43 ± 11</td>
<td>38 ± 9</td>
<td>0.16</td>
</tr>
<tr>
<td>TSH (mIU/mL)</td>
<td>7 (6.21–8.19)</td>
<td>1.24 (0.72–2.78)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FT4 (ng/dL)</td>
<td>0.78 (0.66–0.87)</td>
<td>1.13 (0.99–1.37)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FBG (mg/dL)</td>
<td>87.99 ± 9.95</td>
<td>90.03 ± 7.79</td>
<td>0.43</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>185.00 (175.50–213.50)</td>
<td>185.00 (153.50–210.00)</td>
<td>0.23</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>139.00 (103.50–234.00)</td>
<td>86 (65.00–88.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>51.00 (44.50–59.50)</td>
<td>50.00 (44.00–61.00)</td>
<td>0.92</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>108.00 (91.50–120.00)</td>
<td>107.00 (84.90–131.80)</td>
<td>0.98</td>
</tr>
<tr>
<td>anti TPO (IU/mL)</td>
<td>80.00 (16.80–1032.00)</td>
<td>1.1 (0.50–2.35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fasting insulin (μU/mL)</td>
<td>7.45 (5.20–14.42)</td>
<td>6.00 (5.37–7.5)</td>
<td>0.59</td>
</tr>
<tr>
<td>HOMA index</td>
<td>1.69 (1.06–2.96)</td>
<td>1.34 (1.23–1.43)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

FBG, fasting blood glucose; HOMA, homeostatic model assessment; TSH, thyroid stimulant hormone; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TPO, thyroid peroxidase antibody; N/A, not applicable. Results are given as mean ± SD and median (25th–75th percentile) within 95% confidence interval according to normality test result.
Patient and control groups, respectively (p < 0.0001). Comparisons of serum PON-1 activity between the patient and control groups are demonstrated in Figure 2. A positive correlation was found between TC and LDL-C (p < 0.001, r = 0.760). No statistically significant correlations were found among NOX-2 and other biochemical parameters including PON-1, TSH, FT4, TC, TG, LDL-C and HDL-C.

Discussion

The results of this research demonstrated higher median serum NOX-2 levels in SH patients. Previous experimental and clinical studies have indicated both subclinical and clinical hypothyroidism are associated with increased oxidative stress [7, 8, 12, 20, 21]. Malondialdehyde (MDA) and serum protein carbonyl are indicators of oxidative stress. There have been reported that MDA and serum protein carbonyl levels are higher in SH patients in some studies [8, 20]. Sarandöl et al. found increased oxidative stress in Sprague Dawley rats with experimental hypothyroidism [21]. Santi et al. stated that serum thiorbituric acid reactive substance levels were higher in hypothyroidism [22]. In a study by Ates et al. a higher serum total oxidant status level and oxidative status index were observed in SH patients than control group. In the same study, the authors also speculated that the increased ROS production might be related to increased NOX-2 production [7]. The findings of the current study confirm the validity of this hypothesis.

Endothelial dysfunction is one of the characteristics of the pathophysiology of cardiovascular events in SH. Different factors including elevated oxidative stress, decreased nitric oxide (NO•) levels, low grade inflammation and insulin resistance have been proposed to clarify the mechanism of endothelial dysfunction in SH patients [23, 24]. The endothelium and endothelium-derived nitric oxide (NO•) have a pivotal role in the healing of damaged endothelium, and the regulation of cardiovascular and vascular homeostasis. Decreased levels of nitrite and nitrate have been implicated in the course of SH and it has also been revealed that the measurement of NO• level could be useful to evaluate the cardiovascular risk in SH patients [25]. Increased ROS production related with decreased bioavailability of NO• have been reported in SH [10, 26]. Therefore, in the current study, it was believed that decreased NO• bioavailability related with increased NOX-2-derived ROS production contributes to endothelial dysfunction in SH patients.

In our study, decreased serum PON-1 activity was determined in the patient group. There have been conflicting results reported about PON-1 activity in SH patients [12, 13, 27, 28]. In some previous studies, decreased PON-1 activity and increased oxidative stress have been found in subclinical hypothyroidism patients [12, 27]. In the current study, the finding of serum PON-1 activity is in accordance with the aforementioned studies. In our study the differences between healthy controls and SH patients were so higher compared to previous studies [12, 27]. This finding may indicate that PON-1 activity may have potential biomarker to evaluate the future cardiovascular risk in SH patients. However, Milionis et al. and Kebapcilar et al. observed no difference between control groups and SH patients in terms of PON-1 activity [13, 28]. The differences
between studies might be explained by genetic variants and the difference of serum lipid levels between study populations and the diagnosis criteria of SH.

In the current research, there was not any correlation determined between PON-1 activity and NOX-2 levels in the patients. As far as we are concerned this has been the first in vivo study to evaluate the association between serum NOX-2 and PON-1 activity in SH patients. However, previous in vitro studies have reported an association between NOXs and PON-2 [29, 30]. It has been stated that NOX inhibition by diphenylene iodonium causes PON2 silencing and thus leads to inhibition of ROS production in cell cultures [30]. Shiner et al. indicated that there was a positive correlation between NOX and PON-2 expressions [29]. The differences between the aforementioned studies and the current study can be considered to be related to the differences in the nature of the studies and the PON and NOX types examined. There is a need for further studies of larger patient populations to confirm these results.

No significant correlation was determined between TPOAb levels, NOX-2 and PON-1 activity. Some researchers have reported an association between thyroid auto-antibody positivity and oxidative stress [7, 31, 32]. Rostami et al. reported a negative correlation between glutathione, which has an important role in the first line of the antioxidant system and TPOAb levels in Hashimoto’s thyroiditis [32]. Baser et al. found a negative correlation between total oxidant status and anti TG levels in patients with euthyroid autoimmune thyroiditis [31]. Ates et al. found a negative correlation between log (PON-1) and TPOAb levels in patients with subclinical hypothyroidism [7]. In the same study, no significant correlation was determined between TPOAb and total oxidant capacity in patients. These conflicting results might be related to differences in the disease nature and the duration of positivity of thyroid antibody levels in patients.

The change in serum lipid profile in SH has been evaluated in several reports with conflicting results [15–18]. Canaris et al. and Walsh et al. found higher LDL-C and TC levels in SH patients than in euthyroid subjects [15, 18]. Hueston et al. found only increased levels of TC in SH patients compared to euthyroid subjects [16]. In a study made by Bell et al. no differences were found between SH patients and euthyroid patients in terms of TG, LDL-C, HDL-C, and TC levels [17]. In the current study, higher TG levels were determined in SH patients than in the control group. However, no difference was found between the groups in terms of TC, HDL-C and LDL-C levels. The differences between studies might be related to differences in the study population, nutritional habits, the cut-off value of TSH to define SH, and the duration of the disease.

In conclusion, although sample size is relatively small, the results of this study clearly indicate that over-production of NOX-2 and decreased PON-1 activity may contribute to increased oxidative stress in SH patients. It can therefore be speculated that NOX-2 antagonistic substances may be beneficial to decrease NOX-2 related oxidative stress in SH. However, further clinical studies of prospective evaluation of NOX-2 levels after treatment are needed to confirm this hypothesis. SH is only associated with elevated serum levels of TG. Increased TG levels may enhance the cardiovascular risk in untreated SH patients.

Acknowledgement: None.

Conflict of interest: The authors declare no competing interests.

References

20. Torun AN, Kulaksizoglu S, Kulaksizoglu M, Pamuk BO, Isbilen E, Tutuncu NB. Serum total antioxidant status and lipid peroxidation marker malondialdehyde levels in overt and subclinical hypothyroidism. Clin Endocrinol (Oxf) 2009;70:469–74.