ORİJİNAL ARAŞTIRMA ORIGINAL RESEARCH

The Effects of 8-OHdG and TNF-α Levels on the Development of Malignancy in Patients with Hypoactive Thyroid Nodules (Graves' Disease, Hashimoto's Thyroiditis, and Toxic Multinodular Goiter)

Hipoaktif Tiroid Nodülü Olan Hastalarda (Graves Hastalığı, Hashimoto Tiroiditi ve Toksik Multinodüler Guatr) 8-OHdG ve TNF-α Düzeylerinin Malignite Gelişimi Üzerine Etkileri

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ABSTRACT Objective: This study aimed to evaluate 8-OHdG and TNF-α levels in patients with hypoactive thyroid nodules (toxic multinodular goiter, Graves' disease, and Hashimoto's thyroiditis), as these parameters may be related to oxidative stress and the pathogenesis of cancer. **Material and Methods:** The study included patients diagnosed as Graves' disease (n=20), toxic multinodular goiter (n=20), and Hashimoto thyroiditis (n=20), and 20 healthy controls. TNF-α levels were measured in blood samples and 8-OHdG levels were measured in urine-both via ELISA. **Results:** TNF-α and 8-OHdG levels were significantly higher in the patient groups than in the control group ($p<0.05$). In the Hashimoto's thyroiditis patients a correlation was observed between 8-OHdG and thyroglobulin antibodies ($p=0.03$). Carcinoma was detected in 7 of 43 female patients, but not in any of the male patients. No difference was observed in 8-OHdG or TNF-α levels between the patients with and without papillary carcinoma ($p>0.05$). There wasn't a significant difference in 8-OHdG or TNF-α levels between the patients with biopsy results that were benign, malignant, and non-diagnostic ($p>0.05$). **Conclusion:** Serum TNF-α and urine 8-OHdG levels were significantly higher in the patients with thyroid diseases; however, a relationship with cancer was not observed.

Key Words: Thyroid neoplasms; thyroid nodule; thyroid diseases

ÖZET Amaç: Bu çalışma da, tiroid nodülü olan hastalarda (toksik multinodüler guatr, Graves Hastalığı, Hashimoto troiditi) 8-OHdG, TNF-α gibi parametrelerin düzeylerinin değerlendirilmesi amaçlandı. Bu parametreler, oksidatif stres ve kanser patogenezi ile ilişkili olabilir. **Gereç ve Yöntemler:** Graves Hastalığı (n=20), toksik multinodüler guatr (n=20), Hashimoto tiroiditi (n=20) tanısı almış 60 hasta ve 20 sağlıklı birey çalışmaya dahil edildi. TNF-α düzeyleri, bu bireylerin kan örneklerinden; 8-OHdG düzeyleri ise ELİSA Kit ile idrar örneklerinden tetkik edildi. **Bulgular:** 8-OHdG, TNF-α parametreleri, hasta grubunda kontrol grubundan anlamlı olarak yüksek idi ($p<0,05$). Hashimoto tiroiditi olan hastalarda 8-OHdG ile tiroglobulin antikor düzeyleri arasında bir korelasyon saptandı ($p=0,03$). Kırküç kadın hastanın 7'sinde tiroid karsinomu mevcutken erkek hastalar arasında kanserli olgu mevcut değildi. Papiller tiroid kanseri olan ve olmayan olgular arasında 8-OHdG ve TNF-α düzeyleri açısından fark yoktu ($p>0,05$). Biopsi sonucu benign, malign ve nondiagnostik olan hastalar arasında 8-OHdG ve TNF-α düzeyleri açısından istatistiksel olarak anlamlı fark bulunmadı ($p>0,05$). **Sonuç:** Serum TNF-α ve 8-OHdG düzeyleri tiroid hastalığı olanlarda anlamlı olarak yüksek saptandı. Ancak, kanser ile ilişkisi gözlenmedi.

Anahtar Kelimeler: Tiroid tümörleri; tiroid nodülü; tiroid hastalıkları

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Normal cell growth and proliferation are regulated by many signaling pathways controlled by growth factors (i.e. neurotrophins, cytokines, and hematopoietins) in the extracellular environment.[1] Cyto-

kines are soluble proteins that exhibit heterogeneous characteristics and high biological activity, ensuring communication between cells. These proteins play an important role in the onset and continuation of thyroid autoimmunity. Cytokines simulate T-cells and B-cells via cell injury and antibody production.[2,3] TNF-α contributes to the formation of ground HLA class II structures and adhesion molecules in thyroid epithelium cells.[4] Antigen presentation plays a role in the pathogenesis of Graves' disease and other autoimmune diseases. TNF-α is secreted by epithelium, lymphocytes, and fibroblasts in the thyroid gland.[5,6]

Serological factors regulate inflammatory process and are thought to be associated with angiogenesis, which plays a role in the development and progression of thyroid cancer, and other factors affecting the growth.[7] Anomalies observed in cytokine profiles in benign and malignant thyroid disorders indicate inflammatory dysregulation.[8] It has been shown that there is an increase in TNF-α expression in patients with thyroid carcinoma.[6]

A relationship between oxidative DNA damage, and cancer and inflammatory disorders has been reported. DNA, a stable molecule, can also suffer from spontaneous chemical oxidative damage, such as lipids, carbohydrates, and proteins. The primary factors that cause oxidative damage in DNA are ionized radiation, a high oxygen concentration, chemicals undergoing auto-oxidation, xanthine oxidase, and TNF-α.[9] The use of the urine 8-hidroksideoksiguanozin (8-OHdG) level and leukocyte DNA as an indicator of oxidative damage in DNA is useful for evaluating situations associated with carcinogenic risk.[10,11] Similarly, the indicators of increased DNA damage were observed in lymphocytes obtained from patients with autoimmune disease. 8-OHdG levels were high in patients with autoimmune thyroid disorders, such as Graves' disease and Hashimoto's thyroiditis.[12,13]

Iodine deficiency, goitrogenic foods, and autoimmune events lead to diffuse thyroid hyperplasia. Hydrogen peroxide-induced DNA damage causes thyroid hyperplasia, increased proliferation and mutation, and increased mutational load. Some of these spontaneous mutations stimulate growth via ensuring activation of the cAMP cascade. As a result, gene expression in the structures lead to an increase in proliferation of IGF-1, TGF-β1, and epidermal growth factor, for example. The cells form clones by splitting and small clones that carry mutation may start to proliferate via self-simulation. Afterwards, these small focuses are transformed into a nodule formation. Adenomas that secrete TSH can stimulate nodular formation of thyroid tissue via similar mechanisms and in such diseases as Graves' disease and acromegaly.[14]

We aimed to evaluate the markers of oxidative stress and cancer in patients with toxic multinodular goiter, Graves' disease, and Hashimoto's thyroiditis. Furthermore, we investigated the correlation between serum markers and other data (clinical status, the presence of autoantibodies, cytological, histopathological results).

MATERIAL AND METHODS

PATIENTS

The study included 60 patients (43 females and 17 males; mean age: 50.93±13.68 years) with hypoactive thyroid nodules (single or multiple) that were diagnosed as Graves' disease (n=20, GD group), multinodular toxic goiter (n=20, TG group), and Hashimoto's thyroiditis (n=20, HT group), and 20 healthy controls (control group). The study protocol was approved by the Ege University, School of Medicine Ethics Committee. Patients were screened for other possible autoimmune diseases that may be associated with thyroid autoimmunity. The patients and controls were chosen from among non-smokers. All of the participants provided written informed consent to participate. Pregnant women and individuals in which fine needle aspiration biopsy (FNAB) was contraindicated due to such risk factors as bleeding diathesis and anti-aging drug use were excluded from the study.

Thyroid function and thyroid autoantibodies in the patients were investigated, and parenchyma structure of the thyroid gland, the number, size, echogenicity, and activity of their nodules were determined via ultrasonographic and scintigraphic methods. Free T3, free T4, and TSH levels were

measured using the electrochemiluminescence method: the reported reference ranges were as follows: free T3: 3.1-6.8 pmol L^{-1} (2.0-4.4 pg mL^{-1}); free T4: 12-22 pmol L^{-1} (0.93-1.7 ng dL^{-1}); TSH: 0.274.2 μIU mL^{-1} (Roche Diagnostics, GmbH D-68298 Mannheim, Germany). The reference ranges for Anti-Tg Ab and anti TOB Ab were 40 IU mL^{-1} and 35 IU mL^{-1}, respectively, and these parameters were measured via Immulite Anti-Tg Ab and Anti-TPO Ab assay (Immulite 2000 Systems, Siemens, 2009). TSH receptor Ab levels were measured manually using the radioimmunoassay method; values <1 U L^{-1} were accepted as negative, values of 1.1-1.5 U L^{-1} were accepted as positive at limits, and values >1.5 U L^{-1} were accepted as positive (Immunotech, Beckman Coulter Company, France, 2008).

The patients' thyroid glands were imaged ultrasonographically using a high-resolution broadband linear probe (13-5 MHz frequency) and the nodular formations were measured across their long axes (Siemens, Antares, Germany, 2008). For scintigraphic evaluation of the thyroid gland a static image of 400 s 500 $kcount^{-1}$ was obtained by focusing on the thyroid lodge using a pinhole collimator for 20 min after 5 mCi IV injection using pertechnetate Tc 99m (Elscint NM, Apex model SPX4, Israel, 2008). All patients were brought to the euthyroid state and FNAB of hypoactive thyroid nodules in the patients with Graves' disease and toxic multinodular goiter, accompanied by ultrasonography, was performed according to the ATA guide (2006), and the biopsy specimens were cytologically examined. FNAB of hyperactive nodules in the patients with toxic multinodular goiter was not performed. Nodules were considered hypoactive because the patients had Graves' disease. As hypoactive nodules are associated with a high risk of malignancy (5-10%) and hyperactive nodules a much lower risk of malignancy (<1%), hypoactive nodule samples were selected for both groups. All nodules in the Graves' disease patients were hypoactive. Taken biopsy was eligible from hypoactive nodules in toxic multinodular goiter group.

Cytopathological interpretation of FNAB results was evaluated as malignant/benign/non-diagnostic. Patients with suspicious and malignant cytology results underwent surgery (total/near total thyroidectomy). Microcarcinoma was defined as ≤10 mm in any direction. Blood and urine samples (20 cc) were collected to measure indicators of DNA damage (TNF-α and 8-OHdG, respectively).

TNF-α MEASUREMENT VIA ELISA

Blood samples were collected into plain tubes (1.5 mL) and serum was separated via centrifugation at 1500 rpm for 15 min. Samples were stored at -86 °C until analysis. In order to reduce interassay variance all samples were analyzed in 1 assay; repeated freeze-thaw cycles were avoided. Quantitative measurement of TNF-α was performed using the ELISA method (Bender MedSystems, Vienna, Austria), according to the manufacturer's instructions. A value of the triplicate readings for every standard, and the patient and control groups was calculated.

URINARY 8-OHdG MEASUREMENT VIA ELISA

Into each well of an 8-OHdG-coated microtiter plate, 50 μL of fresh urine sample and 50 μL of reconstituted primary antibody were placed, followed by incubation at 37 °C for 1 h for ELISA (NWLSS™ Urine 8-OhdG ELISA Kit, Northwest Life Science Specialties, LLC). Secondary antibody was added to the plate, followed by incubation at 37 °C for 1 h, the unbound enzyme-labeled secondary antibody was removed, and then the antibodies that bound to the plate were identified using a substrate that contained 3,3',5,5'-tetra-methyl-benzidine. Absorbance was measured using a computer-controlled spectrophotometric plate reader (Multiscan FC-Thermo-Scientific) at a wavelength of 450 nm. The concentration of 8-OHdG in the urine samples was interpolated from a standard curve drawn with the assistance of logarithmic transformation. The detection range of the ELISA assay was 0.5-200 ng mL^{-1}. The urinary 8-OHdG level in each participant was normalized by the creatinine level in urine and was expressed as ng mg^{-1} of creatinine.

STATISTICAL ANALYSIS

After determining whether or not numeric values were normally distributed, Student's t-test was

used to compare 2 groups and one-way ANOVA was used to compare ≥3 groups with normally distributed variables. If ANOVA test results were significant, Dunnet's test was then applied. The Mann-Whitney and Kruskal-Wallis tests were used for variables that were not normally distributed; however, chi-square analysis was used for categorical variables by forming cross tables.

RESULTS

THE MARKERS RELATED TO FUNCTIONAL THYROID DISORDERS

The demographic and clinical features of the patient and control groups were shown in Table 1.

The distribution of age and gender in all groups was similar. TSH receptor antibody levels in the GD group were significantly higher than in the TG and HT groups ($p<0.05$). All autoantibodies in the TG group were significantly lower than those in the GD and HT groups. There wasn't a difference in Anti-Tg or Anti-TPO levels between the GD and HT groups. A correlation between thyroglobulin in the HT group was observed, supporting the existence of autoimmunity with 8-OHdG, an indicator of DNA damage ($p=0.03$).

MARKERS RELATED TO PATHOLOGY

Patients in the GD, TG, and HT groups were compared as a whole to the control group in terms of 8-OHdG ¾ an indicator of DNA damage ¾ and TNF-α, which plays a role in inflammatory process. These parameters were significantly higher in combined patient population than in the control group ($p<0.05$) (Table 2).

There wasn't a difference in 8-OHdG or TNF-α levels between the GD, TG, and HT groups. In each of the 3 patient groups a correlation was observed between TNF-α and 8-OHdG levels ($p<0.01$).

Biopsy results were positive for cancer in 7 of the 60 patients (11.6%) and were confirmed via histopathologic examination, as follows: papillary carcinoma (n=5 [8.3%]), papillary microcarcinoma (n=1 [1.6 %]), and papillary follicular variant (n=1 [1.6%]). The distribution of the cytological findings in FNAB specimens were given in Table 3a.

Cytological distribution of FNAB specimens following repetition of non-diagnostics is shown in Table 3b.

Pre-operative cytology and post-operative histology results are shown in Table 4.

All patients that were diagnosed with carcinoma were female (16.3%). Carcinoma was diagnosed in 5% of the patients in the GD group (n=1), 10% of the patients in the TG group (n=2), and 20% of the patients in the HT group (n=4); the difference between the 3 patient groups was not significant. The Graves' disease and Hashimoto's thyroiditis cases were thought to have autoimmune origin, as compared to the toxic multinodular goiter cases in terms of cancer frequency. The combined carcinoma frequency rate in the GD and HT groups was 12.5%, versus 10% in the TG group, and the difference was not significant. Mean age of the patients diagnosed with carcinoma was 44.28±15.05 years, versus 51.81±13.40 years in those without carcinoma; the difference was not significant.

The three patient groups were evaluated to determine if there was a correlation between TNF-α and 8-OHdG levels, and TSH receptor antibodies, thyroglobulin antibody, and thyroperoxidase anti-

TABLE 1: Demographic and clinical features of the patient and control groups.

	Mean Age	Gender	Number of Nodule		Nodule size		Nodule Localization		
	Mean±SD	(F/M ratio)	1	≥2	20 mm ↓	20 mm ↑	Right	Left	Bilateral
GD Group (n=20)	45.1±12.34	12/8	40%	60%	70%	30%	25%	15%	60%
TG Group (n=20)	57.9±13.57	14/6	_	100%	25%	75%	_	_	100%
HT Group (n=20)	49.70±12.49	17/3	45%	55%	60%	40%	20%	25%	55%
All Patients Combined (n=60)	50.93±13.68	43/17	28,30%	71,70%	51,70%	48,30%	15%	13,30%	71,70%
Control (n=20)	48.05±11.91	15/5							

TABLE 2: Comparison of TNF-α and 8-OHdG levels in the patients and control group.

	All Patients Combined	Control Group	p
8-OHdG (ng mL^{-1} of creatinine) (mean ± SD)	22.26±12.41	5.11±2.44	p<0.05
TNF-α (ng mL^{-1}) (median ± IR)	4.81±72.54	6.01±3.17	p<0.05
n	60	20	-

TABLE 3a: Distribution of cytological findings in hypoactive thyroid nodule FNAB specimens, according to groups.

Biopsy	GD Group	TG Group	HT Group
Benign	95% (n=19)	70% (n=14)	65% (n=13)
Malignant	5% (n=1)	10% (n=2)	10% (n=2)
Non-diagnostic	-	20% (n=4)	25% (n=5)

TABLE 3b: Cytological distribution of FNAB specimens following repetition of non-diagnostics.

Biopsy	GD Group	TG Group	HT Group
Benign	95% (n=19)	90% (n=18)	80% (n=16)
Malignant	5% (n=1)	10% (n=2)	20% (n=4)

TABLE 4: Pre-operative cytology and post-operative histology results.

	Pre-operative cytology	Post-operative malignant	Post-operative benign
Indeterminate	n=11	2/11	9/11
Malignant	n=5	5/5	-

bodies. There was a correlation between thyroglobulin in Hashimoto's thyroiditis patients (HT group), supporting the existence of autoimmunity with 8-OHdG, an indicator of DNA damage (p=0.03). TNF-α and 8-OHdG levels were not correlated with each other in the patient groups. There wasn't a correlation between TNF-α or 8-OHdG levels, and nodule dimension or the number of nodules. Additionally, there wasn't a correlation between cytological findings in FNAB specimens (malignant/benign/non-diagnostic), and the number of nodules or nodule dimension. There wasn't a relationship between patient age, and the number or dimension of nodules, nor between patient age, and TNF-α or 8-OHdG levels. A statistically significant difference was not observed in TNF-α or 8-OHdG levels between the patients with and without papillary carcinoma, nor was there a difference in TNF-α or 8-OHdG levels between the patient groups with biopsy results that were benign, malignant, or non-diagnostic.

DISCUSSION

Most thyroid nodules are histologically diagnosed as thyroid adenoma and differentiated from thyroid cancer. These lesions differ from autonomous functional thyroid nodules ¾ including TSHR mutations ¾ because they are associated with many disorders of genetic origin.[15] Functional disorders associated with cold thyroid nodules are caused by failed iodine transport or organification of iodine. Reduced expression of Na/I carrier system (NIS) and consequent insufficient iodine transport are observed in thyroid cancers, as well as in benign cold thyroid nodules.[16] The present study aimed to investigate the relationship between the indicators of inflammation, DNA damage, and cancer pathogenesis, and the disease and the nodule in patients with thyroid diseases of autoimmune origin having hypoactive thyroid nodule and with toxic multinodular goiter.

In the present study the TG group had the highest mean age, whereas the GD group has the lowest, as previously reported. There were more females than males in each of the 3 patient groups. Nodular disease was observed 5-15 times more in the female patients; therefore, genetic predisposition and the effect of steroid hormones are considered causal factors. The growth stimulation effect of estrogen was reported in thyroid cancer cell lines in rats in vitro.[17,18]

It was reported that the prevalence of thyroid autoimmunity was correlated with increased age, and thyroid nodularity;[19,20] however, no correlation was observed between thyroid autoimmunity, and patient age or thyroid nodularity in the present study. Some studies reported that there is a relationship between oxidative DNA dama-

ge, and cancer and inflammatory diseases. An increase in the formation of oxygen radicals and a reduction in antioxidant enzyme levels, and/or the existence of a defect in DNA repair mechanisms lead to an increase in oxidative DNA damage.[21,22] Human studies showed that DNA damage is an important mutagenic and carcinogenic factor.[23] It is thought that autoimmune thyroid diseases are associated with oxidative stress and 8-OHdG levels in mononuclear cell cultures obtained from patients with Graves' disease and Hashimoto's thyroiditis. In the present study a correlation between the 8-OHdG level and thyroglobulin antibodies was observed, which supports the existence of autoimmunity in patients with Hashimoto's thyroiditis ($p=0.03$).

The level of 8-OHdG in patients with Graves' disease and Hashimoto's thyroiditis, which were untreated, was significantly higher than in healthy controls.[24] Thyroid hyperplasia increased the proliferation together with possible DNA damage due to H_2O_2 action. Similarly, in the present study 8-OHdG, an indicator of DNA damage, was significantly higher in the patients with Graves' disease and Hashimoto's thyroiditis than in the controls ($p<0.05$).

Cytokines are important for the onset and continuation of thyroid autoimmunity, and contribute to the formation of benign and malignant thyroid diseases.[2,3,8] In cases of thyroid carcinoma an increase in TNF-α expression was observed.[2,3,8] Its expression also increased in the cases diagnosed with thyroid carcinoma.[9] Research has shown that cytokines play a role in events related to thyroid autoimmunity.[25-28] Thyroid dysfunction is accompanied by remarkable activation of the TNF-α system. Nielsen et al. reported that TNF-α response in patients with Hashimoto's thyroiditis and Graves' disease was higher than that in healthy controls.[29] Nonetheless, some studies reported that there isn't a relationship between autoimmune diseases and TNF-α.[4,5,28,30-32] In the present study the level of TNF-α in the patient groups was significantly higher than that in the control group ($p<0.05$).

In the present study there wasn't a strong correlation between TNF-α or 8-OHdG levels, and patient age, nodule dimension, and the number of nodules. Additionally, there wasn't a significant difference in TNF-α or 8-OHdG levels between the patients with and without papillary carcinoma that had fine-needle aspiration biopsy results that were benign/malignant/non-diagnostic, or between those diagnosed as Graves' disease, toxic nodular goiter, and Hashimoto's disease. Fiore et al. reported that there was a correlation between thyroid cancer and autoimmunity, whereas another study indicated that thyroid autoimmunity might confer resistance to thyroid carcinoma.[33,34]

In different studies was reported that patients with toxic multinodular goiter had cancer ratio in hypoactive nodules respectively, 7%, 9%, and 21%.[33,35-37] However, the frequency of cancer in patients with non-toxic multinodular goiter was 6.2-9.7% and 10.58% in 2 different studies.[37-39] Studies have reported evidence for and against an increase in cancer development in hypoactive nodules on the basis of autoimmune thyroid disease.[40,41] In the present study no difference was observed in cancer formation between diseases of autoimmune origin (Graves' disease and Hashimoto's thyroiditis) and toxic multinodular goiter. Similarly, differences in the frequency of thyroid cancer in patients with toxic and non-toxic multinodular goiter, according to gender, were reported.[37,42-44]

When the relationship between age and thyroid cancer was examined, Baier et al. observed that cases with malignant cytology were younger (age ≤45 years) and non-diagnostic cytology was more frequent in patients aged >75 years;[43] however, in the present study no correlation was observed between patient age and benign/malignant cytological diagnosis. Raparia et al. reported that the cancer frequency rate was 19% in cases with nodule dimensions <2 cm and 47% in cases with nodule dimensions >2 cm.[44] In the present study there wasn't a relationship between cancer and nodule size, as there was a limited number of cancer cases. The results of thyroid FNAB (malignant, benign, and non-diagnostic) can vary due to many

factors. Nodule dimension, nodule pattern (single or multinodular), individual factors (endocrinologist, pathologist), and FNAB methods (whether or not it is performed with USG) are the most important factors. Ultrasonographic findings of microcalcifications, hypoechogenicity, irregular margins, absence of a halo, predominantly solid composition, and increased intranodular vascularity are associated with malignant thyroid nodules.[42-47] In the present study a high malignancy rate (11.6%) was observed, but reported cytological diagnosis (benign/malignant/non-diagnostic) findings are inconsistent.

The present study aimed to determine if high serum TNF-α and 8-OHdG levels in patients with hypoactive nodules ¾ as a measure of carcinogenic potential ¾ could be used as a marker for the early detection of cancer. Although serum TNF-α and 8-OHdG levels in the patients with thyroid disorders were high, their relationship with the development of cancer was not clear. This outcome may have been due to the limited number of the patients with cancer. Additional researchs are warranted in order to further delineate the relationship between TNF-α and 8-OHdG levels, and the development of cancer in patients with thyroid disorders.

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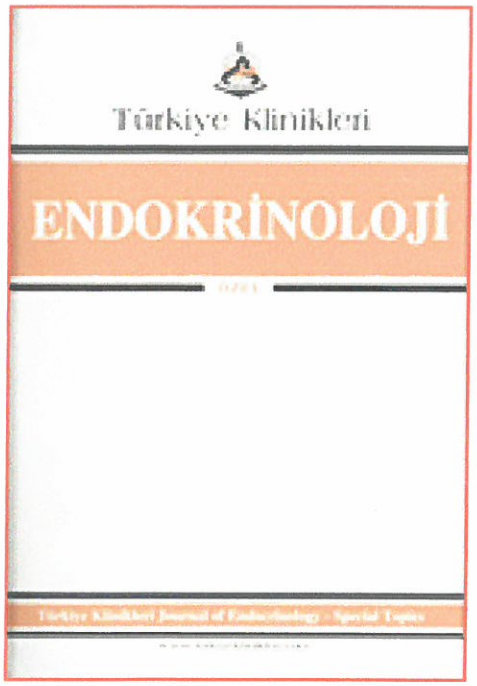

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