



The effect of thyroid hormone status on selected antioxidant parameters in patients with Graves' disease and active thyroid-associated orbitopathy

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Abstract

Introduction: Oxidative stress has been implicated in the pathogenesis of thyroid-associated orbitopathy (TAO) in patients with Graves' disease (GD). This study assessed the effect of thyroid hormone abnormalities on selected antioxidant parameters in patients with active TAO.

Material and methods: The study group consisted of 56 patients with GD and active TAO treated with antithyroid medication. Depending on the thyroid hormone level, they were subdivided into two groups: Group 1 — hyperthyroid patients (n = 34) and Group 2 — euthyroid patients (n = 22). The total oxidant status expressed as the ferric reducing ability of plasma (FRAP) as well as selected enzymatic and non-enzymatic components of the antioxidant system, including the activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), and paraoxonase 1 (PON-1), as well as the levels of vitamin C, uric acid, and lipid peroxidation products: malondialdehyde (MDA) and conjugated dienes (CD) were assessed in all enrolled participants.

Results: The FRAP values in Group 1 were significantly higher than in controls. The FRAP values in Group 2 were lower than in Group 1 and higher than in controls. However, the differences were not significant. In Group 1, the activity of SOD and GPx, as well as serum levels of uric acid, MDA, and CD, were significantly higher than in controls. At the same time, serum PON-1 activity and vitamin C levels were significantly lower in Group 1 than in controls. In Group 2, the SOD activity as well as MDA and CD levels were non-significantly lower than in Group 1 and non-significantly higher than in controls. The activity of GPx in euthyroid patients with TAO was significantly higher than in controls.

Conclusions: Hyperthyroidism is a significant contributor to oxidative stress in patients with active TAO, which manifests as upregulated lipid peroxidation and antioxidant system activation. Euthyroid state restoration leads to a relative reduction in activity and levels of most studied antioxidant parameters, which still remain above the normal values. The autoimmune inflammation of the orbital tissue seems to be a thyroid hormone status-independent modifier of oxidative stress. (*Endokrynol Pol* 2020; 71 (5): 418–424)

Key words: antioxidant parameters; hyperthyroidism; Graves' disease; lipid peroxidation; thyroid orbitopathy

Introduction

Graves' disease (GD) is a chronic autoimmune condition and the most common cause of hyperthyroidism in iodine-sufficient areas [1]. Thyroid hormone overproduction is caused by activation of the TSH receptor (TSHR), which is an autoantigen present on thyrocytes, by the TSH receptor antibodies (TRAb) [2–4]. Thyroid hormone synthesis utilises hydrogen peroxide (H₂O₂) generated by dual oxidases (DUOX1 and DUOX2) and thyrocyte apical membrane enzymes, and its excess is inactivated by the antioxidant enzymes within the thyrocyte [5]. Hyperthyroidism causes increased de-

mand for oxygen and its excessive use and impairs the mitochondrial respiratory chain, thus increasing the use of ATP (adenosine triphosphate), which results in excessive production of reactive oxygen species (ROS) [6]. ROS cause damage to the cell membrane through the peroxidation of lipids and proteins, leading to a loss of their function and enzymatic activity [7].

Thyroid-associated orbitopathy (TAO) is the most common extrathyroidal manifestation of Graves' disease, with an annual incidence rate of 42.2/million [8]. TAO is classed as a rare disease. According to Laurberg et al., the annual incidence rate of moderate-to-severe TAO (assessed as per EUGOGO [European Group on



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Table 1. Demographic and clinical characteristics of patients with active thyroid-associated orbitopathy (TAO) in hyperthyroid (Group 1) and euthyroid (Group 2) state

	Age (years)	Gender male/female	TRAb [IU/L]	TSH [μ IU/mL]	FT4 [ng/dL]	CAS	NOSPECS
Group 1 (n = 34)	53 (SD = 8.4)	6/28 (17.6/82.4%)	24.559 (SD = 57.432)	0.05 (SD = 0.0077)	4.116 (SD = 9.542)	5.714 (SD = 0.956)	4.048 (SD = 1.117)
Group 2 (n = 22)	48 (SD = 12.9)	4/18 (18.2/81.8%)	7.698 (SD = 14.726)	3.121 (SD = 4.481)	1.168 (SD = 0.479)	5.667 (SD = 1.328)	4.118 (SD = 0.781)

TRAb — TSH receptor antibodies; CAS — Clinical Activity Score; NOSPECS — classification of severity of Graves' orbitopathy; SD — standard deviation

Graves' Orbitopathy] criteria) is 16.1/million (women: 26.7; men: 5.4; 4.9% of all patients with GD), with no changes seen after iodine fortification of salt [9]. Another study found similar prevalence of moderate-to-severe TAO [10].

The pathophysiology of TAO is complex and has not been fully explained to date. Orbital fibroblasts show expression of TSHR, which is the main target of autoimmunity [2, 11]. The role of stimulating antibodies against the IGF-1 receptor (IGF-1R) located on the fibroblast surface [12, 13], antibodies against extraocular muscle antigens, or acetylcholine receptor (AChR) antibodies [14] in TAO has also been mentioned. T-cell activation induced by orbital receptor stimulation by the target antibody results in orbital tissue infiltration, triggering a cascade of events which leads to the production of ROS, cytokines, growth factors including proangiogenic factors and inflammatory mediators, many of which act as potent stimulators of glycosaminoglycan accumulation and oedema formation [15–21]. Despite our improving understanding of the role that oxidative stress plays in TAO, there is still much controversy around this subject.

Material and methods

A group of 82 patients referred to the Department of Endocrinology and Neuroendocrine Tumours of the Medical University of Silesia in Katowice with Graves' disease with concomitant TAO were assessed. Out of those, 56 patients with GD diagnosed no later than six months prior to enrolment, and first onset of clinically active TAO were enrolled in the study group. All patients were treated with an antithyroid agent (thiamazole) during the course of the study. Thirty-four patients (Group 1) were still hyperthyroid, whereas the remaining 22 (Group 2) were euthyroid. The control group (Group C) consisted of 20 healthy volunteers matched for age and gender distribution, without clinical features of thyroid disease or thyroid hormonal imbalance. The epidemiological and clinical characteristics of the study group are shown in Table 1.

In the study group, the inclusion criteria were as follows: GD diagnosis confirmed with laboratory tests (TSH, FT4, TRAb) and diagnostic imaging (thyroid ultrasound), clinically active TAO (Clinical Activity Score, CAS > 3) confirmed during an ophthalmic examination, and active orbital tissue inflammation confirmed with magnetic resonance imaging (MRI). All patients enrolled in the study group had clinically active TAO with NOSPECS class 2 to 5, meeting the criteria for moderate-to-severe according to the classification of EUGOGO [22]. The exclusion criteria were as follows:

other orbital diseases, other immune and inflammatory conditions, liver impairment, and previous immunosuppressive treatment. Following an overnight fast, peripheral blood samples (10 mL) for biochemical determinations were collected in the morning. The blood was centrifuged, and the obtained serum and cell mass were stored at -70°C until further biochemical analyses.

The ferric reducing ability of plasma (FRAP) was determined according to the method described by Benzie and Strain [23] using the Technicon RA-XT™ analyser (Technicon Instruments Corporation, USA). The enzymatic activity of superoxide dismutase (SOD) in haemolysed red blood cells was determined by kinetic spectrophotometry according to Oyangui [24] using standard Randox kits.

The enzymatic activity of glutathione peroxidase (GPx) was determined by the kinetic-spectrophotometric method according to the manufacturer's instructions of the Bioxytech kit. The assay utilises the method of Paglia and Valentine [25].

Serum paraoxonase 1 (PON-1) activity was determined by kinetic spectrophotometry according to Eckerson et al. [26] with paraoxon (o,o-diethyl-o-[p-nitrophenyl]-phosphate) as the substrate in glycine/NaOH buffer (pH 10) containing calcium chloride as a PON-1 activator.

The concentration of vitamin C was determined by spectrophotometry [27] and the concentration of uric acid by the enzymatic method with uricase, using the Emapol reagent kit and EM-280 biochemical analyser, respectively (both Emapol, Gdańsk, Poland). In order to evaluate the intensity of lipid peroxidation, the serum concentration of malondialdehyde (MDA) was determined by spectrofluorimetry in reaction with thiobarbituric acid according to the procedure described by Wąsowicz et al. [28]. Additionally, the serum concentration of conjugated dienes (CD) was determined using spectrophotometry according to the procedure described by Corongiu et al. [29]. A Shimadzu A160 spectrophotometer (Shimadzu, Japan) was used for all measurements.

The statistical analyses were carried out using Statistica 10.0 software. Basic descriptive statistics were computed for the studied parameters, including the median, minimum, and maximum values, as well as lower (Q25) and upper (Q75) quartiles. Having verified the normality of distribution assumption using the Kolmogorov-Smirnov test, the Kruskal-Wallis one-way analysis of variance (ANOVA) was used due to unequal group count. For all comparisons, a p-value < 0.05 was considered significant.

All patients gave their formal written consent to participate in the study, which followed the tenets of the Declaration of Helsinki. The study protocol was approved by the Bioethical Board of the Medical University of Silesia (KNW/0022/KB/147/13, NN-6501-34/06).

Results

The FRAP values in Group 1 were significantly higher than in controls (Me: 658 vs. 515 $\mu\text{mol/L}$; $p < 0.05$). The FRAP values in Group 2 were lower than in Group 1 and higher than in controls. However, the differences were not significant (Tab. 2).

Table 2. The ferric reducing ability of plasma (FRAP) in the control group (Group C) and in patients with active of thyroid-associated orbitopathy (TAO) in hyperthyroid (Group 1) and euthyroid (Group 2) state

	n	FRAP [$\mu\text{mol/L}$]					p			
		Min	Me	Max	Q25	Q75	C-1	C-2	1-2	ANOVA
Group C	20	350	515	725	489	591				
Group 1	34	360	658	1125	562	758	< 0.05	NS	NS	0.0001
Group 2	22	320	576	985	452	775				

NS — non-significant

Table 3. The activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), and paraoxonase 1 (PON-1) in the control group (Group C) and in patients with active thyroid-associated orbitopathy (TAO) in hyperthyroid (Group 1) and euthyroid (Group 2) state

	n	Min	Me	Max	Q25	Q75	C-1	C-2	1-2	ANOVA
SOD [NU/gHb]										
Group C	20	845	1250	1800	1110	1420				
Group 1	34	1250	1620	2100	1530	1699	< 0.05	NS	NS	0.0001
Group 2	22	1500	1520	1820	1375	1657				
GPx [IU/gHb]										
Group C	20	25	57	110	46	71				
Group 1	34	60	100	390	80	132	< 0.05	< 0.05	NS	0.0001
Group 2	22	35	88	320	63	112				
PON-1 [IU/L]										
Group C	20	165	296	595	246	421				
Group 1	34	80	222	350	166	266	< 0.05	NS	NS	0.0001
Group 2	22	80	265	475	179	365				

NS — non-significant

In Group 1 the, activity of SOD and GPx was significantly higher than in controls (Me: 1620 vs. 1250 NU/gHb; $p < 0.05$ and Me: 100 vs. 57 IU/gHb; $p < 0.05$, respectively), whereas the activity of PON-1 was significantly lower than in controls (Me: 222 vs. 296 IU/L; $p < 0.05$). In Group 2, the SOD activity was lower than in Group 1, but the difference was not significant. In Group 2 the activity of GPx was significantly higher than in controls (Me: 88 vs. 57 IU/gHb; $p < 0.05$) (Tab. 3).

In Group 1, the vitamin C levels were significantly lower than in controls (Me: 54 vs. 65 $\mu\text{mol/L}$, $p < 0.05$). However, there was no significant difference in vitamin C levels between Group 2 and either Group 1 or controls. In Group 1, the serum levels of uric acid were significantly higher than in both Group 2 and controls (Me: 6.23 vs. 5.32 and 4.65 mg/dL, $p < 0.05$) (Tab. 4).

In Group 1, the MDA and CD levels were significantly higher than in controls (Me: 3.91 vs. 3.2 $\mu\text{mol/L}$, $p < 0.05$ and Me: 141 vs. 114 $\mu\text{mol/L}$, $p < 0.05$, respectively). In Group 2, the MDA levels were also significantly higher than in controls (Me: 4.5 vs. 3.2 $\mu\text{mol/L}$, $p < 0.05$). There was no significant difference in the

measured lipid peroxidation markers between groups 1 and 2 (Tab. 5).

Discussion

Hubner et al. were the first to suggest the role of oxidative stress in hyperthyroidism. They demonstrated an increase in the enzymatic activity of glucose-6-phosphate dehydrogenase in red blood cells of patients with hyperthyroidism. It returned to normal values as the patients were in the euthyroid state [30].

Acarsu et al. demonstrated significantly higher MDA levels in euthyroid patients with GD and active TAO as compared to euthyroid patients with GD without TAO and to controls. There were, however, no significant differences in MDA levels between the two latter groups, which indicates that restoring euthyroid status normalises oxidative stress parameters in the absence of TAO [31]. Another study demonstrated significantly higher levels of MDA and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the tears of patients with GD with and without TAO as compared to controls. There was also a difference between patients with and without TAO,

Table 4. The concentration of vitamin C and uric acid in the control group (Group C) and in patients with active thyroid-associated orbitopathy (TAO) in hyperthyroid (Group 1) and euthyroid (Group 2) state

	n	Min	Me	Max	Q25	Q75	C-1	C-2	1-2	ANOVA
Vitamin C [$\mu\text{mol/L}$]										
Group C	20	43	65	91	56	71				
Group 1	34	30	54	43	50	58	< 0.05	NS	NS	0.0001
Group 2	22	32	57	107	48	75				
Uric acid [mg/dL]										
Group C	20	3.2	4.65	6.5	3.98	5.22				
Group 1	34	2.8	6.23	11.5	5.61	7.21	< 0.05	< 0.05	NS	0.0001
Group 2	22	3.1	5.32	12.5	4.23	6.21				

NS — non-significant

Table 5. Malondialdehyde (MDA) and conjugated diene (CD) levels in the control group (Group C) and in patients with active thyroid-associated orbitopathy (TAO) in hyperthyroid (Group 1) and euthyroid (Group 2) state

	n	Min	Me	Max	Q25	Q75	C-1	C-2	1-2	ANOVA
MDA [$\mu\text{mol/L}$]										
Group C	20	2.7	3.21	4.5	2.98	3.51				
Group 1	34	2.95	4.5	9.25	3.7	6.15	< 0.05	< 0.05	NS	0.0001
Group 2	22	2.2	3.915	7.8	3.185	4.925				
CD [$\mu\text{mol/L}$]										
Group C	20	70	114	165	101	131				
Group 1	34	95	141	235	120	164	< 0.05	NS	NS	0.0001
Group 2	22	70	125	205	104	143				

NS — non-significant

with the former presenting elevated levels of these substances. Furthermore, a correlation was demonstrated between CAS and MDA as well as 8-OHdG levels in patients with TAO [32]. The results of our study are in agreement with these findings. We found significantly higher levels of MDA and CD in hyperthyroid patients with TAO than in euthyroid patients with TAO or in controls.

Bednarek et al. found increased activity of SOD and CAT, reduced activity of GPx and glutathione reductase (GR), as well as elevated ceruloplasmin (CP) levels in patients with GD and TAO as compared to controls, whereas there was no difference in the activity of SOD, CAT GPx, GR, and CP levels between patients with GD without TAO and controls [33]. This supports our finding that that autoimmune orbital inflammation is a thyroid- and metabolism-independent source of intensive oxidative reactions.

In another study, a significantly increased activity of SOD and GPx was demonstrated in patients with hyperthyroidism with and without TAO, as compared to controls. Interestingly, restoring euthyroid status normalised these parameters only in the absence of TAO [34].

The results of our study are consistent with the above. We demonstrated significantly increased activity of SOD and GPx in patients with hyperthyroidism and TAO, as compared to controls. In euthyroid patients with TAO, the SOD activity was non-significantly lower than in those with hyperthyroidism and non-significantly higher than in controls. The activity of GPx in euthyroid patients with TAO was significantly higher than in controls. Therefore, our results support previous findings [34, 35] that excessive thyroid hormone production plays an important role in activating the enzymatic component of antioxidant system.

Paraoxonase 1 is an antioxidant enzyme associated with high-density lipoprotein (HDL), which protects both low-density lipoprotein (LDL) and HDL against oxidative modification. Its activity negatively correlates with levels of oxidative stress parameters measured in serum and macrophages [36]. Lipid oxidation may play an important role in the development of micro- and macro-vascular disease and focal hypoxia [37]. Ikeda et al. found that serum PON activity was one of the significant factors for microvascular and ischaemic complications [38]. In vitro, PON-1 hydrolyses a large variety of endogenous or exogenous substrates, some

of which are clearly involved in the progression of arteriosclerosis. A close relationship between PON-1 deficiency and accelerated progression of arteriosclerosis has been found in animal models [39].

Azizi et al. demonstrated decreased activity of PON-1 in both hyperthyroidism and hypothyroidism [40]. Other researchers found that decreased PON-1 activity in patients with hyperthyroidism returns to normal after the euthyroid state has been restored [41, 42] and demonstrated a positive correlation between TSH levels, PON-1 activity, and total cholesterol levels [43]. To date, only one study has been published to assess the PON-1 activity in euthyroid patients with GD and TAO. It demonstrated significantly lower PON-1 activity in subjects with TAO as compared to controls, with euthyroid state not affecting PON-1 activity levels [44]. To the best of our knowledge, ours is the first study to assess the serum PON-1 activity in patients with GD and TAO depending on their thyroid hormone status. We demonstrated significantly lower PON-1 activity in hyperthyroid patients with TAO as compared to controls. However, in euthyroid patients with TAO, the PON-1 activity was non-significantly lower than in controls. Decrease activity of PON-1 in the group with active TAO indicates an oxido-reduction imbalance leading to increased lipoprotein oxidation and exhaustion of protective antioxidative effect of PON-1 due to increased oxidative stress.

Uric acid is the end product of purine metabolism. It is considered to be the main antioxidant in human serum, which scavenges singlet oxygen, peroxy radicals (RO_2), and hydroxyl radicals (HO) [45]. In patients with active TAO, uric acid levels depend on their thyroid hormone status and oxidative stress severity. Ye et al. found no significant differences in serum uric acid levels between euthyroid subjects, those with subclinical hypothyroidism, and those with subclinical hyperthyroidism [46]. Liu et al. demonstrated higher serum uric acid levels in hyperthyroid patients with GD than in controls and significantly lower serum uric acid levels in hypothyroid patients than in controls [47]. Euthyroid state restoration was associated with a significant reduction of serum uric acid levels from baseline [48].

There are no studies to evaluate serum uric acid or vitamin C levels in patients with GD with active TAO. In our study, we demonstrated significantly higher serum uric acid levels in hyperthyroid patients with TAO than in euthyroid patients with TAO or controls. We also showed that serum uric acid levels did not return to the normal range after euthyroidism had been restored. Seven et al. demonstrated significantly lower vitamin C levels in subjects with hyperthyroidism as compared to controls. They

also found that the oxidative stress levels, assessed using the TBARS/GSH (thiobarbituric acid reactive substances/glutathione) ratio, were reduced during vitamin C supplementation [49]. Rotondo Dottore et al. evaluated the effect of vitamin C on fibroblasts harvested from patients with GD with active TAO exposed to oxidative stress. They demonstrated significantly decreased levels of glutathione disulphide (GSSG), which is a marker of oxidative stress, as well as inhibition of excessive fibroblast proliferation after the specimens were incubated in a vitamin C-containing mixture [50]. In our study, vitamin C levels were significantly lower in hyperthyroid patients with active TAO than in controls. Similarly, the vitamin C levels were lower in euthyroid patients with active TAO than in controls, but the difference was not significant. It seems that the decreased vitamin C levels in patients with TAO may be caused by its increased demand and excessive use during long-term severe oxidative stress during which no additional supplementation has been provided. Vitamin C supplementation commencing at the diagnosis of hyperthyroidism in patients with GD may improve the effect of anti-inflammatory treatment with glucocorticoids, reducing excessive proliferation of orbital fibroblasts in those with active TAO.

Systemic corticosteroids remain the first line of treatment in patients with TAO, although new methods of treatment are being tried such as biological drugs or anti-thymocyte globulin administration [51–53]. Previous studies have also demonstrated that corticosteroid treatment normalises the levels and activity of redox system parameters, which supports their efficacy in oxidative stress reduction in patients with GD and active TAO [33].

Conclusions

Hyperthyroidism is a significant contributor to oxidative stress in patients with active TAO, which manifests as upregulated lipid peroxidation and antioxidant system activation. Euthyroid state restoration leads to a relative reduction in activity and levels of most studied antioxidant parameters, which remain above the normal values. The autoimmune inflammation of the orbital tissue appears to be a thyroid hormone status-independent modifier of oxidative stress.

Declaration of interest

There are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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