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Enhanced oxidative stress in Hashimoto's thyroiditis: Inter-relationships to biomarkers of thyroid function

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ABSTRACT

Objectives: Oxidative stress has been implicated in the pathogenesis of several inflammatory and immune-mediated disorders including Hashimoto's thyroiditis (HT). The objectives of the present cross-sectional investigation were to estimate serum glutathione (GSH) status and the activities of its recycling enzymes in HT and to explore their interrelationships with biomarkers of autoimmunity and thyroid function.

Design and methods: Newly diagnosed females with HT (n = 44) and 58 matched control subjects were recruited. Thyroid hormone profile, anti-thyroperoxidase anti-body (TPO-AB), anti-thyroglublin antibody (Tg-AB), thyroid volume (Tvol), urinary iodine excretion (UIE), GSH and the activities of glutathione peroxidase (GPx), glutathione reductase and gamma-glutamyltransferase were assessed.

Results: Median UIE in HT was slightly but not significantly higher than that of controls. HT group exhibited higher levels of TSH, TPO-AB, Tg-AB and larger Tvol when compared with controls ($P<0.001$). The means of GSH and GPx in HT patients were significantly different from those of controls ($P<0.001$). In HT subjects, significant associations were seen between Tvol on TSH, GSH on TPO-AB, GSH on TSH and TPO-AB titers on TSH, respectively.

Conclusions: This is the first study to demonstrate a substantial reduction in GSH status in HT subjects. Secondly, the interrelationship between the GSH contents and TPO-AB titers in HT provides a preliminary data to support the notion that GSH diminution is a hallmark of in the events leading to oxidative stress activation and the development of immunological intolerance in HT. Further studies are required to elucidate the role of GSH in the etiology of down-regulation of thyroid function.

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Introduction

Growing evidence suggests that several clinical conditions, inflammatory and immune-mediated disorders including Systemic Lupus Erythematosus (SLE) and Rheumatoid Arthritis (RA) are linked with lower cellular redox potential and/or enhanced oxidative stress [1–3]. Increased oxidative stress may induce the expression of a variety of immune and inflammatory molecules leading to tissue damage [4].

Hashimoto's thyroiditis (HT) is a multi-factorial disease and one of the most frequent human thyroid autoimmune diseases [5,6]. Pathogenesis of HT is characterized by progressive thyroid cell destruction [6,7]. Excessive dietary iodine intake has strongly been implicated as an environmental trigger of HT [8–10]. High iodine intake exerts an array of inhibitory effects on thyroid hormone biosynthesis and secretion [11]. It has also been demonstrated that excessive iodine intake attenuates cellular antioxidant capacity in experimental models [12]. Moreover, recent prospective studies have shown that even minimal elevation in iodine levels in iodine-replete regions is associated with increased incidence of HT [5,13,14].

Reduced glutathione (GSH) is the most abundant non-protein thiol in mammalian cells. It represents the first line of cellular antioxidant defense against oxidative damage [15–17]. Several lines of evidence suggest that GSH depletion is implicated in the etiology of an array of clinical conditions including diabetes, neurodegenerative diseases and cancer [17–19]. GSH is also recognized as an important regulator of the immune system [20–25].

There are two studies showing increased oxidative in HT as assessed by elevated lipid peroxidation and/or decreased antioxidant status but information regarding iodine status, glutathione levels and auto-antibodies are lacking [26,27]. Moreover, it has been reported that oxidative stress is slightly but significantly elevated in hypothyroid patients with positive antithyroperoxidase antibody (TPO-AB) compared to negative TPO-AB matched controls [28]. Therefore, the

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current cross-sectional investigation was undertaken to evaluate serum glutathione and its recycling enzymes in newly diagnosed females with HT residing in a border-line iodine sufficient region (100 μg/L > median urinary iodine excretion (UIE) <150 μg/L) and to explore their interrelationship with TPO-AB, anti thyroglubolin antibody (Tg-AB) titers, thyroid volume (Tvol) and thyroid functional status. As a control group, age and gender matched individuals without any history of thyroid- and autoimmune dysfunction were employed.

Materials and methods

Reagents

NADPH, Na_2-EDTA, reduced glutathione, DTNB and glutathione reductase was purchased from Sigma-Aldrech Incorporation (Dorset, UK). All other chemicals were obtained Merck (Darmstadt, Germany).

Subjects and patients

Patient group consisted of 44 newly diagnosed females with HT attending the Endocrinology Outpatient Clinic at Imam Khomeini Teaching Hospital, Urmia, Iran (November-2009 and August-2010). Exclusion criteria were menopausal state, pregnancy, anti-thyroid drug therapy and/or antioxidant therapy within the last 6 months. As a control group, 58 females (-TPO-AB) without any history of hypothyroid and autoimmune dysfunction were employed. This investigation was approved by the ethical committee at the Urmia University of Medical Sciences, Iran. Informed consent was obtained from all patients before entering the study.

Blood collection

Blood samples (5 mL) were collected by venous puncture. The blood sample was allowed to stand for 10 min at room temperature and subsequently centrifuged at 1000 ×g for 15 min. Serum aliquots (250 μL) was transferred into Eppendorf tubes and kept −70 °C until analysis.

Urine collection

Fasting urine samples (10 mL) were collected. Aliquots (1 mL) were transferred into Eppendorf tube and kept at −70 °C until analysis.

Assessment of thyroid volume (Tvol)

Tvol was determined using a 7.5 MHz linear transducer real-time ultrasonography instrument (Toshiba Nemio30, Japan). Examination was carried out in supine position, with neck hyper extended by a senior radiologist. Tvol was calculated as follows: width × length × depth × 0.479 for each lobe [29]. Tvol is referring to the sum of the volumes both lobes.

Thyroid function and antibody tests

Serum free thyroxine (fT_4), free triiodothyronine (fT_3) and thyrotropin (TSH) were estimated by enzyme immunoassay (EIA) (Pishtaz Teb, Tehran, Iran). Normal reference range fT_4, fT_3 and TSH were 0.7–1.8 ng/dl, 1.9–4.3 pg/mL and 0.32–5.2 mIU/L, respectively. TPO-AB- and Tg-AB EIA-kits were obtained from Aeskue (Hamurg, Germany). Recommended range for negative, equivocal and positive TPO-AB were ≤40 IU/mL, 40–60 IU/mL and >60 IU/mL, respectively. Recommended range for negative, equivocal and positive Tg-AB was ≤120 IU/mL, 120–180 IU/mL and >180 IU/mL, respectively.

Assessment of urinary iodine excretion (UIE)

UIE was assessed by the Sandell–Koltoff reaction. Briefly, urine sample was incubated with ammonium persulfate at 100 °C for 40 min to liberate iodine. The decline of the yellow colored cerium solution [cerium (IV)] in the presence of iodine was measured spectrophotometrically at 410 nm [30,31].

Glutathione assay

GSH was determined by the recycling method as described elsewhere [32]. Briefly, serum (100 μL) was mixed with sulfosalicylic acid 5% (100 μL) and subsequently centrifuged for 10 min at 1000 ×g. Supernatant (50 μL) was mixed with phosphate-EDTA buffer (0.1 mol/0.001 mol; pH: 7.4; 200 μL), NADPH (2 mg/mL in 0.1 mmol/L KOH; 100 μL), DTNB (1.5 mg/mL in 0.5% $NaHCO_3$; 20 μL) and glutathione reductase (6 U/mL in 0.1 mol/0.001 mol phosphate-EDTA buffer; 20 μL). Absorbance was read at 412 nm using a double beam UV/Vis Perkin Elmer spectrophotometer.

Glutathione peroxidase (GPx) assay

GPx activity was measured as described previously by Paglia et al. [33]. Serum (50 μL) was mixed with (950 μL) of reaction mixture containing Tris buffer (50 mmol/L; PH 7.6), Na_2EDTA (1 mmol), GPx (2 mmol), NADPH (0.2 mmol), sodium azide (4 mmol) and glutathione reductase (1000 U). The sample was incubated at 37 °C for 5 min and finally was mixed with of H_2O_2 (8.8 mmol/L; 10 μL). Absorbance was read at 340 nm for 3 min.

Glutathione reductase (GR) determination

GR activity was carried out as previously described by Delides et al. [34]. Serum (50 μL) was mixed with reaction mixture (1.4 mL) containing phosphate buffer (0.15 mol/L, pH 7.2), EDTA-disodium salt (15 mmol/L), NADPH-tetrasodium salt (10 mmol/L). Absorbance was read at 300 nm for 5 min.

Gamma-glutamyltransferease (GGT) estimation

Serum (100 μL) was mixed with glycylglycine buffer (1 mL; 14.5gr Tris-base, 11.9gr glycylglycine, 2.44gr $MgCl_2$ ($6H_2O_2$), pH 8.2) and the sample was incubated for 3 min at 37 °C. Subsequently, the reaction was initiated by adding 100 μL of glycylglycine 100 μL gamma-glutamyl p-nitroaniline (1.37gr in 0.15 μM HCL). Absorbance was monitored at 405 nm for 5 min [35].

Statistical analysis

Data handling was carried using SPSS software for windows version 16 (SPSS Inc., Chicago, USA). Quantitative data were expressed as either median or mean ± SD. Normality of data distribution was assessed with Kolmogorov–Smirnov test. Non-parametric data were analyzed by the Mann–Whitney test. Differences between categories were tested with one way ANOVA or Scheffe test for multiple comparisons. Correlations between the different parameters were calculated by linear regression analysis. $P \leq 0.05$ was considered statistically significant.

Results

Table 1 display anthropometric- and biochemical characteristics for control (negative TPO-AB) and HT groups. No significant difference was seen in mean age between control–and HT groups ($P<0.421$). Mean BMI in HT subjects was higher when compared to controls ($P<0.019$). Mean Tvol was larger in HT patients than that of controls ($P=0.001$). The means of Tg-AB titers and TPO-AB titers

Table 1
Demographics and biochemical characteristics of individuals with Hashimoto's thyroiditis and controls.

Parameters	Controls (n = 58)	Hashimoto thyroiditis (n = 44)	P-value
Age (old years)	34.53 ± 10.07	33.00 ± 10.21	P<0.421
BMI (kg/m^2)	25.93 ± 5.24	29.35 ± 9.12	P<0.009
Tvol (mL)	10.15 ± 4.20	17.23 ± 8.62	P<0.001
Tg-AB	19.77 ± 29.68	653.62 ± 954.00	P<0.001
TPO-AB (IU/mL)	4.60 ± 5.65	475.07 ± 293.02	P<0.001
TSH (mIU/L)	1.40 ± 1.16	19.68 ± 13.04	P<0.001
fT_4 (ng/dL)	1.16 ± 0.30	0.80 ± 0.39	P<0.001
fT_3 (pg/mL)	3.66 ± 0.64	3.39 ± 3.43	P<0.561
UIE (μg/L)	130.52 ± 113.58	169.01 ± 133.62	P<0.121

Data are represented as the mean ± SD.

were also higher in HT subjects than that of controls (both P<0.001). HT group exhibited higher TSH but lower fT_4 than that of controls (both P<0.001). Mean UIE was marginally higher in HT subjects relative to than of controls but the difference failed to reach statistical significant (130.52 ± 113.58 μg/L vs. 169.01 ± 133.62 μg/L; P<0.121) (Fig. 1).

HT patients exhibited markedly lower mean serum GSH level when compared to controls (P<0.001). A significant difference was also seen in mean GPx activity between HT cases and control group (P<0.001). On the other hand, no differences were seen in either the mean of GR activities or the mean of GGT activities between HT group and control subjects. Mean levels of GSH, GPx, GR and GGT in HT patients and controls is shown in Table 2.

As shown in Fig. 2A, Tvol was correlated with TSH levels in individual with HTs (P<0.001). A moderate association was also seen between TSH levels and TPO-AB titers (Fig. 2B). Moreover, significant relationships were obtained between GSH levels on TPO-AB titers and GSH levels and TSH levels in individuals with HT (Fig. 2C and D). No significant correlations were seen between Tg-AB with either thyroid hormones or GSH levels and its recycling enzymes.

Discussion

In the present investigation, we have employed a battery of biochemical tools to explore the relationship between oxidative stress as assessed by serum glutathione and its related enzymes including GPx, GR or GGT and thyroid functional status in newly diagnosed but untreated HT patients. This study extends beyond that of Alsayed et al. [36] in the terms that we have employed HT subject residing in moderate iodine sufficient region (100 μg/L > median UIE < 150 μg/L) to minimize the deterioration effect of excess on cellular antioxidant

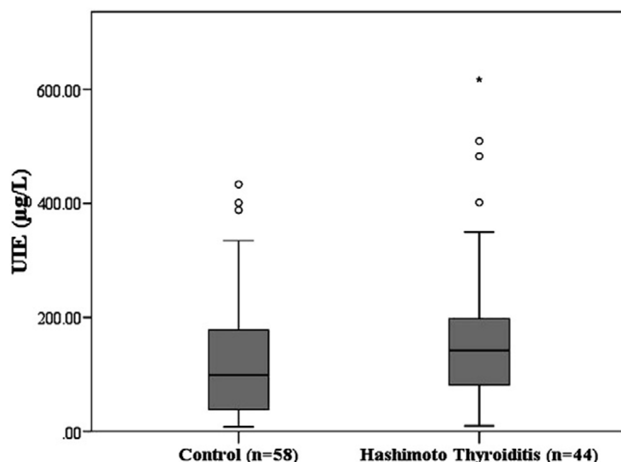

Fig. 1. Median urinary iodine excretion (UIE) levels in subjects with Hashimoto thyroiditis (n = 44) and control subjects (n = 58).

Table 2
Glutathione levels and the activities of glutathione reductase, glutathione peroxidese and gamma-glutamyl transferease in sera from patients with Hashimoto's thyroiditis (n = 44) and matched control subjects (n = 58). Data are presented as the mean ± SD.

	Control group (n = 58)	Hashimato's thyroiditis (n = 44)
GSH (μmol/L)	6.2 ± 4.1*	2.4 ± 2.2
GPx (IU/L)	276.5 ± 45.5*	329.5 ± 65.2
GR (IU/L)	36.9 ± 15.5	35.2 ± 11.1
GGT (IU/L)	19.4 ± 12.2	18.6 ± 7.3

Data represents the mean ± SD.
* P<0.05; Control group vs. Patient group.

capacity. We report that median UIE in the patient group was marginally but not significantly higher than that of controls subjects. An important finding from the current investigation is that mean GSH levels in HT patients is markedly lower compared to healthy controls. The fall in GSH levels coincided with a marked elevation of GPx activities in HT patients. On the other hand, no differences were seen in either GR activities or GGT activities between HT patients and healthy matched controls. Another important observation from the current investigation is that GSH levels were inversely and significantly correlated with TPO-AB titers as well as TSH activities in HT patients. The levels of TSH in HT patients were also strongly and positive correlated with TPO-AB titers.

Mechanism(s) linking deterioration of cellular antioxidant defense to the pathogenesis of autoimmune thyroiditis is not fully clear [6]. However, it has postulated that overproduction of ROS is main event leading to apoptosis and cell necrosis and eventually thyroid dysfunction [37,38]. Elevated ROS beyond that needed for the activation of normal biochemical processes would deplete whole body non-enzymatic cellular antioxidants including GSH and/or up-regulate the activity of enzymatic cellular antioxidants such as GPx. Moreover, excess ROS would increase the likelihood of polyunsaturated fatty acid auto-oxidation and generation of an array of aldehydes. Of these, 4-hydroxy-2-alkenals (4-HNE) has received the most attention because of its adverse biological effects. Interaction between 4-HNE and native proteins yields aldehyde protein adducts with the capability to induce pathogenic antibodies which also directly or indirectly participates in the generation of ROS [39,40].

In the current study, we report that whole body iodine store as estimated by UIE was 30% higher in HT patients relative to that of control groups. Our hypothesis is that a sub-marginal increase in whole body iodine store may overwhelm enzymatic and/or non-enzymatic antioxidant systems against oxygen reactive species in autoimmune susceptible individuals. The exact mechanism for excess iodine induced oxidative stress in not fully understood. However, when iodide (I^-) is present in excess relative to tyrosine residues, the iodide reacts with the iodinium cation (I^+) produced during enzymatic iodide oxidation by thyroperoxidase to yield molecular iodine (I_2). Interaction between endogenous peroxides and I_2 may lead to the generation of ROS [41,42]. Excess ROS will also lead to augmentation of cellular antioxidant capacity and thyroid dysfunction.

GSH is a reliable predictor of the antioxidative capacity of the whole body. In contrast to high intracellular GSH concentration (mM), its extracellular GSH concentration is kept at low μM range because of its rapid turnover [15]. GSH is essential during T lymphocyte proliferation, cell cycle progression from the G1 to S phase and DNA synthesis [43]. Moreover, considerable evidence has built up from experimental as well as clinical studies to support the notion that GSH depletion in the pathogenesis of autoimmune diseases through inhibition of IL-1 and T-cell receptors-mediated transduction signaling [20,22]. Low GSH has also been linked with apoptosis and cell death [22,44].

In the current study, we report that serum GSH is 62% lower in HT patients than that of matched healthy controls. This is markedly lower than previously reported values (18–26%) reported for a

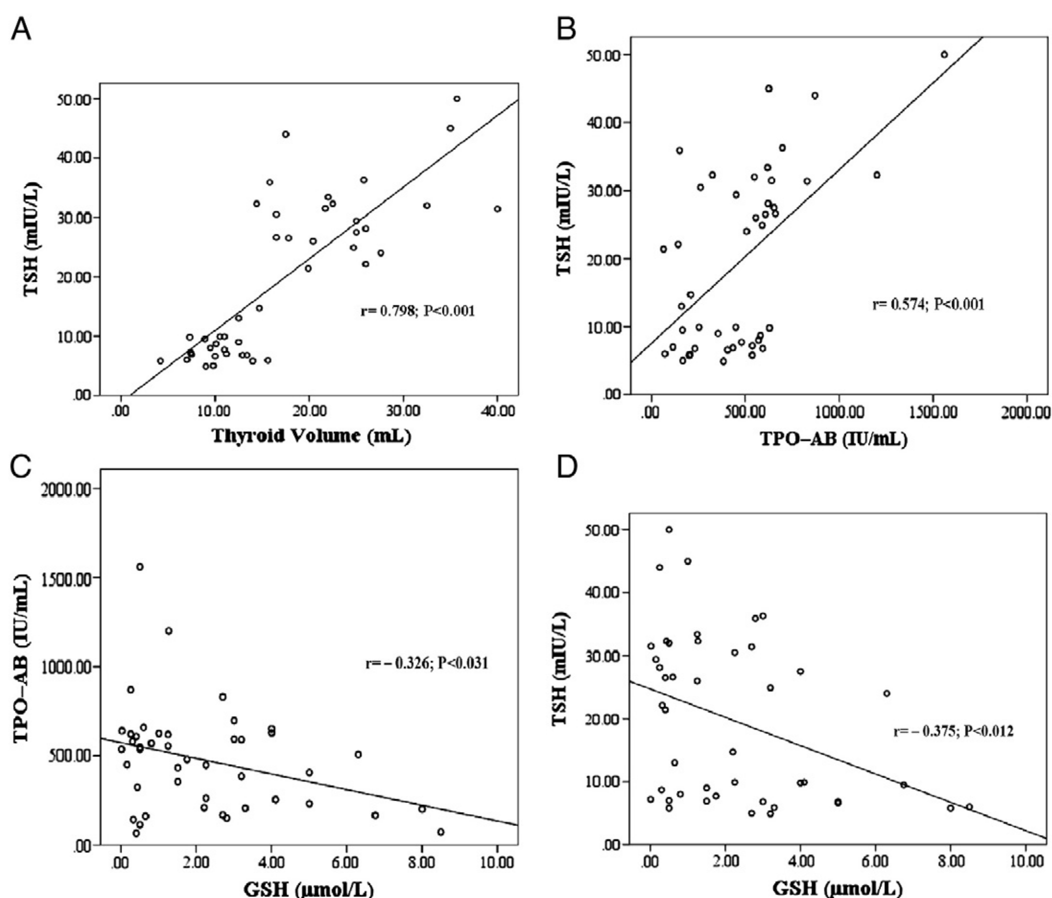

Fig. 2. Associations between markers of thyroid malfunction and/or oxidative stress in individuals with Hashimoto thyroiditis (n = 44). A: Thyroid volume (Tvol) and thyroid stimulating hormone (TSH) levels; B: Thyroid stimulating hormone (TSH) levels and anti-thyroperoxidase antibody (TPO-AB) titers; C: Glutathione (GSH) levels and anti-thyroperoxidase antibody (TPO-AB) titers; D: Glutathione (GSH) contents and thyroid stimulating hormone (TSH) levels.

comparison of serum GSH levels between healthy controls and those with hypothyroidism [45,46]. These findings indicate that the presence of autoimmune antibodies is likely to be an important factor for the enhancement of ROS production which eventually leads to cellular antioxidant depletion. Indeed, we have found a significant inverse association between GSH contents and TPO-AB titers in HT subjects. The latter finding provides the first clinical support for the theory that GSH is capable of inhibiting complement-mediated damage in autoimmune diseases [24]. Hence, we suggest that GSH diminution is possibly a hallmark of in the events leading to oxidative stress activation and the development of immunological intolerance in HT. Further studies with larger sample size are required to reestablish the relation between GSH status and TPO-AB titers and to evaluate the impact of therapeutic antioxidant on the appearance of TPO-AB titer in HT subjects.

Another finding from the present investigation supporting increased oxidative burden in HT patients is up-regulation of extracellular GPx activity. GPx is a seleno-cysteine protein which is responsible for the enzymatic reduction of catalyzes H_2O_2 [47]. The physiological functions of thyroidal GPx are protection of thyrocytes from oxidative damage and modulation of thyroid hormone biosynthesis [48]. Information regarding GPx activity in individual with hypothyroidism is conflicting; some have reported an increase while others reported a decline or no changes [45,46,27]. However, in the current investigation we reveal that GPx activity in HT patients is 19% higher than that of healthy control subjects. The up-regulation of GPX activity in HT patients reflects enhancement of oxidative stress. Mechanism of H_2O_2 production in the HT subjects is intricate. The proposed pathways for endogenous H_2O_2 production in the presence of excess iodine are over expression of NADPH oxidase and reaction between excess iodide and tyrosine resides on thyroglublin [41,44,49,50].

H_2O_2 itself is not highly reactive as an oxidant, but it can be activated {i.e. converted to hydroxyl radicals (HO·)} in the presence of transition metal ions such as Fe^{3+} and Cu^{2+}. Hydroxyl radical reacts with many cellular components including polyunsaturated fatty acids from the follicular cell membrane leading to increased production aldehydes with the capability to induce pathogenic antibodies [4]. The absence of inter-correlations between GPx activities in HT patients and GSH, TSH, Tg-AB or TPO-AB suggest that the enzyme is probably acting beyond it V_{max} and thus exhibiting a lower capacity to neutralize accumulated H_2O_2 in the cell [51]. Abundance generation of ROS can act as a second messenger to stimulate nuclear factor kappa B-dependent expression of pro-inflammatory cytokines such as I-CAM1 and IL-6. These events form an augmentation loop that feeds back to further stimulate the production of additional ROS. ROS levels in cells are influence by GSH viability. ROS levels can either activate or inactivate specific redox sensitive targets at cell cycle checkpoints, thereby influencing cell destiny [6].

In conclusion, this study has revealed that sera antioxidant capacity as estimated by GSH content and GPx activity is substantially reduced in individuals with HT. Secondly; the interrelationship between the GSH contents on TPO-AB titers or TSH levels and TSH levels on TPO-AB titers in HT provides direct support the notion that GSH diminution is a hallmark of in the events leading to oxidative stress activation and the development of immunological intolerance in HT. Further studies with larger sample size are required to elucidate the role of GSH in the etiology of down-regulation of thyroid function.

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