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Oxidative DNA Base Damage, Antioxidant Enzyme Activities and Selenium Status in Highly Iodine-deficient Goitrous Children

BELMA GIRAY and FILIZ HINCAL*

Department of Toxicology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey

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The objective of this study was to investigate oxidative DNA damage, and the levels of antioxidant enzymes (AOE) and selenium (Se) in relation to iodine deficiency and/or goiter in children. The study was performed in a group of goitrous high school children (15–18 years of age) ($n = 14$) with severe or moderate iodine deficiency. Thyroid hormones (TSH, FT_4, TT_4, FT_3, TT_3), urinary iodine (UI) and plasma Se levels, and erythrocyte glutathione peroxidase (GSHPx), superoxide dismutase (SOD) and catalase (CAT) activities were determined and compared with those of a control group consisting of non-goitrous high school children ($n = 14$) with normal UI levels or mild iodine deficiency. In the goitrous group, concentrations of FT_4, TT_4, plasma Se and UI, and activities of GSHPx and SOD were found to be significantly lower. Six typical hydroxyl radical-induced base lesions in genomic DNA of peripheral blood were identified and quantified by gas chromatography/isotope-dilution mass spectrometry (GC/IDMS), and higher levels of DNA base lesions were observed in the goitrous group. The results suggest that highly iodine-deficient goitrous children may be under oxidative stress, which may lead to greater level of oxidative damage to DNA. This study supports the evidence for the reported relationship between iodine deficiency and the increased incidence of thyroid malignancies.

Keywords: Oxidative DNA damage; Antioxidant enzyme activities; Selenium deficiency; Iodine deficiency; Thyroid hormones; Goiter

Abbreviations: Se, selenium; AOE, antioxidant enzymes; GSHPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase; UI, urinary iodine; TSH, thyroid stimulating hormone; T4, thyroxine; T3, 3,5,5′-tri-iodothyronine; GC/IDMS, gas chromatography/isotope-dilution mass spectrometry; 5-OH-Cyt, 5-hydroxycytosine; 8-OH-Gua, 8-hydroxyguanine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 2-OH-Ade, 2-hydroxyadenine; FapyAde, 4,6-diamino-5-formamidopyrimidine; IDD, iodine deficiency disorders; SMOID-G, severely plus moderately iodine deficient goitrous children; NMID-C, non-goitrous control children with normal UI levels plus mildly deficient children; $^{\cdot}OH$, hydroxyl radical; BPH, benign prostatic hyperplasia

INTRODUCTION

Free radicals generated by endogenous or exogenous sources, most notably hydroxyl radical, can cause damage to biological molecules including lipids, proteins and DNA.[1–3] Oxidative damage to DNA produced by free radicals has been implicated in mutagenesis, carcinogenesis, reproductive cell death and aging.[4] Antioxidant enzymes (AOE) exist in cells to protect against the toxic effects of free radicals and other oxygen-derived species produced during normal cellular metabolism or exogenously generated oxidative stress.[5] Selenium (Se) is an integral component of the peroxide metabolizing antioxidant enzyme, glutathione peroxidase (GSHPx). Thus, it plays an important role in the defense system of the cell.[6] On the other hand, it has been shown that three isozymes of iodothyronine 5′-deiodinase are also selenoenzymes.[7,8] These enzymes catalyze the metabolic conversion of thyroxine (T_4), the principal hormone secreted by thyroid, to the major biologically active hormone, 3,5,5′-tri-iodothyronine

*Corresponding author. Tel.: +90-312-305-18-71. Fax: +90-312-310-09-06. E-mail: hincal@tr.net

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(T_3). These findings indicate that, besides iodine, Se is also involved in thyroid functions, more specifically, in the regulation of thyroid hormone metabolism.

Similar to iodine, Se is inadequately available for man and livestock in many parts of the world, and the relationships between Se and thyroid function are complex and dual. Se is involved in thyroid hormone metabolism and thus may provide sparing of iodine by decreasing the catabolism of prohormone, T_4, when a shortage of iodine intake exists.[9] But the effects of Se deficiency on thyroid gland may also be related to the damage induced by H_2O_2, the level of which is increased in thyroid cells by the lack of protection due to defective GSHPx. Therefore, the thyroid as a major site of H_2O_2 generation might be a possible source of oxygen-derived radicals in iodine and Se deficiency, although the high concentration of intracellular H_2O_2 as a co-factor of thyroperoxidase allows a higher efficiency of thyroid hormone synthesis. Existing data suggest that long-term residency in iodine-deficient areas and a history of benign thyroid disease (mostly goiter, adenomas or hyperthyroidism) are associated with an increased risk of thyroid malignancies.[10,11] In this study, we investigated AOE activities, Se and iodine status and the levels of modified DNA bases in genomic DNA in goitrous high school children living in an endemic goiter area. The objective was to understand the alterations of AOE and the Se status and the oxidative DNA base damage in iodine deficiency and the possible link between them.

MATERIALS AND METHODS

Materials

Selenium dioxide, cyclohexane, EDTA, 2,3-diaminonaphthalene, 5,5′-dibromo-o-cresolsulfonphthalein, proteinase K, sodium dodecyl sulfate, xanthine, xanthine oxidase, nitroblue tetrazolium, bovine serum albumin, bovine erythrocyte superoxide dismutase (SOD) were obtained from Sigma (St. Louis, USA). Cerium ammonium sulfate, potassium chlorate and arsenic trioxide were from Riedel (Seelze, Germany). Hydrochloric acid, ammonia, nitric acid, perchloric acid were obtained from BDH (Poole, Dorset, UK). Hydrogen peroxide was from Aldrich (Dorset, UK). All other chemicals were from Merck (Darmstadt, Germany). Commercial kits for thyroid stimulating hormone (TSH), total and free thyroxine (TT_4, FT_4) and total and free tri-iodothyronine (TT_3, FT_3) were purchased from Roche Diagnostics (Mannheim, Germany) and the RANSEL glutathione peroxidase kit was from RANDOX (Crumlin, UK). Materials for gas chromatography/isotope-dilution mass spectrometry (GC/IDMS) were obtained as described.[12]

Subjects

The study was conducted in supplementation of an earlier, more comprehensive study, which covered the whole high school student population of two towns in the East Black Sea Region, where the prevalence of endemic goiter is one of the highest in Turkey. As described elsewhere,[13] after screening the whole student population ($n = 502$) of the two schools for goiter by inspection and palpation, the overall prevalence of goiter was found to be 39.6%. Groups of goitrous and non-goitrous children were selected by the simple random technique. Both groups were later re-classified as normal, mildly deficient, moderately deficient and severely deficient groups according to the degree of iodine deficiency based on urinary iodine (UI) levels as recommended by WHO.[14] The goiter group ($n = 14$) of the present study was selected by a further simple random technique from the reformed group of "severely plus moderately iodine deficient goitrous children" (SMOID-G). A control group ($n = 14$) was formed by the same random technique from the group of "non-goitrous control children with normal UI levels plus mildly iodine deficient control children" (NMID-C).

Both groups consisted of seven males and seven females, otherwise healthy individuals at the age of 15–18 years. The subjects were all from the urban population of the two towns (which are ~50 km apart from each other) and socioeconomically homogenous (lower-middle class). Dietary information relevant to Se and antioxidant nutrients, and possible goitrogenic food intake including Brassicaceae family vegetables was collected through a standard food-frequency questionnaire. Hence the attempt was undertaken to establish the pattern of food consumption in terms of the list of foods (cereals, meats and fish, vegetables and fruit, dairy products and eggs) and the frequency of consumption. Smokers and subjects taking medications or vitamin supplements were excluded. The heights and weights of all subjects were also recorded.

The study was approved by the Ethical Review Board of Karadeniz Technical University, Faculty of Medicine, Trabzon. Written consent was obtained from the community school boards, as well as the parents of the children involved.

Sample Collection

Venous blood samples were collected in heparinized tubes in the morning after breakfast for the determination of Se status and AOE activities. Centrifugation was performed at 800 g, plasma was

separated, and erythrocyte packages were prepared as recommended. Spot urine samples were collected at the same time for UI measurements. All samples were immediately aliquoted and stored in a freezer at 20°C until analysis. For DNA isolation, venous blood samples were drawn into EDTA-coated tubes and stored at −20°C until extraction.

Thyroid Hormone Parameters and Urinary Iodine Levels

The thyroid hormone status was determined by measuring the plasma FT_4, TT_4, TT_3, FT_3 and TSH concentrations by radioimmunoassay using commercial kits supplied by Roche. UI concentrations were measured using a modification of the Sandell Kolkoff reaction as described by Dunn *et al.*[15]

Se Status and Antioxidant Enzyme Activities

The activity of GSHPx was determined by using "RANSEL glutathione peroxidase kit" which is based on an enzymatic cycling assay as described by Paglia and Valentine.[16] Cumene hydroperoxide was used as the substrate. One enzyme unit was defined as the amount of enzyme that transforms 1 μmol of NADPH to $NADP^+$ per minute at 37°C and the specific enzyme activity was expressed in units per gram of hemoglobin. The activity of SOD (CuZnSOD) was measured in the samples freed of hemoglobin, according to the method of Sun *et al.*[17] The assay involves the inhibition of nitrobluetetrazolium reduction with xanthine–xanthine oxidase system, which is used as a superoxide generator. Specific enzyme activity was expressed as unit per mg hemoglobin. One unit was defined as the amount of enzyme required to inhibit the rate of reaction by 50%. Catalase (CAT) activity was determined by the method of Aebi[18] as the decrease in the absorbance of hydrogen peroxide as monitored at 240 nm in a spectrophotometer. The specific activity was expressed as K per g hemoglobin (K: rate constant of the first order reaction as defined by Aebi[18]).

Plasma Se levels were measured by the spectrofluorometric method described by Lalonde *et al.*[19] Calibration of the spectrofluorometric method and the instrument quality assessment of the analytical data, verification of precision, accuracy and sensitivity were accomplished by the direct use of Standard Reference Material (SRM) (Seronorm by Nycomed, Oslo, Norway). Results were in good agreement with certified values. Limit of detection of the method was 0.7 μg/l; within-day precision was 2.4% CV, between-day precision was 2.6%, and recovery was determined to be 98.10 ± 0.04%.

Isolation of DNA and Analysis of DNA Base Damage

Genomic DNA was extracted from whole blood using a standard protocol.[20] The concentration of DNA was estimated by the measurement of absorbance at 260 nm (absorbance of 1 = 50 μg of DNA/ml). Aliquots of stable isotope-labeled analogues of modified DNA bases were added as internal standards to 50 μg of DNA. An aliquot of 2′-deoxyguanosine-$^{15}N_5$ was also added for quantification of guanine to assess by mass spectrometry the DNA amount in each sample. Upon hydrolysis, 2′-deoxyguanosine-$^{15}N_5$, yields guanine-$^{15}N_5$, which is used as an internal standard for guanine in DNA.[21] Samples were dried under vacuum in a Speed VAc and then hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed tubes at 140°C for 30 min. The hydrolyzates were lyophilized in vials for 18 h. Lyophilized hydrolyzates were derivatized and then analyzed by GC/IDMS with selected ion monitoring as described.[22]

Statistical Analysis

All data are expressed as mean ± standard deviation. Differences between goitrous and control groups were determined by Student's t-test. For parameters with non-Gaussian distribution (UI, TSH), Mann Whitney U test was used.

RESULTS AND DISCUSSION

There was no significant difference between the two groups in physical development and the data collected by a standard food-frequency questionnaire did not show any significant difference with respect to dietary habits, including highly consumed *Brassica oleracea var. acephala* and antioxidant nutrients. The hemoglobin levels of the subjects were in the reference limits.

Table I summarizes plasma thyroid hormone concentrations. Figure 1 shows the plasma Se concentrations and erythrocyte GSHPx, SOD and CAT activities measured in SMOID-G and NMID-C children. All goitrous children were in the state of euthyroid. TSH levels did not differ, but in agreement with the general features of endemic goiter, FT_4 and TT_4 concentrations were lower than in non-goitrous children. These comparisons also revealed that highly iodine-deficient goitrous children had significantly lower Se levels, and erythrocyte GSHPx and SOD activities than those of non-goitrous control children.

Six modified DNA bases 5-hydroxycytosine (5-OH-Cyt), 8-hydroxyguanine (8-OH-Gua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-

TABLE I Thyroid hormone concentrations of plasma in goitrous and control children (values are given as mean ± SD and ranges were given in parentheses)

Groups	UI (μg/dl)	TSH (mU/l)	FT_4 (pmol/l)	TT_4 (nmol/l)	FT_3 (pmol/l)	TT_3 (nmol/l)
Severely and moderately UI deficient goiter (SMOID-G) (UI < 5 μg/dl) (n = 14)	2.0±0.9* (1.0–3.4)	2.2±1.8 (0.8–7.9)	14.0±2.5* (9.0–18.7)	109.0±27.8† (53.5–150.3)	5.7±1.3 (3.4–7.9)	2.7±0.6 (1.6–3.7)
Normal and mildly UI deficient control (NMID-C) (UI≥5 μg/dl) (n=14)	12.3±7.3 (5.9–27.8)	2.3±1.6 (0.01–5.9)	19.3±3.4 (12.6–25.1)	134.3±20.9 (102.8–172.3)	5.6±1.2 (3.8–8.0)	2.4±0.4 (1.8–3.0)

*$p < 0.001$.
†$p < 0.02$.

Gua), 8-hydroxyadenine (8-OH-Ade), 2-hydroxyadenine (2-OH-Ade) and 4,6-diamino-5-formamidopyrimidine (FapyAde) were identified and quantified in genomic DNA of peripheral blood of goitrous children and goiter-free control children. The mean levels of these modified DNA bases are given in Fig. 2. It was found that, of the six damaged DNA bases quantified, the means of three bases were significantly higher in the goitrous group. In the case of 5-OH-Cyt and 8-OH-Ade, the increases were about 50%, and over 30% of increase was determined for 8-OH-Gua. No significant differences for the mean values of 2-OH-Ade, FapyAde and FapyGua between the two groups were observed. Although the group sizes were limited, no statistical differences were detected for levels of modified DNA bases between females and males. Figure 3 illustrates the levels of six modified DNA bases in the peripheral blood of individual goitrous and control children. These plots demonstrate the differences in 8-OH-Gua, 8-OH-Ade and 5-OH-Cyt levels between the groups. Substantial individual variations among the goitrous children were noted. Although the mean FapyGua and FapyAde values were not significantly different from those of control children, some goitrous children had markedly high levels. Therefore, it seems possible that if the sample size were greater, those differences would become more obvious.

Iodine deficiency is a worldwide problem. A quarter of the world's population subsist on a diet that is deficient in iodine and are at risk for iodine deficiency disorders (IDD).[23] Goiter is the earliest and the predominant clinical sign of iodine deficiency and endemic goiter occurs when the prevalence of thyroid enlargement in the population of an area exceeds 10%.[23] Existing data indicate that endemic goiter prevails in all geographical regions of Turkey and the East Black Sea Region is recognized as one of the highest prevalence rate region of the country.[24]

A history of benign thyroid diseases, mostly goiter and nodules has now been considered as established risk factors for thyroid cancer.[10,25–27] Long term residence in regions with iodine imbalance[10,28] and poor nutrition (a diet poor in vegetables and fruits, and hence in antioxidant nutrients)[29] are other recognized risk factors for thyroid tumors.[25,26] There are also experimental evidences suggesting that iodine deficiency is a risk factor for thyroid cancer by possibly favoring both initiation, promotion and progression of thyroid tumors.[30,31] In addition, in iodine-deficient thyroid glands, the highly stimulated thyrocytes synthesize, under TSH control, an increased amount of H_2O_2 for the production of thyroid hormones.[32] Thyrocytes, as other cells, are protected by several forms of GSHPx family, SOD and CAT. However, significantly lower SOD activity in endemic goiter tissue was reported

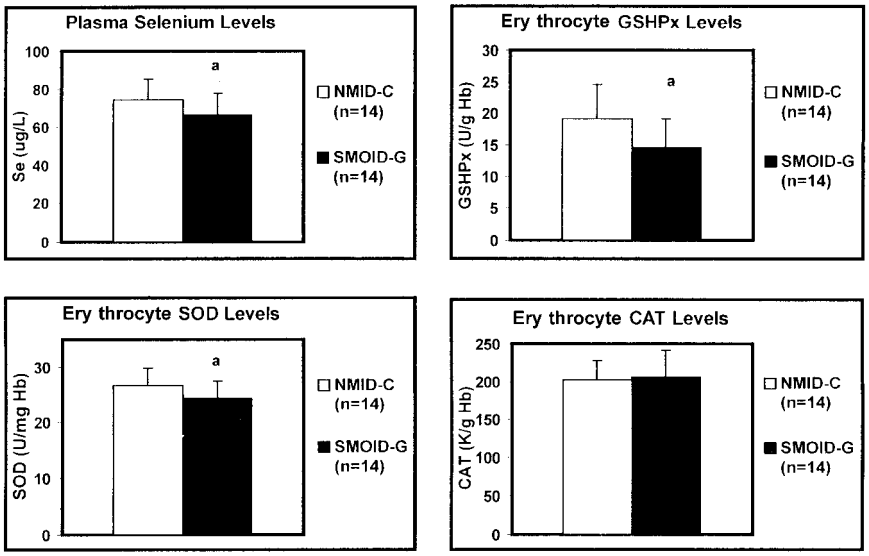

FIGURE 1 Plasma selenium concentrations and erythrocyte antioxidant enzyme activities in goitrous (SMOID-G) and control (NMID-C) children. $^{a}p < 0.05$ by Student's t-test.

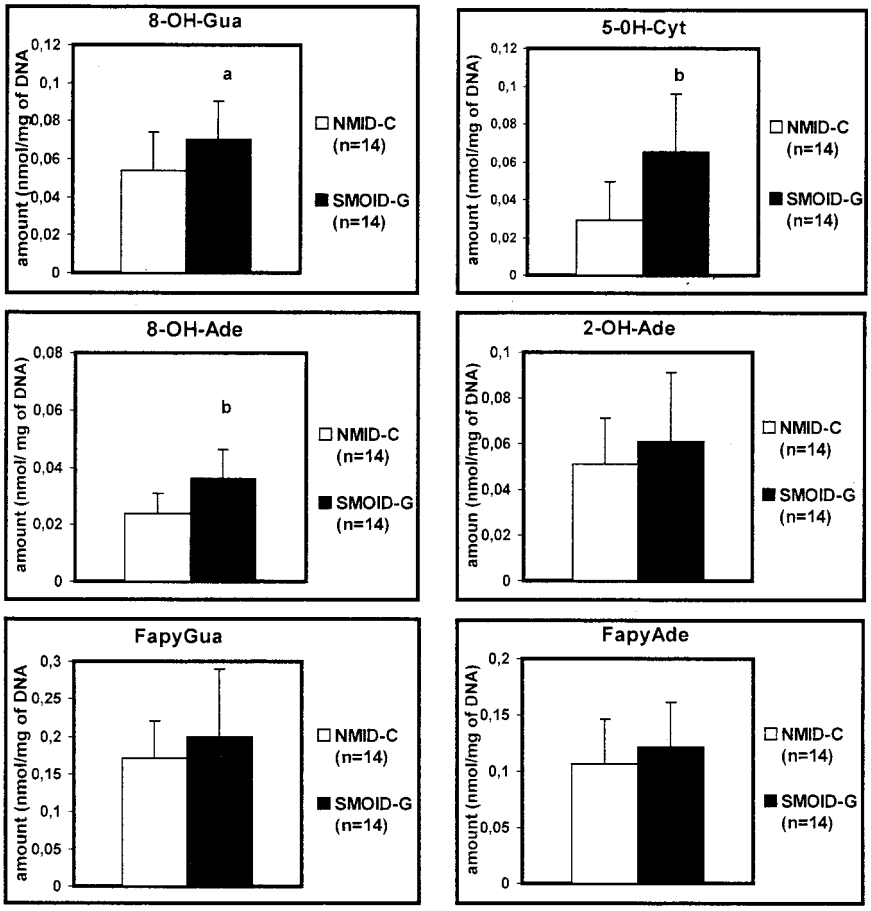

FIGURE 2 The amount of modified DNA bases in total genomic DNA of peripheral blood of goitrous (SMOID-G) and control (NMID-C) children. 1 nmol / mg of DNA corresponds to approximately 308 lesions / 10^6 DNA bases. $^{a}p < 0.05$, $^{b}p < 0.01$ by Student's t-test.

previously.[33] Moreover, when Se deficiency coupled with iodine deficiency, through an increased availability of H_2O_2 and a decrease in thyroid GSHPx activity, the stimulated thyroid gland is possibly exposed to greater levels of H_2O_2, and in turn, to highly reactive peroxides.[9] It is, therefore, plausible that deficits of antioxidant status may lead to the exposure of thyroid cells to increased oxidative stress, and may eventually contribute to the occurrence of malignant transformations.

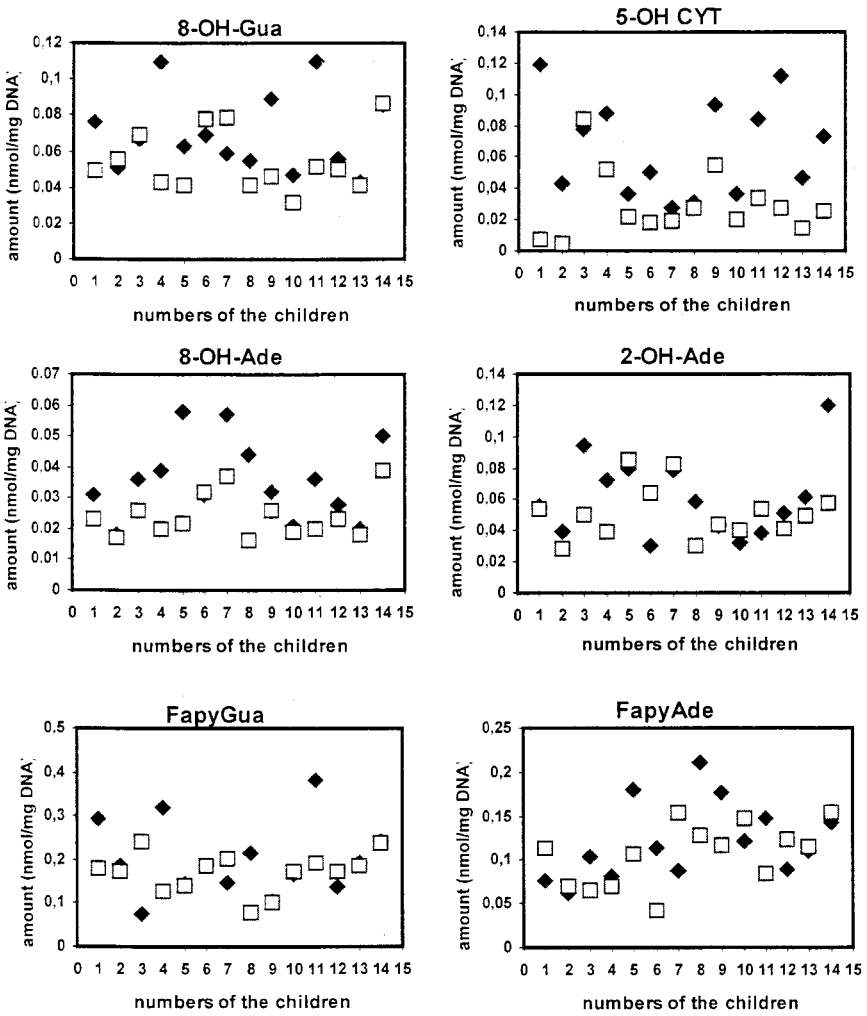

FIGURE 3 Levels of modified DNA bases in peripheral blood of individual goitrous and control children. Each data point corresponds to one individual. The same individual as the same number in each plot and the numbers were given arbitrarily. (◆) Goitrous children; (□) control children. 1 nmol / mg of DNA corresponds to approximately 308 lesions / 10^6 DNA bases.

We observed significantly lower activities of GSHPx and SOD in erythrocytes, lower plasma Se concentrations, and higher level of DNA lesions in a group of highly iodine deficient high school children with goiter coming from an endemic goiter area. In addition, not only goitrous children, but also our overall study group had borderline, if not deficient, Se intake. The daily intake of Se, estimated using an algorithm given by Longnecker et al.[34] was lower than the RDA value of 50 μg Se / day in both goitrous and control groups. These findings suggest a possible link between increased DNA damage and decreased activities of AOE, and lower Se levels in goiter. Six modified bases were quantified. The levels of three of these lesions (5-OH-Cyt, 8-OH-Ade and 8-OH-Gua) in goitrous children were significantly higher than those found in controls. Since the modified DNA bases observed were typical products of hydroxyl radical ($^{\cdot}$OH) attack on DNA, their elevated levels might indicate the participation of $^{\cdot}$OH. Some of these identified DNA base lesions are known to possess premutagenic properties and may play a role in carcinogenesis.[35–40] In fact, pathogenesis of thyroid disease is associated with several genetic alterations.[41,42] Various previous studies clearly showed elevated levels of typical $^{\cdot}$OH-modified DNA bases in various cancerous tissues than in their surrounding normal tissues.[43–45] There is also evidence of a correlation between increased levels of modified DNA bases and decreased levels of AOE (GSHPx, SOD and CAT) in human cancerous tissues,[44] including lymphocytes of acute lymphoblastic leukemia patients.[46] Furthermore, human benign prostatic hyperplasia (BPH) tissues have been shown to have higher oxidative DNA base damage and lower AOE activities than surrounding normal prostate tissue.[47] Thus, the results of the present study are in accordance with the general trends observed in the afore-mentioned studies, and suggest that goitrous subjects might have a predisposition for thyroid malignancies.

Although we have not found a decrease in the activity of CAT in this study, it was significantly

lower in goitrous group in our previous study[13] carried out on a larger group. Furthermore, in that survey, we observed that the status of AOE and Se in highly-iodine deficient non-goitrous children was not different from that of "non-goitrous children with normal UI levels or mild iodine deficiency".[13] Therefore, if we consider our previous findings together with those of the present study, it appears that goiter development is more likely to occur in individuals having lower status of AOE and Se. The observed higher level of DNA lesions in the goitrous group indicates that free radical reactions are increased in the goitrous state. It is likely that goitrous children with both decreased activity of AOE and Se, and increased levels of modified DNA bases are at greater risk of thyroid malignancies.

In summary, a possible association between increased level of modified DNA bases and decreased activity of AOE and Se in highly iodine deficient goitrous children compared to non-goitrous children is reported here for the first time. However, in order to reach a better understanding, large-scale studies would be needed in the future.

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