High Thyroid Stimulating Hormone Level Contributes to Nitric Oxide and Superoxide Anion Overproduction in Women with Hypothyroidism

Fadia Bekhti-Sari1, Nassima Mokhtari-Soulimane1, Hafida Merzouk1, Lotfi Loudjedi2, Sabah Ghedouchi1, Baya Guermouche1, Sid Ahmed Merzouk3, Necib Berber2

1Laboratory of Physiology, Physiopathology, and Biochemistry of Nutrition, Department of Biology, Faculty of Natural and Life Sciences, Earth and Universe.
2Nuclear Medicine Department, Tlemcen University Hospital Center, Tlemcen 13000, Algeria.
3Department of Technical Sciences, Faculty of Engineering, University Abou-Bekr Belkaïd, Tlemcen 13000, Algeria.

ABSTRACT

Objective: Hypothyroidism has been found to increase oxidative stress parameters and to disturb lipid profiles. The aim of this study was to determine the effect of high thyroid stimulating hormone level (TSH) on lipid profile and oxidative stress biomarkers in women particularly nitric oxide (NO•) and superoxide anion (O2•-).

Patients and methods: 31 healthy women and 35 women freshly diagnosed with hypothyroid were included in the study. Their body mass index (BMI), TSH, glucose, plasma and erythrocyte levels of NO•, O2•-, malondialdehyde (MDA) and carbonyl proteins (CP) were estimated in the two groups. Superoxide dismutase activity (SOD) and scavenging capacity of plasma were also evaluated.

Results: The present study showed a significant elevation in both plasma and erythrocyte NO•, MDA, CP and erythrocyte O2•- level in hypothyroid women compared to controls. However plasma total antioxidant capacity (ORAC) was significantly decreased, whereas, SOD activity was found higher in patients. Disequilibrium in lipid profile is noticed by elevation in total and LDL cholesterol, and LDL/HDL cholesterol ratio in hypothyroid women compared to healthy women. The level of TSH correlated significantly and positively with plasma and erythrocyte NO•, erythrocyte O2•- and LDL/HDL cholesterol, while plasma O2•- correlated negatively with TSH.

Conclusion: Hypothyroidism leads to oxidant/antioxidant imbalance and lipid disturbance in women.

Keywords: Hypothyroidism; oxidative stress; lipid profile.

INTRODUCTION

Hypothyroidism is associated with the presence of both traditional risk factors like dyslipidemia [1] and oxidative stress. [2] It is defined as a deficiency of both triiodothyronine (T3) and thyroxine (T4) concentration and leads to hyper secretion of pituitary TSH and an amplified increase in serum TSH levels. This is a key laboratory finding, particularly in the early detection of thyroid failure. [3] Hypothyroidism is more prevalent in women than men and increases with age. [4]

In hypothyroidism, the elevated LDL cholesterol level may occur as a result of increased cholesterol synthesis and absorption, decreased hepatic lipase and lipoprotein lipase activities and defects in the receptor mediated catabolism of LDL cholesterol. [5]
Thyroid hormones regulate mitochondrial respiration and oxidative metabolism, and thus play an important role in free radical production. Therefore, variations in the thyroid hormone level may modulate in vivo cellular oxidative stress (OXS). [6]

Nitric oxide (NO•) and superoxide anion (O2• -), are important in pathophysiological events such as inflammation, cancer, atherosclerosis, and aging. [7,8] Nitric oxide (NO•) is an endogenous free radical formed in a variety of cell types by NO-synthase mediated oxidation of L-arginine to L-citrulline. [9] It is a biological messenger mediating many important physiological functions but also pathological processes. It plays a vital role in host defense and immunity by modulating inflammatory processes. [10] Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated first after oxygen is taken. [11] The prooxidant agent NO• and Superoxide O2• - induce oxidation of macromolecules such as lipids and proteins, and produce MDA and CP. 

Malondialdehyde is an end-product of lipid peroxidation and is frequently measured as an index of these processes. Lipid peroxidation is associated with a wide variety of toxic effects, including decreased membrane fluidity and function, impaired functions of the mitochondria and Golgi apparatus and inhibition of enzymes. [12] Carbonyl Proteins is an early marker of OXS. It has been depicted in the literature that oxidative modification of protein occurs whenever lipid peroxidation products such as 4-hydroxy nonenal, MDA, etc. bind covalently to amino acid residues on proteins. [13]

Several defense mechanisms have evolved to protect against oxidant injury. Central to these defenses are the antioxidant enzymes. [14] The antioxidant enzyme superoxide dismutase, participates in the detoxification of reactive oxygen species by catalyzing the dismutation of superoxide radicals. Three SOD isoenzymes are known in humans including CuZn-SOD found in the cytoplasm and nucleus, a mitochondrial Mn-SOD, and extracellular SOD (EC-SOD). [15]

The mechanism of increased oxidative stress in hypothyroidism is controversial. Several studies suggest that an insufficient antioxidant defense system is thought to be a factor. Although, it has been indicated that the hypometabolic state due to hypothyroidism is associated with a decrease in oxidative stress. [16] Some authors reveal that hypothyroidism induce disequilibrium in oxidative stress. [17,18] However the mechanism of initiation of oxidative stress in hypothyroidism is unknown. The present work is an attempt to explore an effect of TSH on oxidative stress parameters (particularly NO• and O2• -) and LDL/HDL atherogenic fraction in hypothyroidism women. The oxidative stress status was evaluated by assaying, plasma total antioxidant capacity (ORAC), markers of lipid (MDA) and protein oxidation (CP), pro-oxidant agents (NO• and O2• -) and blood antioxidant defenses (superoxide dismutase activity). Also lipid profile, total cholesterol, LDL/HDL cholesterol and triglycerides were estimated.

MATERIALS AND METHODS

Sixty six women were recruited from the nuclear medicine department. This work was realized during the period of February 2011 up to February 2014. The control groups consisted of clinically normal subjects without any infectious disease or chronic ailments. The women with hypothyroidism were selected based on their TSH level (TSH ≥ 20 mUI/L). [19] Exclusion criteria were as follows: previous treatment for hypothyroidism, antioxidant vitamin supplements, acetylsalicylic acid, inflammatory diseases, antihypertensive, exposure to high-iodine condition, smokers, alcoholics, pregnant, hormone replacement therapy,
diabetes mellitus, kidney failure, and acute chronic or malignant diseases. The participation in this study was voluntary and all subjects gave their written, informed consent.

**Anthropometric measurements**

Body weight was measured while the subjects were wearing light clothing without shoes to the nearest 0.1 kg. Height was measured to the nearest 0.5 cm, without shoes. BMI was calculated as weight (in kilograms) divided by height (in meters squared).

The population characteristics are reported in Table 1.

**Blood Sampling**

Blood samples were collected after fasting for at least 12 h into EDTA tubes, at the same time (from 08:00 A. M. to 8:30 A. M.). The samples were centrifuged at 3000 g for 15 min and plasma was separated for TSH, glucose, lipid profile and oxidative stress markers determination. The remaining erythrocytes were washed two times with physiological saline and hemolyzed by the addition of cold distilled water. Cell debris was removed by centrifugation (3000 g for 15 min). The hemolysates were assayed for SOD activity, NO•-, O•2-, MDA and CP levels.

**Biochemical measurements**

**TSH determination:** TSH was measured by immunoassay analyzer using chemiluminescence assay on ARCHITECT system (Abbot Ireland Diagnostics Division Lisnamuck, Longford Co. Longford Ireland)

**Glucose Determination:** Plasma glucose was determined by glucose oxidase method using a glucose analyzer (CHRONOLAB AG, Switzerland, Barcelona, SPAIN).

**Lipid Profile:** Plasma total cholesterol and triglycerides concentrations were measured using standard enzymatic methods (Kits SIGMA Chemical Company, St Louis, MO, USA). High-density lipoprotein cholesterol and Low-density lipoprotein cholesterol were measured in the supernatant plasma after the precipitation of lipoproteins with dextran sulfate and magnesium chloride as previously described by Burstein et al. [20]

**Oxidative stress measurements**

**Determination of superoxide anion:** The spectrophotometric determination of the O•2- was based on the reduction of nitroblue tetrazolium (NBT) in the presence of superoxide anion (O•2- ), a chromophor that absorbs at 550 nm. [21]

**Determination of nitric oxide NO•:** Plasma and erythrocyte NO• was determined by the method of Guevara et al [22] after deproteinization using methanol: diethyl ether (3:1, v/v). Nitrite and nitrate levels were measured together, nitrate being previously transformed to nitrite by cadmium reduction. Nitrite was assayed directly spectrophotometrically at 540 nm, using the colorimetric method of Griess.

**Determination of malondialdehyde:**

Plasma malondialdehyde (MDA) levels, a marker of lipid peroxidation, were determined by the reaction of MDA with thiobarbituric acid (Sigma Aldrich kit; St. Louis, MO, USA).

**Determination of Carbonyl Proteins:**

Plasma carbonyl proteins (marker of protein oxidation) were determined by the derivatization of protein carbonyl groups with 2, 4-dinitrophenylhydrazine leading to the formation of stable dinitrophenyl hydrazone adducts (Sigma Aldrich kit).

**Superoxide dismutase activity: **

Superoxide dismutase (SOD) activity was measured by spectrophotometric method as described by Elstner et al. [23] The technique is based on NADPH oxidation mediated by superoxide radical. The chemical reaction generates superoxide anion (O2• -) from molecular oxygen in the presence of EDTA, MnCl2 and mercaptoéthanol. NADPH oxidation is proportional to superoxide anion disponability in the medium. The addition of plasma or hemolysate sample containing SOD to the reaction mixture causes a proportionate inhibition of NADPH oxidation. The absorbance is read
at 540 nm. Standards for activity were prepared using bovine erythrocytic SOD for each set of samples.

Scavenging capacity of plasma: The oxygen radical absorbance capacity of plasma (ORAC) employs the oxidative loss of the intrinsic fluorescence of allophycocyanin (APC) as we have previously described in ref. [24] APC fluorescence decay shows a lag or retardation in the presence of antioxidants, related to the antioxidant capacity of the sample. Trolox was used as a reference antioxidant for calculating the ORAC values, with one ORAC unit defined as the net protection area provided by 1 mM final concentration of trolox.

Statistical analysis: The analyzed parameters were statistically evaluated using Statistica (version 4.1; Statsoft, Paris, France). Results are expressed as means ± standard deviation (SD). Comparisons between the two groups; hypothyroidism women and healthy subjects were performed by the use of Student’s t test. Differences were considered statistically significant at P<0.05. To determine whether statistically significant correlation exist between the different parameters, correlation coefficient was calculated.

RESULTS

Our results showed a significant elevation in TSH during hypothyroidism, the baseline characteristics of the population are profiled in table 1. Regarding BMI and glucose, no difference was observed in hypothyroidism women compared to controls.

Concerning lipid profile, total cholesterol, LDL cholesterol and VLDL cholesterol levels showed an increase in hypothyroidism women compared to the control group (table 2). However, LDL/HDL ratio was highly elevated in patients, while triglycerides level presented no difference in patients and controls.

While pro-oxidant agents, plasma and erythrocyte NO• was higher in patients compared to controls, whereas O2• - level increased in erythrocyte but not in plasma of hypothyroidism women compared to controls. Concerning MDA and CP high levels was observed in both plasma and erythrocyte (table 3 and 4).

Table 1: Characteristics of the study groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>31</td>
<td>35</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Female</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.63 ± 0.79</td>
<td>46.24 ± 0.87</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.66 ± 0.77</td>
<td>28.27 ± 0.81</td>
</tr>
<tr>
<td>TSH</td>
<td>1.48 ± 0.02</td>
<td>22.74 ± 1.68***</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.53 ± 0.10</td>
<td>5.24 ± 0.17</td>
</tr>
</tbody>
</table>

Values are presented as means±SD. * P<0.05, **P<0.01, ***P<0.001; BMI : body mass index (weight/height²), TSH: Thyroid stimulating hormone.

Table 2: Lipid profile of the study groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>3.53 ± 0.14</td>
<td>3.98 ± 0.18***</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.31 ± 0.10</td>
<td>0.78 ± 0.07***</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>1.13 ± 0.06</td>
<td>1.49 ± 0.11**</td>
</tr>
<tr>
<td>VLDL cholesterol (mmol/L)</td>
<td>0.25 ± 0.02</td>
<td>0.56 ± 0.04***</td>
</tr>
<tr>
<td>LDL/HDL cholesterol</td>
<td>1.07 ± 0.06</td>
<td>1.78 ± 0.17***</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.00 ± 0.16</td>
<td>1.05 ± 0.08</td>
</tr>
</tbody>
</table>

Values are presented as means±SD. * P<0.05, **P<0.01, ***P<0.001; HDL: high-density lipoprotein, LDL: low-density lipoprotein, VLDL: very low-density lipoprotein.

Table 3: Plasma oxidative stress biomarkers of the study groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2• - (µmole/L)</td>
<td>2.89 ± 0.24</td>
<td>2.53 ± 0.26</td>
</tr>
<tr>
<td>NO• (µmole/L)</td>
<td>86.54 ± 9.19</td>
<td>113.37 ± 4.54*</td>
</tr>
<tr>
<td>MDA (µmole/L)</td>
<td>1.03 ± 0.05</td>
<td>1.53 ± 0.16**</td>
</tr>
<tr>
<td>CP (µmole/L)</td>
<td>5.99 ± 0.27</td>
<td>6.87 ± 0.31*</td>
</tr>
</tbody>
</table>

Values are presented as means±SD. * P<0.05, **P<0.01, ***P<0.001; O2• -: superoxide anion, NO•: nitric oxide, MDA: malondialdehyde, CP: carbonyl proteins.

Table 4: Erythrocyte oxidative stress biomarkers of the study groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2• - (µmole/L)</td>
<td>15.01 ± 0.76</td>
<td>29.36 ± 3.45***</td>
</tr>
<tr>
<td>NO• (µmole/L)</td>
<td>1.05 ± 0.70</td>
<td>132.44 ± 6.93*</td>
</tr>
<tr>
<td>MDA (µmole/L)</td>
<td>4.54 ± 0.17</td>
<td>7.19 ± 0.35***</td>
</tr>
<tr>
<td>CP (µmole/L)</td>
<td>1.50 ± 0.07</td>
<td>1.90 ± 0.16*</td>
</tr>
</tbody>
</table>

Values are presented as means±SD. * P<0.05, **P<0.01, ***P<0.001; O2• -: superoxide anion, NO•: nitric oxide, MDA: malondialdehyde, CP: carbonyl proteins.

The antioxidant SOD activity in both plasma and erythrocyte were significantly higher in patients (figure1). However, ORAC presented low level in hypothyroidism women compared to controls (figure 2).
Figure 1: Plasma and erythrocytes levels of superoxide dismutase activity (SOD). Values are presented as means±SD, ***P<0.001 indicates a highly significant difference between healthy controls and hypothyroidism patients.

Figure 2: ORAC: oxygen radical absorbance capacity which represents total plasma antioxidant capacity

Table 5: Pearson correlations of prooxidant agents and atherogenic risk with thyroid stimulating hormone in freshly diagnosed hypothyroid women

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma NO•</td>
<td>0.46</td>
<td>0.01</td>
</tr>
<tr>
<td>Erythrocyte NO•</td>
<td>0.56</td>
<td>0.01</td>
</tr>
<tr>
<td>Plasma O2•</td>
<td>-0.51</td>
<td>0.01</td>
</tr>
<tr>
<td>Erythrocyte O2•</td>
<td>0.51</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL/HDL cholesterol</td>
<td>0.41</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Values are presented as correlation coefficients (r). Statistically significant: *P < 0.01; **P < 0.001; P < 0.0001.

In the hypothyroid women, TSH was statistically and positively correlated with plasma and erythrocyte NO• (P < 0.01), erythrocyte O2• - (P < 0.001) and LDL/HDL cholesterol (P < 0.01). In contrast, the r values of TSH and plasma O2• - were statistically and negatively correlated (P < 0.01) (table 5).

**DISCUSSION**

The thyroid disorders are known to alter the lipid metabolism and oxidative stress balance. \(^{[2,25-27]}\) The results of this study showed an increase in total cholesterol, and LDL/ HDL cholesterol ratio in patients. Hypothyroidism leads to increased intestinal cholesterol absorption due to thyroid hormone actions on Niemann-Pick C1-like 1 protein in the gut. \(^{[28]}\) The thyroid hormone effects on LDL cholesterol receptor expression and cholesterol absorption outweigh the effects of decreased hepatic cholesterol synthesis, leading to a net accumulation of serum LDL cholesterol in hypothyroidism. Cholesteryl ester transfer protein (CETP) transfers cholesterol from HDL cholesterol to LDL cholesterol and very low density lipoprotein (VLDL). Plasma CETP concentrations are decreased in hypothyroidism, which may lead to alterations in serum HDL-C concentrations. \(^{[29,30]}\) The increased LDL cholesterol and decreased HDL cholesterol levels enhance LDL/ HDL cholesterol ratio, which are characteristic of atherogenic risk. In fact, TSH was positively correlated with LDL/HDL cholesterol. High level TSH in body causes endothelial dysfunction and increased serum levels of IL-6, TNF-α, CRP and several indices of oxidative stress which link to atherosclerosis. \(^{[31]}\)

On the other hand, a rise of NO• and O2• - levels induces lipoprotein oxidation which contributes to the atherosclerotic plaque formation. \(^{[32]}\)
Delconstantinos et al. [33] suggest that an increase in cholesterol content of endothelial cell membranes could participate in impairment of membrane-bound (NO•) synthase activity. [10]

The increase in erythrocyte O2•-, plasma and erythrocyte NO• in hypothyroidism women seen in the present study was associated with enhanced levels of TSH. In fact, the NO• level was found higher in hypothyroidism. [18] This prooxidant agent is generated by two distinct isoforms of nitric oxide synthases: inducible (iNOS) and endothelial NOS (eNOS). [34] According to Naseem [32] when the levels of O2•- increase, the interaction with NO• becomes the favored reaction and NO• is able to outcompete SOD for O2•-. Superoxide radicals and NO• can lead to the formation of many other reactive species, including hydroxyl radicals (OH•), which can readily start the free-radical process of lipid peroxidation. [35] This explains the elevation of MDA level in hypothyroidism women. Indeed, hypothyroidism raises MDA levels and leads to protein carbonylation. [13,36,37] This pro-oxidant marker reflects the long exposure to oxidative stress. [38]

In this study, TSH was statistically and negatively correlated with plasma O•2- . No difference was observed concerning plasma O•2- level in hypothyroidism women compared to controls. This result may be explained in part by action of TSH on O•2-, short half-life (few seconds) of O•2-, [39] and this instability and reacting. Firstly, depression of metabolism due to hypothyroidism has been reported to decrease oxidant production. [40] Also, a high SOD activity neutralized O•2- by its conversion to H2O2 and the dioxygen molecule. [15] On the other hand O•2- reacts with NO•, this reaction induces potential damage to lipids, proteins and DNA. Lipid peroxidation, and oxidative degeneration of polyunsaturated fatty acids leads to the formation of highly reactive aldehydes such as malondialdehyde (MDA) which can bind covalently to proteins resulting in their structural modifications and affecting biological function. [41] This explains high plasma MDA and CP levels in hypothyroidism women compared to controls.

Concerning SOD, it was reported that this enzyme is important to oxygen radical-scavenging. [42] The enzyme is both constitutive and inducible under oxidative stress. [43] The increase in SOD activity in hypothyroidism indicates the presence of oxidative stress due to an overproduction of O•2- and NO• found in patients compared to the control group. The reaction of NO• with superoxide depends on superoxide concentration, which in turn, is controlled by SOD; this may be a reason for enhanced SOD activity. [44] Superoxide dismutase catalyzes the dismutation of the superoxide anion into hydrogen peroxide (H2O2), which is then deactivated to water (H2O) by catalase or glutathione peroxidase (GPx). [45,46] Cytosolic CuZn-SOD and mitochondrial Mn-SOD react with intracellular O•2-, while extracellular O•2-- level was limited by extracellular SOD (EC-SOD), and is particularly abundant in the interstitium of the arterial wall. [39]

Previous studies have suggested that plasma ORAC levels have been modified during oxidative stress situation. [47] Our data revealed that the ORAC was decreased in patients which presents an evident oxidative stress marked by an elevation in O•2-, NO•, MDA, CP levels. The free radical accumulation in hypothyroidism could be due to various reasons such as decreased clearance of oxidants, increased pro-oxidant agents and disorders of lipid profile providing substrate for enhanced lipid peroxidation.

All these results suggest that during hypothyroidism, different risk factors lead to atherogenic risk associated with enhanced oxidative stress. Since it is caused by overproduction of O•2- and NO• leading principally to the formation of MDA and CP. Then, the decrease of
ORAC suggest more exceeding SOD activity due to outcompete NO• for O•2-.

CONCLUSION
The present study shows that hypothyroidism in women was marked by NO• with O•2- reaction, leading macromolecule (MDA and PC) oxidation and SOD activity exceeding. These findings indicate that TSH seems to have an impact on oxidative stress, antioxidant system and atherogenic risk induction. Oxidant and antioxidant status in hypothyroid subjects should be carefully considered, and appropriate management should be organized, including antioxidant supplementation. However, such a suggestion needs more investigation to confirm it.

ACKNOWLEDGMENTS
This research was supported by a financial support from the Algerian Research investigation (PNR 185 ANDRS, 2011). The authors are grateful to the patients and subjects who volunteered to participate in this study and all the members of nuclear medicine department of Tlemcen University Hospital Center (Algeria).

Conflicts of interest: nil

Abbreviations: BMI, body mass index; TSH, thyroid stimulating hormone; NO•, nitric oxide; NOS, nitric oxide synthase; O2•-, superoxide anion; OH•, hydroxyl radicals; MDA, malondialdehyde; CP carbonyl proteins; ORAC, total antioxidation capacity; SOD, superoxide dismutase; HDL, high density lipoproteins; LDL, low density lipoprotein; VLDL, very low density lipoprotein; T3, triiodothyronine; T4, thyroxine; OXS, oxidative stress; NADPH, oxidase, nicotinamide adenine dinucleotide phosphate-oxidase; GPx, glutathione peroxidase.

REFERENCES


