Original Article

# Persistence of Oxidative Stress in Newly Diagnosed Hypothyroid Patients Despite Effective Thyroxin Therapy

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## Abstract

**Background and Aim:** We have earlier reported increased oxidative stress (OS) in newly diagnosed hypothyroid patients. However, the comprehensive effect of thyroxin supplementation on OS, insulin resistance, inflammation, and glycation levels has not been analyzed. Therefore, in the present study, we have analyzed the effect of thyroxin therapy in newly diagnosed hypothyroid patients on OS and various biochemical markers after normalization of thyroid profile. **Methods**: Out of 67 recently diagnosed primary hypothyroid patients, 37 patients were recruited for this study based on the criteria of strict adherence to thyroxin treatment protocol and regular follow-up. Venous blood samples were analyzed before and 6 months after initiation of therapy for glucose, thyroid and lipid profiles, insulin, ultrasensitive C-reactive protein (usCRP), and anti-thyroperoxidase (TPO) antibody. Antioxidants such as glutathione, glutathione peroxidase, catalase, and glutathione S-transferase, and oxidized products such as malondialdehyde (MDA), and protein carbonyl (PCO) levels were analyzed as parameters of OS. HbA1 and fructosamine were assayed as glycation indices, and lipid risk factor for coronary artery disease was calculated from lipid profile. The parameters were re-assayed 6 months later after normalization of thyroid profile. **Results:** OS (MDA; $P < 0.01$ and PCO; $P < 0.01$) did not come back to normal level despite attainment of normal thyroid profile following treatment. Dyslipidemia ($P < 0.05$) and inflammation ($P < 0.05$) were significantly associated with OS. Furthermore, levels of triglyceride, anti-TPO antibody, and usCRP were higher in patients even after successful treatment. **Conclusion:** OS in treated hypothyroid patients despite normalization of thyroid profile persists longer which could partly be due to the residual inflammation and/or dyslipidemia.

**Keywords:** Dyslipidemia, hypothyroidism, inflammation, oxidative stress, treatment

***Received:*** 02nd May, 2018; ***Revised:*** 28th May, 2018; ***Accepted:*** 31st May, 2018

## Introduction

Hypothyroidism in India is among the most common endocrine disorders. Various nationwide studies reveal that thyroid disorder is on the rise, particularly in adolescents and young adults of India.[1] On the long run, hypothyroidism associated with chronic complications such as hyperlipidemia, secondary obesity, and atherosclerotic cardiovascular disease. The major component of the body is protein and lipids. Therefore, oxidative damage to body protein and lipids due to ensuing oxidative stress (OS) can exert its long-term health effects. We earlier reported the presence of OS in our studies[2] which corroborates with other studies.[3,4] Hypothyroidism is a treatable endocrine condition where thyroid profile normalizes with proper treatment, and patients need to review their thyroid profile at regular intervals, thereafter. However, there are reports available which suggest that thyroid-stimulating hormone (TSH) level and hyperlipidemia in hypothyroidism normalize slowly with thyroxin replacement therapy.[5,6] OS is known to be associated with obesity,[7] hyperlipidemia,[8] atherosclerosis, etc.[9] Furthermore, one of the previous reports suggests that treatment with thyroxin itself produces OS.[10]

Through this study, we intended to analyze whether thyroxin therapy normalizes OS parameters along with thyroid profile, and if not, then what are the plausible factors responsible for the persistence of OS.

Access this article online

Quick Response Code:

Website: www.ijcep.org

DOI: 10.4103/ijcep.ijcep_22_18

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**How to cite this article:** Nanda N, Bobby Z, Hamide A. Persistence of oxidative stress in newly diagnosed hypothyroid patients despite effective thyroxin therapy. Int J Clin Exp Physiol 2018;5:70-4.

## Materials and Methods

### Participants and study design

Clinically diagnosed primary hypothyroid patients were contacted from the Outpatient Services of Department of Medicine, Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), for this study. A total of 37 patients, of which 26 were females and 11 males, were recruited for the study. All the patients had a TSH level >10 μU/ml. The newly diagnosed hypothyroid patients who were not on any medication were included in this study before their thyroxin supplementation. Hypothyroid patients already on treatment for the disease, patients taking lipid-lowering drugs or antioxidant vitamin supplements, smokers, alcoholics, hypertensives, diabetics, patients with impaired fasting glucose, pregnant women, women on hormone replacement therapy, and patients suffering from diseases other than hypothyroidism, also patients with a family history of diabetes mellitus were excluded from the study. Two female patients had a family history of thyroid dysfunction, and two other had attended menopause. Thirty-six healthy, euthyroid participants of similar age and gender were taken as controls. Control group consisted of clinically screened euthyroid participants without any infectious or inflammatory disease or chronic ailments. The study was approved by the Research Council and Human Ethics Committee of JIPMER. Written informed consent was obtained from all the participants of the study groups before their enrollment into this study.

### Sample collection

Venous blood from the antecubital vein was collected from patients after overnight fast (before they started L-thyroxin therapy). Three millimeters of blood was collected in ethylenediaminetetraacetic acid (EDTA) bottle and the rest was kept aside for serum separation. The whole blood was used for the immediate assay of reduced glutathione (GSH) and hemoglobin (Hb). The red blood cells were separated by washing the EDTA whole blood thrice (5000 rpm for 5 min each) at 4°C using a cooling centrifuge (Kubota 6930), with cold normal saline. The packed cells were used for the subsequent analysis of erythrocyte antioxidant enzymes. Serum samples were separated from the blood collected in a separate tube and part of it was used for the immediate estimation of glucose and lipid profile. Remaining serum and plasma samples were stored in different vials for the estimation of various parameters by refrigeration at −40°C till the estimations of thyroid profile, protein carbonyls, malondialdehyde (MDA), ultrasensitive C-reactive protein (usCRP), and anti-thyroperoxidase (TPO) antibody were carried out.

### Thyroid profile

Thyroid profile was assessed by estimation of serum $T_3$, $T_4$, and TSH. $T_3$ and $T_4$ were assayed by radioimmunoassay and TSH by immunoradiometric kits procured from Bhaba Atomic Research Center, Mumbai, India. The intra- and inter-assay coefficient of variations was 3.3% and 7.3%, respectively, for T3; 4.7% and 8.2%, respectively, for T4; and 3.6% and 7.8%, respectively, for TSH assay.

### Glucose, insulin, and lipid profile

Fasting serum glucose was estimated by glucose oxidase method using kits from Dr. Reddy's Lab (Hyderabad, India). Insulin was estimated using IRMA kit for human following manufacture's (Immunotech, Beckman Coulter Co, Czech Republic) instructions. Lipid profile was estimated by enzymatic method using kits from Biocon (Germany) for total cholesterol and Accurex (Mumbai, India) for triglyceride (TG). High-density lipoprotein (HDL) cholesterol-precipitating reagent and enzymes were obtained from Agappe diagnostics (Thane, India). Low-density lipoprotein (LDL) cholesterol was calculated employing the Fridewald formula,[11] as the total TG level was below 400 mg/dl for the cases. All these analyses were performed using a calibrated autoanalyzer (550 Express Plus, Ciba Corning Diagnostics, Oberlin, OH, USA).

### Calculation of lipid risk factors

Various lipid risk factors of atherosclerosis such as non-HDL cholesterol, total cholesterol/HDL-cholesterol, triacylglycerol/HDL cholesterol, LDL-cholesterol/HDL-cholesterol, and atherogenic index (AI) were calculated from the estimated lipid profile.[12-14] The AI was expressed as $\log_{10}$ (TG/HDL-C) where TG and HDL-C were expressed in mM/L.

### Assay of erythrocyte antioxidants

Whole blood reduced GSH, glutathione peroxidase (GPx), glutathione S-transferase, and catalase activities were assayed using the standard protocol described in detail previously.[2]

### Assay of serum markers of oxidation

Protein carbonyl (PCO) was estimated as a marker of protein oxidation by the 2,4-dinitrophenylhydrazine by Levine.[15] It was expressed as nmol/mg protein. Thiobarbituric acid reactive fraction of MDA was measured as an estimate of lipid peroxidation expressed as μM/L of serum.[16]

### Assay of markers of glycation, autoimmunity, and inflammation

Glycated Hb expressed as HbA1 and glycated albumin expressed as fructosamine were assayed by ion exchange chromatography columns (Biocon, Vohl-Marienhagen, Germany) and by p-iodonitrotetrazolium violet kinetic method using Riachem kit (Hemagen Diagnostics, San Diego, CA, USA), respectively. Anti-TPO antibody was assayed by ELISA (Varelisa, Pharmacia and Upjohn, Germany) and usCRP by turbidimetric immunoassay kit adapted to autoanalyzer (Aptec Diagnostics, Belgium), respectively.

### Statistical analysis of data

All parameters are expressed as a mean ± standard deviation. Statistical analyses were performed using the IBM SPSS version 13 (Chicago Inc, IL, USA) program. Normality of the data distribution was checked by Kolmogorov–Smirnov test. Significance of the differences between control and test groups before and after therapy was done by one-way ANOVA followed by Tukey-Kramer *post hoc* test. The association between MDA and various parameters was done by Pearson's correlation analysis. The $P < 0.05$ was considered statistically significant.

## Results

The changes in anthropological parameters, lipid profile, OS parameters, and its complications in patients treated for 6 months with thyroxin are included in Table 1. OS parameters, parameters of inflammation, autoimmunity, glycation, and insulin resistance are enlisted in Table 2.

With regular treatment, euthyroidism was attained in the study group in about 6 months. It was also accompanied by complete reversal of previously increased levels of body mass index (BMI), thyroid profile, lipid profile (except TG), and non-HDL cholesterol [Table 1]. Level of all these parameters in the study group after treatment was not different from control group. The thyroxin treatment for 6 months reversed the levels of coronary lipid risk factors, Non-HDL-C, and LDL-C/HDL-C levels to that of the healthy control. However, despite significant decrease, other lipid risk factors such as TG/HDL-C, TC/HDL-C, AI were still higher than the mean of the control group.

Similarly, the treatment resulted in complete normalization in the levels of antioxidant enzymes such as GSH, GPx, and catalase similar to the level of the healthy controls [Table 2]. On the other hand, there was no statistical difference in insulin resistance (Homeostasis Model Assessment-Insulin Resistance [HOMA-IR]), protein glycation (HbA1c and fructosamine), and autoimmunity (Anti-TPO) among the three groups.

Despite a significant reduction in OS parameters (MDA and PCO) and marker of inflammation (usCRP), these levels did not reach the healthy control levels [Table 2]. Especially usCRP level despite significant decrease still remained higher than control [Table 2]. Furthermore, there was a significant positive correlation between CRP and MDA even after treatment [Table 3].

## Discussion

As a result of the treatment with thyroxin, the thyroid profile was reverted to that of the healthy control group. Although the BMI, TC, and LDL-C levels of the patients also reverted to the level of the healthy control, the posttreatment values for the other lipid risk factors such as TG/HDL-C, TC/HDL-C, and AI were still higher than the healthy controls. These results corroborate with previous reports.[5,6]

This could be due to the continuation of some inflammatory components and some degrees of hyperlipidemia. Our findings corroborate with the report of a recently conducted study in which MDA was found to be decreased but still remained high compared to control following 6 months of treatment.[17,18] However, previous studies did not study the battery of antioxidant enzymes unlike us. For the first time in our study, four major antioxidants and protein carbonyl (PCO), a sensitive marker of protein oxidation, were analyzed before and after treatment.

**Table 1: Comparison of anthropological parameters and routine biochemical parameters of hypothyroid patients before and after (thyroxin therapy for 6 months) treatment with euthyroid control subjects**

| | Control group ($n=37$) | Hypothyroid group ($n=37$) | |
|---|---|---|---|
| | | Before treatment | After treatment |
| General parameters | | | |
| Age (years) | 33±11.41 | 34.00±11.70 | 34.64±12 |
| BMI (kg/m$^2$) | 23.16±3.92 | 26.84±3.99*** | 24.06±3.50$^{\#\#}$ |
| Thyroid profile | | | |
| T3 (ng/dl) | 128.02±29.12 | 66.78±29.77*** | 121.97±34.76$^{\#\#\#}$ |
| T4 (µg/dl) | 8.98±2.10 | 4.24±2.34*** | 9.02±2.56$^{\#\#\#}$ |
| TSH (µIU/ml) | 2.22±1.14 | 55.95±38.29*** | 2.92±2.34$^{\#\#\#}$ |
| Lipid profile | | | |
| TC (mM/L) | 4.36±0.81 | 6.19±1.52*** | 4.87±1.19$^{\#\#\#}$ |
| HDL-C (mM/L) | 1.35±0.26 | 1.27±0.30 | 1.21±0.24 |
| LDL-C (mM/L) | 2.45±0.79 | 3.87±1.44*** | 2.87±1.16$^{\#\#}$ |
| TG (mM/L) | 1.20±0.33 | 2.26±0.81*** | 1.71±0.11**,$^{\#\#}$ |
| VLDL-C (mM/L) | 0.24±0.06 | 0.45±0.16*** | 0.34±0.14**,$^{\#\#}$ |
| Lipid risk factors | | | |
| Non-HDL-C (mM/L) | 3.00±0.84 | 4.91±1.58*** | 3.66±1.23$^{\#\#\#}$ |
| TG/HDL-C | 0.91±0.30 | 1.93±0.94*** | 1.50±0.77**,$^{\#}$ |
| TC/HDL-C | 3.34±0.87 | 5.20±1.93*** | 4.22±1.53*,$^{\#\#}$ |
| LDL-C/HDL-C | 1.92±0.79 | 3.31±1.62*** | 2.53±1.31$^{\#}$ |
| Atherogenic index | 0.06±0.14 | 0.23±0.21*** | 0.11±0.23***,$^{\#}$ |

Data are presented as mean±SD. Analysis of data was done by one-way ANOVA and *post hoc* by Tukey-Krammer multiple comparison test. The *Mark represents comparison with control, and the $^{\#}$Mark represents comparison with hypothyroids before treatment. *$P$<0.05, **$P$<0.01 and ***$P$<0.001 and $^{\#}P$<0.05 and $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001. Male: female is male-female ratio, Atherogenic index=$Log_{10}$ (TG/HDL-C) when TG and HDL-C are expressed in mM/L. BMI: Body mass index, TSH: Thyroid stimulating hormone, TC: Total cholesterol, HDL-C: High-density lipoprotein-cholesterol, LDL-C: Low-density lipoprotein-cholesterol, TG: Triglyceride, VLDL-C: Very-LDL, SD: Standard deviation

**Table 2: Comparison of oxidative stress parameters, markers of insulin resistance, inflammation, autoimmunity, and glycation indices of hypothyroid patients before and after (thyroxin therapy for 6 months) treatment with euthyroid control subjects**

| | Control group ($n=37$) | Hypothyroid group ($n=37$) | |
|---|---|---|---|
| | | Before treatment | After treatment |
| OS parameters | | | |
| GSH (µM/g Hb) | 9.89±2.99 | 7.86±2.20** | 9.45±1.96$^{\#}$ |
| GPx (U/g Hb) | 54.38±11.56 | 71.71±15.84*** | 60.99±14.61$^{\#\#}$ |
| Catalase (K/ml) | 24.34±7.11 | 28.08±7.53 | 28.04±9.97 |
| GST (µM/min/mg Hb) | 4.58±1.38 | 4.27±1.32 | 4.67±1.08 |
| MDA (µM/L) | 1.35±0.42 | 2.90±1.28*** | 2.16±0.94**,$^{\#\#}$ |
| PCO (nM/mg protein) | 1.18±0.41 | 2.20±0.92*** | 1.65±0.69*,$^{\#\#}$ |
| Parameters of insulin resistance | | | |
| Fasting glucose (mM/L) | 4.24±0.68 | 4.54±0.63 | 4.44±0.51 |
| Insulin (µU/ml) | 6.85±5.77 | 9.36±8.66 | 5.82±5.60 |
| HOMA-IR | 1.75±133 | 1.97±1.95 | 1.15±1.09 |
| Parameters of glycation | | | |
| Hb A1 (g%) | 7.34±0.37 | 8.19±0.85*** | 7.60±0.76$^{\#\#}$ |
| Fructosamine (µM/L) | 2.08±0.45 | 2.69±0.68** | 2.07±0.63$^{\#\#}$ |
| Fructosamine/albumin (mM/g) | 0.05±0.01 | 0.07±0.01** | 0.05±0.02$^{\#}$ |
| Inflammatory marker | | | |
| usCRP (mg/L) | 0.17±0.07 | 0.44±0.18*** | 0.32±0.15***,$^{\#\#}$ |
| Immunological marker | | | |
| Anti-TPO Ab (IU/mL) | 34.38±43.33 | 494.68±524.23*** | 270.63±219.92 |

Data are presented as mean±SD. Analysis of data was done by one-way ANOVA and *post hoc* by Tukey-Krammer multiple comparison test. The *Mark represents comparison with control, and the $^{\#}$Mark represents comparison with hypothyroids before treatment. *$P$<0.05, **$P$<0.01 and ***$P$<0.001 and $^{\#}P$<0.05 and $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001; Hb A1: Glycated hemoblobin, usCRP: Ultrasensitive CRP, TPO: Thyroperoxidase, OS: Oxidative stress, GSH: Glutathione, GST: Glutathione-S-transferase, MDA: Malondialdehyde, PCO: Protein carbonyl

**Table 3: Correlation of malondialdehyde with thyroid-stimulating hormone, protein carbonylation, lipid profile, lipid risk factors, anti-thyroperoxidase Ab, and ultrasensitive C-reactive protein in hypothyroid patients after attainment of euthyroid state following thyroxin therapy for 6 months ($n=37$)**

| Parameters | $r$ | $P$ |
|---|---|---|
| TSH | −0.127 | 0.455 |
| PCO | 0.580 | 0.000 |
| TC | 0.353 | 0.032 |
| TG | 0.331 | 0.045 |
| Non-HDL-C | 0.393 | 0.016 |
| TG/HDL | 0.397 | 0.015 |
| TC/HDL | 0.401 | 0.014 |
| LDL/HDL | 0.360 | 0.029 |
| AI | 0.345 | 0.037 |
| Anti-TPO Ab | 0.317 | 0.342 |

Data was analyzed by Pearson's correlation analyses. AI: Atherogenic index, BMI: Body mass index, TSH: Thyroid-stimulating hormone, TC: Total cholesterol, HDL-C: High-density lipoprotein-cholesterol, LDL-C: Low-density lipoprotein-cholesterol, TG: Triglyceride, VLDL-C: Very-LDL, SD: Standard deviation, TPO: Thyroperoxidase, PCO: Protein carbonylation

The increased serum protein carbonyl level in hypothyroid patients had a positive correlation with the increased MDA level [Table 3]. When products of lipid peroxidation such as 4-hydroxy nonenal and MDA covalently attach to the amino acid residues of proteins, they lead to oxidative modification.[19] Therefore, we postulate that low-grade OS associated with hypothyroidism if persists for a prolonged period, the resulting oxidative modifications to body lipids and proteins might be deleterious in these participants.

Baskol *et al*.[18] analyzed only paraoxonase which is associated with HDL apolipoprotein. Furthermore, they did not analyze for the effect of treatment on inflammatory marker, glycated proteins, and autoimmune parameter and insulin resistance in their group of patients. In our study, the response of glycated proteins, anti-TPO antibody titer, and insulin resistance (HOMA-IR) was analogous to the euthyroid effect of thyroxin treatment. However, posttreatment decrease in usCRP, measured as an inflammatory marker, continued to be higher when compared to the control value [Table 2]. The regular marker of inflammation called usCRP, also known as hsCRP, is also recognized as an independent marker of cardiovascular disease.[20] Moreover, CRP as an inflammatory mediator is recently recognized as an adjunctive marker for the global assessment of cardiovascular risk.[14,21,22] Therefore, a higher level of usCRP in our study group despite regular treatment and normalization of thyroid profile indicates the persistence of the risk for coronary artery disease.

One of the previous reports suggests that treatment with thyroxin itself produces OS.[10] Flynn *et al*. in 2006 reported that, despite treatment in primary hypothyroidism, patients

are still at increased risk of morbidity associated with various circulatory disease, ischemic heart disease, dysrhythmias, cerebrovascular diseases, etc.[23] However, the present study is the first of its kind to assess the level of OS in hypothyroidism estimating parameters of oxidant and antioxidant parameters before and after normalization of thyroid profile along with markers of inflammation, lipid risk factors, and other indices such as insulin resistance and autoimmunity. Furthermore, the analysis of correlations with parameters such as fructosamine, PCO, CRP, TPO, and *us*CRP before and after treatment has not been done in any prior study. Therefore, our study attempts at a comprehensive analysis of various factors associated with OS which was found to be persisting in hypothyroid patients who had attained thyroid profile due to their sincere compliance with treatment protocol. These results suggest that, despite normalization of TSH following prescribed thyroxin treatment, OS in hypothyroidism persists for a longer period. From the results, we also postulate that OS in these posttreatment euthyroid participants may considerably be contributed by dyslipidemia and/or inflammation.

## Conclusion

Hypothyroidism is a chronic disease and requires prolonged therapy for full recovery. In view of negative implications of OS, dyslipidemia, and inflammation on health, we suggest that regular monitoring of lipid profile, inflammatory markers, and OS status should be considered in hypothyroidism management. In addition, there is a need to complement the traditional treatment of these patients with antioxidants and lifestyle modification to bring down the ill effects of persisting OS.

### Informed consent

Informed consent was obtained from each participant after they voluntarily agreed to participate in this study.

### Acknowledgment

We acknowledge the research grant from Indian Council of Medical Research in the form of junior research fellowship followed by senior research fellowship to Dr. Nivedita Nanda and as adhoc research grant to Dr. Zachariah Bobby. We also acknowledge the technical assistants from Mr. R. Vengattraman for RIA and ELISA assays.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

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