

THYROID HORMONES IN EXCESS INDUCE OXIDATIVE STRESS IN RATS

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Abstract

Thyroid hormones play a crucial role in the regulation of the mitochondrial oxidative metabolism. Hyperthyroidism caused by the acceleration of the energy metabolism leads to the occurrence of cellular oxidative stress.

The aim is to evaluate the pro-oxidant / antioxidant balance and the effect of vitamin E supplementation in damage caused by the excessive administration of thyroid hormones.

Materials and Methods. White, male Wistar rats were used in the study. Thirty male Wistar rats were divided into three groups (1:control group, 2:animals treated with L-Thyroxine 10 µg/animal/day for 30 days, 3:L-Thyroxin treated rats protected with vitamin E 10 mg/animal/day). Malondialdehyde (MDA), the marker of lipid peroxidation, carbonyl proteins, SH groups, glutathione (GSH) and superoxide dismutase (SOD) were determined from the serum, while MDA, carbonyl proteins, SH groups and GSH were determined from the thyroid tissue homogenates.

The results showed increased levels of carbonyl proteins (1.31 ± 0.33 nmol/mg protein, $p=0.0001$) in serum in thyrotoxic group versus control, while MDA levels did not differ significantly from the control. Significantly low values of the SH groups, GSH and SOD were found ($p<0.001$) in the plasma of Thyroxin treated rats. Vitamin E supplementation significantly increased plasma MDA levels in the Thyroxin treated group as compared with the control group ($p=0.01$) and with the animals treated only with Thyroxin ($p=0.04$). Carbonyl protein levels in plasma of the hyperthyroid supplemented rats were also increased as compared to controls ($p=0.0002$). Antioxidant capacity markers in plasma of group 3 were decreased compared with group 1. The marker of lipid peroxidation (MDA) significantly decreased in thyroid homogenates of the group 2 as compared with group 1 ($p=0.004$). Significantly high levels of the SH groups ($p=0.0006$) and low levels of GSH ($p=0.0001$) were found in thyroid homogenates of the L-Thyroxin treated group as compared with controls.

These results suggest that experimental hyperthyroidism is accompanied with increased oxidative stress and with the consumption of antioxidant enzymes in induced oxidative aggressions. No protective effects of vitamin E on oxidative stress induced by excessive administration of thyroid hormones were detected.

Key words: Hyperthyroidism, Oxidative stress; Vitamin E, Carbonyl proteins, Antioxidant capacity.

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INTRODUCTION

Oxidative stress accompanying hyperthyroidism is caused by increased synthesis of reactive oxygen species (ROS) and changes in the antioxidant defence system (1). ROS have a high reactivity potential, therefore they are toxic and can lead to oxidative damage in cellular macromolecules such as proteins, lipids and DNA (2). In fact, the cell contains a variety of substances capable of scavenging the free radicals, protecting them from harmful effects. Among the enzymatic antioxidants, are glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), while the non-enzymatic antioxidants are glutathione (GSH), vitamin E, vitamin C, β -carotene, and flavonoids (3). When ROS generation exceeds the antioxidant capacity of cells, oxidative stress develops (4). This phenomenon has been related to many pathological conditions (5,6) and it has also been suggested that some complications of hyperthyroidism are due to thyroid-hormone-induced oxidative stress in target tissues (7).

Life means a continuous struggle for energy, which is required to fight against entropy. The most effective way to obtain energy is oxidation. Oxidative processes predominantly occur in mitochondria (8). On the other hand, mitochondria are one of the favorite targets of thyroid hormones. During thyroid hormone synthesis, there is a constant production of hydrogen peroxide, which is absolutely indispensable for iodine oxidation in the presence of thyroid peroxidase. In recent years, the possible correlation between impaired thyroid gland function and reactive oxygen species has been increasingly taken into consideration (9). Thyroid hormones are the most important factors involved in the regulation of the basal metabolic state, as well as in the oxidative metabolism (7). Thyroid hormones can cause many changes in the number and activity of mitochondrial respiratory chain enzymes, which may result in the increased generation of ROS (10,11). Experimental studies and epidemiological data suggest that hyperthyroidism is associated with a general increase in tissue oxidative stress. Great controversy exists as to whether hyperthyroidism is associated with an increase or a decrease in the activities of antioxidant enzymes (12-14). Vitamin E is a potent lipid soluble antioxidant in biological systems with the ability to directly quench free radicals and function as membrane stabilizer (15). Antioxidants treatments might be helpful in reducing the oxidative damage due to hyperthyroidism.

This study aims at investigating oxidative stress parameters, antioxidant status markers and their response to vitamin E supplementation in rats treated with thyroxin.

MATERIALS AND METHODS

White, male, Wistar rats weighing between 220 and 240 g were used. They were divided into 3 groups of 10 animals each: group 1 — controls (M), group 2 (T) — animals treated with L-thyroxine 10 μ g/animal/day for 30 days and group 3 (T+E) — L-thyroxine treated rats protected with 10 mg/animal/day of vitamin E administered

intramuscularly, for 30 days. The L-thyroxine quantity dissolved in 2 mL of milk was administered by gavage in the morning on an empty stomach. All animals were kept under the same environmental conditions, at a room temperature of 23 ± 1 °C, with an artificial lighting cycle (lights on 08.00-20.00 h) and water ad libitum. The experimental procedures used in this study met the guidelines of the Animal Care and the “Iuliu Hatieganu” University of Medicine and Pharmacy ethics committee approved the study.

Thyroid tissue preparation

After thirty days, blood was collected from the retroorbital sinus and the rats were sacrificed by cervical dislocation following ether anaesthesia. The thyroids were immediately dissected out and placed into ice-cold isolation medium. Tissue homogenates were used for analytical procedures.

Analytical procedures

The lipid peroxides level was assessed by fluorescence according to the Conti and Moran method (16), based on the reaction between malondialdehyde, the marker of lipid peroxidation and thiobarbituric acid, measured spectrophotometrically at 534 nm. Plasma or tissue homogenates were boiled in 2-thiobarbituric acid solution 10mM in K_2HPO_4 75mM PH_3 and extracted on n-butanol consecutively. Concentration values of MDA are expressed in nmol / ml based on specific calibration curves.

Protein oxidation was determined through the estimation of carbonyl groups photometrically with dinitrophenylhydrazine according to the Reznick method (17) and expressed as nmol per mg of protein (nmol/mg protein). Serum samples were submitted to a reaction with 2, 4- dinitrophenylhydrazine 10 mM in HCL 2.5 N, and treated with 20% trichloroacetic acid; the precipitate obtained by centrifugation was washed with a 1:1 (v/v) mixture of ethyl acetate and absolute ethyl alcohol and dissolved in guanidine chlorhydrate 6M. In the samples thus obtained the protein concentration was determined by measuring extinction at 280 nm. The carbonyl concentration was given by the formula:

$$C = Abs_{355} \times 45.45 \text{ nmol/ml}$$

The thiol content of samples was determined with dithionitrobenzoic acid (DTNB), according to the Hu method (18). One plasma volume was mixed with Tris (0.25M)- EDTA buffer 20 mM pH 8.2, absorbance being read at 412 nm. The Ellman (DTNB)10mM reagent was added, which produces a staining reaction, and the absorption was determined again at the same wavelength. The results were expressed as nmol SH per milligram of protein (nmol/mg protein).

Fluorescence was used to determine the glutathione (GSH) values (18). For the GSH dosage one plasma volume was mixed with TCA 10% and then centrifuged, the supernatant separated and additioned with 1.7 ml phosphate buffer pH 8 and 1 ml o-phthalaldehyde. Emission intensity was measured at 420 nm at an excitation of 350 nm. Glutathione concentration was determined using a calibration curve made with known concentrations of glutathione processed in the same way. The results were expressed as micromoles per litre ($\mu\text{mol/L}$).

Superoxide dismutase (SOD) activity of the samples was evaluated using the

Flohe method (19) and expressed as U SOD per milligram of protein (U/mg protein). Dosage was performed on lysed erythrocytes at 25°C. Superoxide-dismutase (SOD) catalysed the superoxide radical ($\bullet\text{O}_2^-$) dismutation in peroxide (H_2O_2) and oxygen (O_2). The superoxide radical ($\bullet\text{O}_2^-$) reacts with C ferricytochrome, which can be continuously monitored by recording the absorbance at 550 nm. Superoxide-dismutase reduces the concentration of superoxide ions and thus inhibits the reduction of the C cytochrome and the SOD amount may be thus calculated from the degree of inhibition of the C cytochrome using a calibration curve achieved by the SOD standards known. One unit of SOD activity is defined as the amount of enzyme able to inhibit the reduction rate of cytochrome C by 50%.

Serum free-thyroxine (FT_4) concentrations were measured with an enzyme immunoassay kit (EIAgen Free T4 Kit, Adaltis Italy).

Statistical analysis

Data are given as mean \pm standard deviation (S.D.). Statistical analyses were performed using the one-way ANOVA and paired T-test for normally distributed samples, and the Kruskal-Wallis and Wilcoxon tests for data which were not normally distributed. Linear relations among variables were examined with Pearson's Correlation Analysis. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Significantly higher FT_4 ($p < 0.001$) values were observed in the L-thyroxine administered group as compared with the control group (Table 1). FT_4 values of the L-thyroxine and vitamin E-administered group were significantly decreased in respect to those of the L-thyroxine only administered group (Table 1).

In the hyperthyroid rats, the MDA levels did not differ significantly from euthyroid values ($p > 0.05$) while in the thyroid tissue, the MDA levels were significantly decreased ($p < 0.01$) as compared with euthyroid values. We found that carbonyl proteins levels

Table 1. Plasma lipid peroxides (MDA), carbonyl proteins (PC), antioxidant status parameters and FT_4 values in the experimental groups (mean \pm SD)

	Control group(C)	Hyperthyroid group (T)	Hyperthyroid + vitamin E group (T+E)
MDA (nmol/mL)	1.51 \pm 0.23	1.56 \pm 0.3	1.94 \pm 0.43 ^b
PC (nmol/mg protein)	0.69 \pm 0.17	1.31 \pm 0.33 ^a	1.36 \pm 0.38 ^a
SH (mmol/L)	0.27 \pm 0.02	0.20 \pm 0.03 ^a	0.15 \pm 0.05 ^b
GSH ($\mu\text{mol/L}$)	65.71 \pm 16.06	32.96 \pm 19.60 ^a	28.83 \pm 25.37 ^a
SOD (U/g protein)	6928.94 \pm 1031.47	5114.16 \pm 1136.15 ^a	4523.30 \pm 873.33 ^a
FT_4 (ng/dL)	1.48 \pm 0.21	3.34 \pm 0.40 ^a	2.22 \pm 0.42 ^{a*}

Malondialdehyde=MDA, Thiol groups=SH, Glutathione=GSH, Superoxide dismutase=SOD, free-T4= FT_4 , ^aSignificant($p < 0.001$) versus control group; ^bSignificant($p < 0.05$) versus hyperthyroid group, ^{a*} Significant($p < 0.001$) versus hyperthyroid group.

Table 2. Lipid peroxides (MDA), carbonyl proteins (PC) and antioxidant status parameters in the thyroid gland (mean \pm SD)

	Control group (C)	Hyperthyroid group (T)	Hyperthyroid+vitamin E group (T+E)
MDA(nmol/mL)	0.30 \pm 0.06	0.21 \pm 0.05 ^a	0.28 \pm 0.12
PC(nmol/mg protein)	1.42 \pm 0.32	1.67 \pm 0.66	2.46 \pm 0.9
SH(mmol/L)	0.02 \pm 0.008	0.08 \pm 0.03 ^{a*}	0.03 \pm 0.01 ^b
GSH(μ mol/L)	93.34 \pm 10.54	65.04 \pm 12.64 ^{a*}	62.09 \pm 15.4 ^{a*}

Malondialdehyde=MDA, Thiol groups=SH, Glutathione=GSH, ^aSignificant($p < 0.01$) versus control group; ^{a*}Significant($p < 0.001$) versus control group, ^bSignificant($p < 0.01$) versus hyperthyroid group

were significantly higher (1.31 \pm 0.33 nmol/mg protein, $p = 0.0001$) in the plasma of Thyroxin treated rats, while in the thyroid homogenates, the levels of carbonyl proteins did not differ significantly from the control group (Tables 1, 2).

Thiol groups (SH), superoxide dismutase (SOD) and glutathione (GSH) were lower in the L -thyroxine-administered group in comparison to the control group ($p < 0.001$) (Table 1). A significantly high SH level and a significantly low GSH level were observed in the thyroids of the L-thyroxine-administered group in comparison to the control group ($p < 0.001$) (Table 2).

We also investigated the relation between the mean values of FT4 and the mean values of MDA in the L-thyroxine-administered group. There was a significant positive correlation between hyperthyroidism and oxidative stress ($p < 0.5$; $r^2 = 0.70$).

DISCUSSION

In the plasma of L-thyroxine-treated rats, the marker of lipid peroxidation (MDA) levels did not differ significantly from the euthyroid values ($p > 0.05$). This result of unchanged lipid peroxidation level can be correlated with the observations of Asayama *et al.* (20) who found no change of MDA in liver homogenates from hyperthyroidism induced rats rendered hyperthyroid by administration of T4 to their drinking water over a 4-week period. However, our results are not in concordance with the findings of Seven *et al.* (21) who found a significant increase in MDA levels in the plasma of rats rendered hyperthyroid by administration of T4 in their food for 24 days and Venditti *et al.* (22) who noticed that hyperthyroidism induced in rats by T3 daily i.p. injections for 10 days caused significantly increased MDA levels in liver homogenates.

These discrepancies among results seem to reflect a dependence of peroxidative processes on various factors, such as tissue, species, the iodothyronine used and treatment duration. On the other hand, it is not possible to exclude the fact that some conflicting results depend on the different accuracies of the methods used for lipid peroxidation determination. For example the method for evaluating thiobarbituric acid reactive substances (TBARS) is inaccurate, and give results

which differ according to the assay conditions used (23).

In the present study, the increased levels of protein-bound carbonyls in plasma of L-thyroxine-treated rats is in agreement with the earlier reports (15,24) suggesting the role of free radicals in the pathogenesis, which demand the need for studies assessing the therapeutic role of antioxidants in hyperthyroidism. We found a positive association between hyperthyroidism and lipid peroxides correlated by linear regression which clearly suggest induction of oxidative stress. Such an effect may be related to the enhanced metabolic rate generated by thyroid hormone administration, leading to an accelerated ROS production (2). In the thyroid homogenates of the L-thyroxine treated rats, the MDA values were significantly decreased and carbonyl proteins levels did not show significant changes.

These results show that hyperthyroid state is not accompanied by oxidative stress in the thyroid gland and contradict the results of Joanta *et al.* (25) who observed an increase in lipid peroxides and carbonyl proteins in the same tissue in experimental hyperthyroidism. The synthesis of thyroid hormones crucially depends on H_2O_2 , which works as a donor of oxidative equivalents for thyroperoxidase (26). Because of its great toxicity, H_2O_2 synthesis must always remain in equilibrium with the hormonal synthesis and strictly contained at the apical pole of the cell. Thyrocytes possess various enzymatic systems, such as GPx, catalase, superoxide dismutases, and peroxiredoxins that contribute to limit cellular injuries when H_2O_2 or other ROS are produced in excess (27 - 29).

Our findings may be explained by the fact that the external administration of thyroid hormones usually inhibits pituitary secretion of TSH and indirectly hormonal synthesis (30). It is therefore possible that decreased oxidative stress observed in thyrocytes, is due in part to the absence of H_2O_2 . Significantly high levels of the SH groups ($p=0.0006$) and low levels of GSH ($p=0.0001$) were found in thyroid homogenates of the L-Thyroxin treated group as compared with the control group, reflecting reduced oxidative stress and low antioxidant capacity. Similar results were described at the level of expression, by Western blot in a recent paper (31) where in T4 treated rats there was a decrease in the level of oxidative stress and in the level of GPx. Antioxidant status parameters, namely thiol groups (SH), superoxide dismutase (SOD) and glutathione (GSH) were significantly decreased in the plasma of hyperthyroidism-induced rats in comparison to the control group ($p<0.001$).

GSH is a tripeptide widely distributed and involved in many biological activities including neutralisation of ROS, detoxification of xenobiotics, and maintenance of —SH levels in proteins (32). The reduction of GSH levels in hyperthyroid rats reflects its consumption through the oxidative stress. In contrast with our results, Seven *et al.*(12) and Morini *et al.*(33) have demonstrated increased levels of GSH in the blood from hyperthyroid rats. Our results of decreased SOD activity in plasma of rats following L-thyroxine treatment are not in line with other studies (12, 34) which found increased levels of SOD in experimental hyperthyroidism.

These differences in antioxidant enzyme activity may be caused by various mechanisms. The reactive oxygen species contribute to an intensified synthesis of

antioxidant enzymes in tissues and hence their elevated activity may be a manifestation of adaptation mechanisms in response to oxidative stress. A decreased activity of antioxidant enzymes or a decreased non-enzymatic antioxidant concentration may be caused by their intensified utilization in protection against oxidative tissue damage (14, 35). There are a number of factors that may influence antioxidant system activity: the physiological state of the thyroid gland, the dose and the duration of treatment. In experimental studies, antioxidant enzyme activity was affected by the age of the animals with induced hyperthyroidism (36).

Data on the effects of vitamin E supplementation on thyroid hormone levels are limited. As far as the impact of vitamin E on thyroid status in L-thyroxine-treated rats is concerned, vitamin E supplementation caused a decrease in FT₄ levels (p=0.001). These results show that Vitamin E has a thyroid function suppressing action. This is in line with the report of Seven *et al.* (12) who found decreased T4 and T3 levels in vitamin E-supplemented euthyroid rats and suggested that vitamin E supplementation in the euthyroid state decreases either T4/T3 synthesis or T4-T3 conversion. Further studies on deiodinase activity in the liver tissue of hyperthyroidism-induced vitamin E-supplemented rats will clarify the crucial impact of vitamin E on T4-T3 conversion.

Vitamin E supplementation significantly increased plasma MDA levels in the Thyroxin treated group compared with the control group and with the only Thyroxin treated animals (p=0.04). Carbonyl proteins levels in the plasma of the hyperthyroid supplemented rats were also increased compared with the controls (p=0.0002). Antioxidant capacity markers in the plasma of group 3 were decreased compared with group 1. This could be explained by the relative doses of vitamin E administered as compared with other studies (12, 21) which were not enough to suppress the oxidative stress in hyperthyroidism. Messarah *et al.* (3) observed an increase in vitamin E concentrations in rats suffering from hyperthyroidism, which might be due to an adaptation against the oxidative stress provoked by the thyroid hyperactivity and could be the answer to our results.

In conclusion, our results suggest that thyroid hormones in excess are accompanied by increased oxidative stress and impairment of the antioxidant system. Vitamin E supplementation in hyperthyroidism could exert beneficial effects in favour of the diminution of thyroid hormone levels. These findings indicate that thyroid hormones have a strong impact on oxidative stress and the antioxidant system.

Acknowledgments

This study was supported by a research grant for young PhD students, offered by The National Board For Scientific Research In Higher Education (CNCSIS). The authors are grateful to Professor L. Gozariu for critical evaluation of this manuscript and would like to acknowledge the support of Nicoleta Decea, G. Dragotoiu and Georgeta Hazi, for their valuable assistance in oxidative stress markers and hormonal measurements.

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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