Chapter 8

Oxidative Stress and Antioxidant Status in Hypo- and Hyperthyroidism

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/51018

1. Introduction

Thyroid hormones are involved in the regulation of basal metabolic state and in oxidative metabolism [1]. They can cause many changes in the number and activity of mitochondrial respiratory chain components. This may result in the increased generation of reactive oxygen species (ROS) [2,3]. Oxidative stress is a general term used to describe a state of damage caused by ROS [4]. 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 [5].

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 peroxydase (GPx), catalase (CAT), superoxide dismutase (SOD), while the non-enzymatic antioxidants are glutathione (GSH), vitamin E, vitamin C, β-carotene, and flavonoids [6]. When ROS generation exceeds the antioxidant capacity of cells, oxidative stress develops [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 the favorite targets of thyroid hormones. During thyroid hormone synthesis, there is a constant production of oxygenated water, which is absolutely indispensable for iodine intrafollicular 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].

Experimental studies and epidemiological data suggest that hyperthyroidism is associated with increase in free radical production and lipid peroxide levels [10,11].

In hypothyroidism, a decrease in free radical production is expected because of the metabolic supression brought about by the decrease in thyroid hormone levels [12,13,14].

The changes in the levels of the scavengers α-tocopherol, glutathione [15] and coenzyme Q[16] and activities of antioxidant enzymes [11]were found to be imbalanced and often opposite.

It is worth mentioning that some of the antithyroid drugs have antioxidant effects[17]. It was shown that both methimazole and propylthiouracil abolished or reduced radical production by complement attacked thyroid cells and decreased cytokine production[18].

Antioxidants treatments might be helpful in reducing the oxidative damage due to hypothyroidism and hyperthyroidism.

The available data concerning oxidative stress in both hypothyroidism and hyperthyroidism are scarce and controversial. Reviewing the most recent data on the subject, this study aims at investigating oxidative stress parameters, antioxidant status markers and their response to vitamin E supplementation in hyper- and hypothyroid rats.

2. Oxidative stress

A major threat to homeostasis and therefore to the integrity of aerobic organisms arises from chemical species possessing one or more unpaired electrons in their outer orbital, called free radicals [19]. Oxygen free radicals can develop during several steps of normal metabolic events. Although free radicals (FR) have the potential to damage the organism, their generation is inevitable for some metabolic processes. The main endogenous sources of free radicals are the mitochondrial electron transport chain, the microsomal membrane electron transport chain, reactions of oxidant enzymes and auto-oxidation reactions [20,21,22].

Oxidative stress is a term that was introduced by Sies in 1985 and refers to any situation where there is a serious imbalance between the production of FR or reactive oxygen species (ROS), called the oxidative load, and the antioxidant defense system. The oxidative load is described as "a measure of the steady-state level of reactive oxygen or oxygen radicals in a biological system"[23]. Oxidative stress has been defined as "a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage"[24].

Cells can tolerate moderate oxidative loads by increasing gene expression to up-regulate their reductive defense systems and restore the oxidant/antioxidant balance. But when this increased synthesis cannot be achieved due to damage to enzymes, or substrate limitations, or when the oxidative load is overwhelming, an imbalance persists and the result is oxidative stress [25]. Superoxide and hydroxyl radicals, along with non-radical oxygen species such as hydrogen peroxide (H_2O_2) are commonly termed reactive oxygen species (ROS) and have the highest biological activity. ROS are produced in all cells, depending on the intensity of aerobic metabolism, especially in activated neutrophils, monocytes, smooth muscle cells and in endothelial cells [26]. Disequilibrium between ROS production and inactivation leads to oxidative stress. ROS also cause injury to the basic cell structures. They readily react with macromolecules, such as lipid, protein and DNA molecules, which results in degradation of cell membranes and excessive activation or inactivation of enzymes[27]. The ultimate effects of ROS activity include mutations, metabolic dysfunction and cell ageing. They in turn are a cause of development of inflammatory processes, oncogenesis and impaired organ functioning [28, 29].

Oxidative stress is considered to play a pivotal role in the pathogenesis of aging and several degenerative diseases, such as atherosclerosis, cardiovascular disease, type 2-diabetes and cancer [30,31,32]. In order to cope with an excess of free radicals produced upon oxidative stress, humans have developed sophisticated mechanisms in order to maintain redox homeostasis [33].

These protective mechanisms either scavenge or detoxify ROS, block their production, or sequester transition metals that are the source of free radicals, and include enzymatic and non enzymatic antioxidant defenses produced in the body, namely, endogenous[34,35], and others supplied with the diet, namely exogenous[36,37].

Antioxidant enzymes act to scavenge free radicals by converting them to less harmful molecules [38]. Among the most known enzymatic antioxidants, we notice superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxydase (GPx) and catalase (CAT). SOD catalyzes the dismutation of superoxide anion radical to peroxide (H_2O_2) and molecular oxygen (O_2). Catalase, an iron-containing hemoprotein, converts hydrogen peroxide to water and oxygen [39]. GPx is an enzyme containing a selenium ion as a cofactor [40], and for the catalyzed reaction it requires reduced glutathione (GSH), which is provided by glutathione reductase. GPx is one of the most effective antioxidants in erythrocytes. A reduction in GPx activity results in increased H_2O_2 levels and hence severe cellular damage is observed [41].

Non-enzymatic antioxidants, such as glutathione, tocopherols, retinols, and ascorbate, play an important role in scavenging ROS.

3. Toxic effects of H_2O_2

The levels of H_2O_2 reached physiologically in cells vary from a low 0. 001 μM to a maximum of 0. 7 μM. When H_2O_2 is applied to the exterior of cultured cells, the intracellular concentrations are approximately 10-fold lower than the extracellular concentrations [42, 43]. Because there are great variations in the rate of H_2O_2 degradation in different cell types and models, it is difficult to compare concentration-effect relations. In most cell cultures, H_2O_2 in the medium disappears in less than 1 h. At higher concentrations than those that have a signaling role, H_2O_2 induces oxidative stress, DNA oxidation and damage, and consequent mutagenesis and apoptosis [42]. For the phagocytes, H_2O_2 has been designated as “the enemy within” [44]. Oxidative stress involves the oxidation of various cellular components, proteins, lipids, nucleic acids, etc. The accumulation of oxidatively damaged proteins accelerates chaperone-mediated autophagy, which will degrade them [45]. Oxidative damage to DNA produces adducts (including 8-oxo-deoxyguanosine and thymine glycol), single-strand breaks, and at high levels double-strand breaks [46]. Positive Comet assays demonstrate these breaks. The half-life of these damages varies for the various lesions (from 9–62 min for the adducts, more for the breaks) [47]. The positive Comet assays for thyroid cells incubated with 50 μM H_2O_2 disappear by 80% in 2 h [48]. Mutagenesis, if it leads to constitutive activation of a protooncogene or to inactivation of tumor suppressor genes is carcinogenic, especially if it is combined to a proliferative effect. Thus, H_2O_2 is carcinogenic and has been found to play a role in several human cancers (7) even if it may not be sufficient [49]. Conversely, selenium, the essential constituent of protective enzymes, prevents tumor development in rats submitted to chemical carcinogenesis [50]. Lack of protective systems in knockout mice such as lack of peroxiredoxin or glutathione (GSH) peroxidases indeed leads to malignant cancers [51,52]. Transfection of an H_2O_2-generating system transforms epithelial cells [53]. High-level acute H_2O_2 treatment of various cells in vitro leads to apoptosis [54]. This effect has been linked to a loss of GSH and reduced glutaredoxin and consequent activation of apoptosis signal-regulating kinase (ASK) and of an apoptosis program [55]. These effects are stronger in actively proliferating cells [56]. Chronic H_2O_2 administration at low levels induces senescence in cultured cells in vitro in human fibroblasts [57,58]. H_2O_2 favors inflammation [59], and its inhibitory effect on indoleamine dioxygenase, which by depriving lymphocytes of tryptophan is immunosuppressive, would enhance immune reactions. It is therefore not astonishing that even in relatively short-lived (7 h) neutrophils [60] and macrophages, H_2O_2 generation is tightly regulated by a synergic two-pronged mechanism involving both intracellular calcium and diacylglycerol protein kinase C[58,61].

4. Thyroid hormone synthesis

The thyroid is a shield-shaped organ in the neck region composed of an outer layer of follicular cells and c-cells, which surrounds a lumen that contains colloid. It contributes to the body's energy output by regulation of cardiac rate and output, lipid catabolism, heat production, and skeletal growth [62], which explains the wide range of symptoms related to thyroid abnormalities. The colloid contains thyroglobulin which is converted into thyroid hormone (TH). The luminal side of the follicular cell membrane contains microvilli, which greatly increase the surface area of the cell to facilitate transfer of colloid into the follicular cell.

TH includes thyronine (T_3), and thyroxine (T_4). T_4, and to a lesser extent T_3, is synthesized in the follicular cell and is propagated by thyroid stimulating hormone (TSH) secreted by the pituitary gland. TSH synthesis is propagated by thyrotropin releasing hormone (TRH) secreted by the hypothalamus. Several of these processes deal with direct or indirect collaboration between the thyroid, hypothalamus, pituitary, or pineal glands [63, 64].

TH synthesis includes a radical intermediate, creating a need for a ROS reaction as part of the organ's function to maintain homeostasis. Iodination of tyrosine residues, catalyzed by a peroxidase enzyme, occurs on the endoplasmic reticulum of the thyroid gland cells. Coupling forms various THs [65]. H_2O_2 is required by peroxidase, and is formed by an enzyme from NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) and Ca^{2+} ions.

Beginning with active transport of dietary iodide (the rate limiting substrate) into the cell by sodium-iodide symporter [65], iodide oxidation and hormone synthesis occur at the apical membrane of the follicular cell. Iodination (organification) and the coupling reaction of iodotyrosines require the presence of thyroperoxidase (TPO), a hemoprotein located in the apical plasma as well as in the adjacent cytoplasm, endoplasmic reticulum, Golgi complex, nuclear envelope.

The molecular mechanism of iodination consists of a series of successive stages, having extremely reactive free radicals as intermediate products. Following the addition of oxygenated water (H_2O_2) to thyroperoxidase (TPO), compound I is formed, which oxidizes iodine (I^+), and the active iodine form results: the iodinium ion (I^+) or the hypoiodite ion (IO^-). These remain bound to thyroperoxidase. Tyrosine residues also bind to thyroperoxidase, which favors the organification of iodine with the formation of iodotyrosines: monoiodotyrosine (MIT) and diiodotyrosine (DIT). In the absence of iodine, compound I is spontaneously converted into a stable compound, compound II, which catalyzes the coupling reaction of iodotyrosines, resulting in the formation of thyroid hormones. The excess of oxygenated water (H_2O_2) determines the conversion of compound II to inactive compound III. Inactivation is prevented by iodine [66].

The generation of oxygenated water, as an electron acceptor, is absolutely indispensable for thyroperoxidase activity [67]. H_2O_2 is produced by an NADPH-dependent process on the external aspect of the apical plasma membrane of follicular cells. Although various enzyme systems, including cytochrome reductases, can support H_2O_2 production in the thyroid, an NADPH-dependent, H_2O_2 –generating system was detected in thyroid particulate fractions that appears to be distinct from cytochrome c reductase. The activation of this NADPH oxidase requires Ca^{2+} ions.

The mechanism of formation of oxygenated water (H_2O_2) is controversial; there are two theories: The superoxide anion is the primary product of the enzymatic conversion of oxygen which, under the action of superoxide dismutase (SOD) will be transformed into oxygenated water. The superoxide anion, produced inside the cytoplasm, close to the apical membrane, under the action of NADPH-oxidase, is released outside the thyroid follicular cell only after its transformation into oxygenated water [68]. Other data suggest that oxygenated water is the primary product of the NADPH-oxidase system, and is produced outside the thyroid follicular cell via the two-electron reduction of O_2 [69].

The production of oxygenated water is stimulated by TSH-cAMP and phosphatidylinositol-Ca^{2+}. Other enzymatic systems capable of generating oxygenated water have been evidenced: monoamine oxidase, xanthine oxidase, glucose oxidase [66].

It is necessary to prevent excess or deficiency of H_2O_2, anything but optimal levels are linked to several thyroid diseases and disorders, such as congenital hypothyroidism, tumorigenesis, myxedematous cretinism, thyroiditis, and cancer [70, 71].

Various reports deal with thyroid disorders and H_2O_2. Normal levels of H_2O_2 in the body vary from 0. 001 mM to 0. 7 mM, but excess “induces oxidative stress, DNA oxidation and damage, and consequent mutagenesis and apoptosis” [71]. Several selenoproteins act as a protective barrier for thyrocytes from endogenous H_2O_2 [72]. If DNA damage is perpetuated, it can lead to carcinogenesis. Also, increased levels of H_2O_2 inhibit iodide uptake and organification [73]. Several genetic disorders have been shown to decrease H_2O_2 production by creating a partial iodide-organification defect and reducing or eliminating hormone production [74]. This led to permanent congenital hypothyroidism in non-TH producing individuals, and mild, transient hypothyroidism in low hormone level subjects. As an autoregulatory effect, H_2O_2 production is diminished by high iodide concentration, but mildly stimulated by low iodide levels [75].

As stimulation by TSH permits, monoiodotyrosine (MIT) and diiodotyrosine (DIT) are released from the lumen into the follicular cell. Here, ferric TPO product (oxidized by H_2O_2) reacts with DIT to form a radical stabilized by the aromatic ring. Oxidation of either MIT or another DIT, followed by coupling, yields T_3 and T_4, respectively. Coupling in this reaction is catalyzed by TPO. TH inhibits production of TSH and TRH, an autoregulatory effect.

After hormone synthesis, any free iodotyrosine derivative left over is deiodinated quite rapidly due to excess iodotyrosine deiodinase, avoiding formation of other iodoamino acids, and recycled back into the thyroid. Thus, only T_3 and T_4 can be found in the thyroid vein's blood supply [76]. T_3 is more potent than T_4, more rapid in its reaction, and may be the active form of excreted T_4 that is deiodinized by the target cells [75]. Two general effects of TH are described. First, altering the natural level by injection or thyroidectomy showed altered metabolism rates for several organs, suggesting the need for TH for energy metabolism. This includes the diaphragm, epidermis, gastric mucosa, heart, kidney, liver, pancreas, salivary gland, and skeletal muscles. There are also effects of TH on development. Hypothyroidism proved to have an effect on the rate and result of development; yet these observations were described as quantitative rather than qualitative, and are generally more easily reversed than are developmental inadequacies [77]. Still, although TH affects many of the body's cells, it is not considered necessary to the survival of the organism, and removal is not uncommon.

5. Oxidative stress in experimental hyperthyroidism and hypothyroidism

Thyroid hormones regulate several essential physiological processes such as energy metabolism, growth and formation of the central nervous system, tissue differentiation and reproduction. The molecular action of thyroid hormones is mediated via the thyroid hormone receptors which, after ligand binding, activate genes by binding to the thyroid hormone response elements [79].

Thyroid hormones control the intensity of basal metabolism. They are calorigenic and, consequently, they increase oxygen consumption and heat production. Basal metabolism decreases in hypothyroidism and increases in hyperthyroidism. In the second case, an increase in the number and size of mitochondria, particularly of their cristae, has been seen, concomitantly with the increased concentration and the intensified activity of oxidative phosphorylation enzymes. T_3 and T_4 have been found to stimulate in vitro protein synthesis in mitochondria, ADP capture, ATP formation and oxygen consumption. The primary ligands of T_3 are the nucleus and the mitochondrion. In fact, thyroid hormones have primary actions in several cell organelles, in a coordinated succession: binding to the cell membrane as a substrate, to mitochondria, through which metabolic energy required for nuclear transcription and posttranscription is released, and the specific synthesis of structures and functions is directed. Mitochondria are particularly important for the action mechanism of thyroid hormones, representing the final step of oxygen transfer in the respiratory chain [67].

Mitochondrial respiration is a complex metabolic process by which hydrogen from the reduced forms of dehydrogenases is oxidized to proton (H^+) and molecular oxygen from air is reduced to anion, which allows for the formation of water. NADPH+H^+-dehydrogenase, flavoproteins ($FMNH_2$/$FADH_2$), non-porphyrin iron-sulfur proteins, ubiquinones (Q), and certain cytochromes participate in the main oxidoreduction reactions of the respiratory chain [79]. In mitochondrial respiration, significant amounts of hydrogen superoxide and peroxide radicals are formed, probably due to the auto-oxidable nature of the enzymatic system components (coenzyme Q, NADH+H^+-dehydrogenase, cytochrome b), on the one hand, and to the incomplete reduction of the oxygen molecule ("trivalent" reduction occurs instead of "tetravalent" reduction), on the other hand. Superoxide formation is continuous in the respiratory chain, approximately 1-2% of the electrons that participate in the chain form superoxide and its dismutation product – hydrogen peroxide [80].

Thyroid hormones increase the concentration and activity of Na^+-K^+ dependent ATP-ase, as well as Na^+ and K^+ permeability. 15% to 40% of the basal energy used by the cell is used for the maintenance of an electrochemical gradient. Thyroid hormones concomitantly stimulate the activity of cellular anabolic and catabolic enzymes, determining in this way the intensification of energy consumption [81].

Data from in vivo and in vitro studies indicate that thyroid hormones have a considerable impact on oxidative stress [11]. The great majority of the reactive oxygen species (ROS) are generated at mitochondrial level, via oxidative phosphorylation. Thyroid hormones act on mitochondria by regulating energy metabolism, and mitochondria are a major source of intracellular free radicals [82,83]. During thyroid hormone synthesis, there is a constant production of oxygenated water, which is absolutely indispensable for iodine intrafollicular 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].

The aim of our study was to evaluate oxidative stress parameters, antioxidant status markers and their response to vitamin E supplementation in experimental hyperthyroidism and hypothyroidism.

White, male, Wistar rats weighing between 220 and 240 g were purchased from The Iuliu Hatieganu University of Medicine and Pharmacy,Cluj-Napoca biobase. All animals were kept under the same environmental conditions, at a room temperature of 23±1ºC, with an artificial lighting cycle (lights on 08. 00-20. 00 h) and water ad libitum.

They were divided into 5 groups of 10 animals each: group 1– controls, group 2 – animals treated with L-thyroxine 10μg/animal/day for 30 days and group 3 – L-thyroxine treated rats protected with 10 mg/animal/day of vitamin E administered intramuscularly, for 30 days, group 4 – animals treated with Propylthiouracil (5mg/100g animal /day), for 30 days and group 5 – Propylthiouracil treated rats protected with 10 mg/animal/day of vitamin E administered intramuscularly, for 30 days. The L-thyroxine and Propylthiouracil quantity dissolved in 2 ml of milk was administered by gavage in the morning on an empty stomach.

Thirty days into the experiment, blood was taken from the retro orbital sinus and the rats were sacrificed by cervical dislocation following ether anaesthesia.

Thyroid gland was immediately dissected out and placed into ice-cold isolation medium. Tissue homogenates were used for analytical procedures.

Malondialdehyde (MDA), the marker of lipid peroxidation, carbonyl proteins, SH groups,reduced glutathion (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 lipid peroxides level was assessed by fluorescence according to the Conti and Moran method [84], based on the reaction between malondialdehyde, the marker of lipid peroxidation and thiobarbituric acid, measured spectrophotometrically at 534nm.

Plasma or tissue homogenates were boiled in 2-thiobarbituric acid solution 10mM in K2HPO4 75mM PH3 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[85] 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 2,5N, and treated with 20% trichloracetic acid; the precipitate obtained by centrifugation was washed with a 1: 1 (v/v) mixture of ethyl acetate and absolute ethylic 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

$$C = Abs_{355} x\ 45{,}45 nmol / ml$$

The thiol content of samples was determined with dithionitrobenzoic acid (DTNB), according to the Hu method [86]. One plasma volume was mixed with Tris (0,25M)-EDTA20mM pH 8,2 buffer, 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 wave length.

The results were expressed as nmol SH per milligram of protein (nmol/mg protein).

Fluorescence was used to determine the glutathione (GSH) values [86]. 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 (μmol/l).

Superoxide dismutase (SOD) activity of the samples was evaluated using the Flohe method [87] and expressed as U SOD per milligram of protein (U/mg protein). Dosage was performed on lysed erythrocytes at 25^0C. Superoxide-dismutase (SOD) catalysed the superoxideradical ($O_2^{\bullet -}$) dismutation in peroxide (H_2O_2) and oxygen (O_2).

The superoxide radical ($O_2^{\bullet -}$) reacts with C ferricytochrome, which can be continuously monitored by recording the absorbance at 550 nm. Superoxid-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 known SOD standards. 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 T_4 Kit, Adaltis Italia).

Significantly higher FT_4 ($p<0.001$) values were observed in the L-thyroxine administered group as compared with the control group. 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.

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 were significantly higher (1. 31±0. 33, $p=0.0001$) in the serum of Thyroxin treated rats, while in the thyroid homogenates, the levels of carbonyl proteins did not differ significantly from the control group.

Thiol groups (SH), superoxide dismutase (SOD) and reduced glutathione (GSH) were lower in the L -thyroxine-administered group in comparison to the control group ($p<0.001$).

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 ($p<0.001$).

We also investigated the relation between the mean values of FT_4 and the mean values of MDA in the L-thyroxine-administered group. There was a significant positive correlation between hyperthyroidism and oxidative stress. ($p>0.5$; $r^2=0.70$).

Significantly low FT_4 ($p<0.001$) values were observed in the Propylthiouracil administered group as compared with the control group.

In serum and thyroid tissue of the hypothyroid rats, the MDA levels did not differ significantly from euthyroid values ($p>0.05$).

We found that carbonyl proteins levels were significantly higher (0. 99±0. 27, $p<0.05$) in serum, and the thyroid tissue (1. 99±0. 61, $p<0.05$) of the Propylthiouracil treated rats, as compared with the control group.

Vitamin E supplementation increased significantly the carbonyl proteins levels as compared with the hypothyroid rats.

Thiol groups (SH), superoxide dismutase (SOD) and reduced glutathione (GSH) levels in the hypothyroid group did not differ significantly from the control group.

Administration of Vitamin E to hypothyroid rats resulted in a significant decrease in serum antioxidant status parameters (SH, SOD, GSH) levels as compared with the Propylthiouracil treated rats.

Thyroid hormones, of which T3 is the major active form, exert a multitude of physiological effects affecting growth, development and metabolism of vertebrates [88], so that they can be considered major regulators of their homeostasis. On the other hand, elevated circulating levels of thyroid hormones are associated with modifications in the whole organism (weight loss and increased metabolism and temperature) and in several body regions. Indeed, low plasma lipid levels, tachycardia, atrial arrhythmias, heart failure, muscle weakness and wasting are commonly found in hyperthyroid animals. Plasma membrane [89], endoplasmic reticulum [90] and mitochondria [91] have been considered as potential cellular sites of action of thyroid hormone. However, it is now generally accepted that most of the actions of thyroid hormone results from influences on trascription of T_3-responsive genes, which are mediated through nuclear thyroid hormone receptors [92]. It is worth noting that the idea that oxidative stress underlies dysfunctions produced by hyperthyroidism is not in contradiction with mediation of T_3 action through nuclear events. Indeed, it is conceivable that some of the biochemical changes favouring the establishment of the oxidative stress (increase in mitochondrial levels of electron carriers, NOS activity and the unsaturation degree of lipids) are due to stimulation of the expression of specific genes initiated through T3 binding to nuclear receptors. Thyroid hormone induces upregulation of NOS gene expression in rat hypothalamus [93], and it is conceivable that this also happens in other tissues in which T_3-induced NO• overproduction has been shown [94, 95, 96].

The superoxide anion, hydrogen peroxide and the hydroxyl radical are the major reactive oxygen species in our body. Free radicals are produced as a consequence of normal metabolism and their levels and activities are controlled by enzymatic defense mechanisms, such as the SOD, GPx and CAT, and nonenzymatic defense mechanisms, such as ascorbic acid, Vitamin E, and GSH [97,98,99]. Oxidative damage arises when an imbalance occurs in this system, i. e. over-production of free radicals and/or a decrease in antioxidant defence mechanisms [100].

Disturbances of the oxidant/antioxidant balance resulting from the increased production of ROS are causative factors in the oxidative damage of cellular structures and molecules, such as lipids, proteins and nucleic acids [101]. In particular, biological membranes rich in unsaturated fatty acids are cellular structures susceptible to free radical attack [102].

Among the mediators involved in the pathophysiology of hyperthyroidism and subsequent tissue injury in animal models, free radical-mediated lipid peroxidation plays a pivotal role. Oxygen free radicals react with all biological substances. Lipid peroxidation is an autocatalytic mechanism leading to oxidative destruction of cellular membranes. Such destruction can lead to cell death and to the production of toxic and reactive aldehyde metabolites called free radicals [103]. 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. Malondialdehyde (MDA) is an end-product of lipid peroxidation and is frequently measured as an index of these processes [104].

Thyroid stimulating hormone (TSH) affect metabolism and may be affected by the thyroxine secretions. High concentrations of thyroid hormones stimulate free radical formation in mitochondria by affecting oxygen metabolism [18]. Although reactive oxygen species play an important role in physiological mechanisms, extremely reactive oxygen radicals can cause severe oxidative damage to molecules [110]. If cellular mechanisms cannot scavenge these reactive oxygen species, toxicity is found in biomembranes and lipid peroxidation occurs. This damage is usually more evident in cellular membranes.

Triiodothyronine (T_3) and thyroxin (T_4) circulating hormones are involved in the modulation of the physiological mitochondrial respiration process [105]. These agents were reported to change the number and activity of the mitochondrial respiratory chain components. The up regulating of these hormones can result in a mitochondrial respiration perturbation and a consequent increase in ROS generation [107]. These ROS would lead to oxidative damage to biological macromolecules, including lipids, proteins and DNA [108]. In contrast, in the case of hypothyroidism, there is a suppression of the metabolic rate and decline in ROS release [109].

Recently, increasing experimental and clinical studies have shown that free radicals play a key role in the etiology of many diseases. Thyroid hormones cause oxidative stress as they increase ROS, while activating metabolic systems of the body in general. [10]

Effects of thyroid hormones on lipid peroxidation have been subject of investigation in several laboratories but the results are rather contradictory. It was reported that hypermetabolic condition in hyperthyroidism was associated with an increase in free radical formation and lipid peroxidation levels [10, 11,110]. In previous studies, there are conflicting results about oxidative stress in hyperthyroidism. In some studies, it was demonstrated that the products of lipid peroxidation were decreased [111,112]. On the contrary, Fernandez et al. [10] and Dumitriu et al. [113] found high products of lipid peroxidation. Similarly, Iangalenko et al. [114] found that lipid peroxidation was increased in hyperthyroid patients. Asayama et al. [115] showed that the damaging effect of lipid peroxidation was increased in liver, heart and some skeletal muscles of rats, diminishing antioxidant enzymes in experimental hyperthyroidism.

Peroxidative effects elicited by thyroid hormones were found in the brain of newborn [116] and adult [117] rats. Such effects were also found in heart homogenates [110, 11, 118, 119] from young rats. However, increased lipid peroxidation in hearts from old (1. 5 years) but not from young (8 weeks) hyperthyroid rats was also reported [120]. Thyroid hormone treatment was found to increase lipid peroxidation in lymphoid organs such as mesenteric lymph nodes and thymus, without major effects in the spleen [12], a thyroid hormone-unresponsive tissue [121]. Thus, no significant change (TBARS) or decrease (HPs) were observed in lipid peroxidation level in the testis from adult hyperthyroid rats [122], and the thyroid hormone-induced increase in lipid peroxidation was found to be confined to some skeletal muscles. In both rat [11, 12] and cat [123], such an increase was found in the soleus, a red muscle mainly composed of slow-twitch oxidative glycolitic fibres (type I). Conversely, no change was found in the extensor digitorum longus (EDL) [11, 123], a white muscle mainly composed of fast-twitch glycolitic fibres (Type IIb). These results are consistent with early observations that red, but not white muscles, are sensitive to thyroid hormones [124, 125]. Lipid peroxidation was also increased by thyroid hormone in rat gastrocnemious [110,126], a mixed fibre muscle also containing fast-twitch oxidative glycolytic fibers (type IIa), but was decreased in the white portion of such a muscle [12]. On the other hand, it is surprising that in kidney from hyperthyroid rats the lipid peroxidation level does not change [127], although the tissue exhibits a calorigenic response to thyroid hormone similar to that elicited in liver [121].

Studies on the mouse showed lower susceptibility to thyroid hormone-induced lipid oxidative damage. Indeed, levels of lipid peroxidation were found to be increased in hindlimb muscles [128], unchanged in heart [129] and decreased in liver [107] from hyperthyroid mice. The results concerning liver were attributed by the authors to the animal species or long-term (4-5 weeks) treatment they used, because a laboratory study describing no increase in index of lipid peroxidation in hyperthyroid rat liver used the same long-term treatment [11]. Although this may be true, it is interesting that in both mouse and rat hyperthyroidism was induced by T4, whose biological activity can differ from T_3 in some tissues. Indeed, recent studies have shown that T4, but not T3, increases lipid peroxidation in rat interscapular brown adipose tissue [130].

Although the pathophysiological consequences of the accelerated lipid peroxidation are not yet fully elucidated, this biochemical change is thought to be responsible for some complications of hyperthyroidism. However, it is still to be determined whether the various target tissues of thyroid hormone undergo other biochemical changes that either predispose to free radical-mediated injury, or oppose it.

Despite some contradictory reports, the aforementioned results provide strong evidence that thyroid hormones induce oxidative stress in target tissues. Oxidative stress results from a disturbance of the normal cell balance between production of ROS and the capacity to neutralize their action.

In aerobic cells O_2 is mainly consumed through its four-electron reduction to water by cytochrome c oxidase. This reaction occurs without release of any intermediate in the O_2 reduction. However, despite the efficiency of the mitochondrial electron transport system, the nature of the alternating one-electron oxidation-reduction reactions it catalyses predisposes electron carriers to side reactions, in which an electron is transferred to O2 directly, instead of the next electron carrier in the chain, generating $O_2^{\bullet}$ [131]. This radical is then converted by spontaneous or catalysed dismutation into hydrogen peroxide (H_2O_2) [132], which can be turned into highly reactive hydroxyl radical ($^{\bullet}OH$).

Numerous oxidases in the cytosol, endoplasmic reticulum and outer mitochondrial membrane also contribute to O2 consumption and lead to $O_2^{\bullet}$ and H_2O_2 generation [133].

Major complications of hyperthyroidism are the myopathy and cardiothyreosis [81].

Joanta et al. [134] revealed an increase of the lipid peroxides content and carbonyl proteins level in blood, liver, thyroid, heart and skeletal muscle in experimental hyperthyroidism, suggesting that thyroid hyperfunction is accompanied by oxidative stress. R. Shinohara et al. [120] have investigated how thyroid function might influence the production of oxygen free radicals, the lipid peroxidation process and antioxidant activity in muscle of rat myocardium. It was found that the degree of lipid peroxidation, assessed by measuring substances that react with thiobarbituric acid, significantly increases in animals with hyperthyroidism than euthyroidiene.

Also the antioxidant enzyme activity changed: increased the xanthine oxidase and superoxiddismutase and decreased the glutathione peroxidase. These changes in the prooxidant/antioxidant balance, caused by thyroid hormones excess could be involved in myocardial dysfunction.

Zaiton et al. [123] revealed increased concentration of lipid peroxidation products in the myocardium and solear muscle in rat, but not in liver tissue. Conflicting results obtained Fernandez et al. [10] : increased liver content in lipid peroxides induced by thyroid hormones. Tapia et al. [135] studied the influence of thyroid hormones on Kupffer cells activity in isolated liver, perfused with colloidal carbon solution. The conclusion was that hyperthyroidism increase Kupffer cells activity and the production of oxygen free radicals at this level. Therefore liver macrophages could be an alternative source of reactive species.

Retroocular fibroblast proliferation is involved in the pathogenesis of ophthalmopathy in Basedow-Graves disease. H. Burch et al. [136] studied the way in which the superoxide radical, generated by the xanthine oxidase/hypoxanthine system, can induce cell proliferation in fibroblast cultures from patients with severe ophthalmopathy, as well as from control patients, in whom the excision of retroorbital tissue was performed. The authors found that the superoxide radical determined fibroblast proliferation, the intensity of this phenomenon depending on the concentration of reactive oxygen species. The effectiveness of some pharmacological agents on retroocular fibroblast proliferation induced by the superoxide radical was also monitored. For this, retroocular tissue was incubated with methimazole, propylthiouracil (synthesis antithyroid drugs), allopurinol (a xanthine oxidase inhibitor), and nicotinamide (an antioxidant). The most effective regarding the inhibition of superoxide radical production and implicitly, that of fibroblast proliferation, were methimazole, allopurinol and nicotinamide. These results suggest the implication of reactive oxygen species in retroocular fibroblast proliferation in Basedow-Graves disease [137].

Mitochondria are particularly susceptible to ROS-induced damage because they are a major site of oxygen free radical production [138] and contain great amounts of high and low molecular weight Fe^{2+} complexes, which promote the oxidative damage of membrane lipids [139,140]. Thyroid state-linked changes in the balance between ROS production and antioxidant capacity should result in changes in the damage to mitochondrial components. Therefore, we investigated the effects of altered thyroid states on the extent of oxidative damage of mitochondrial lipids and proteins.

It is well known that MDA is a terminal product of lipid peroxidation. So the content of MDA can be used to estimate the extent of lipid peroxidation. The latter can indirectly reflect the status of the metabolism of free radicals, the degree to which the tissue cells are attacked by free radicals and the degree to which lipid is peroxidated.

In our study in the plasma of L-thyroxine-treated rats, the marker of lipid peroxidation (MDA) levels did not differ significantly from the euthyroid values. This result of unchanged lipid peroxidation level can be correlated with the observations of Asayama et al. [11] who found no change of MDA in liver homogenates from hyperthyroidism induced rats rendered hyperthyroid by administration of T_4 to their drinking water over a 4-week period. However our results are not in concordance with the findings of Seven et al. [141] who found a significant increase in MDA levels in the plasma of rats rendered hyperthyroid by administration of T_4 in their food for 24 days and Venditti et al. [110] who noticed that hyperthyroidism induced in rats by T3 daily i. p. injections for 10 days caused significantly increased MDA levels in liver, heart and skeletal muscle 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 returns results which differ according to the assay conditions used [19].

The high increase in the level of MDA and hydroperoxides in hyperthyroidism might be due to the possible changes in the cellular respiration of target tissues, which are undoubtedly related to any alteration in the thyroid function, knowing the major role of the thyroid hormones in the control (acceleration) of the mitochondrial respiration rate [108], [2] and [28]. From a biochemical point of view, the provoked hyperthyroidism, and via a variety of mechanisms, mitochondrial respiratory chain activity is altered, leading to an increase in electrons transfer from the respiratory chain through the acceleration of the cellular metabolism rate, resulting in the increased generation of superoxide ($O_2^{\bullet-}$) at the site of ubiquinone [7]. Superoxide radicals can lead to the formation of many other reactive species, including hydroxyl radicals ($OH^{\bullet}$), which can readily start the free-radical process of lipid peroxidation [3] and [6].

Proteins are also sensitive to oxidative damage which leads to alteration in their structure and ability to function [142]. Protein oxidation can lead to a loss of critical thiol groups (SH) in addition to modifications of amino acids leading to the formation of carbonyl and other oxidized moieties[143,144,145].

Oxidative cleavage of proteins by either the alpha-amidation pathway or by oxidation of glutamyl side chains leads to formation of a peptide in which the N-terminal amino acid is blocked by an alpha-ketoacyl derivative. However, direct oxidation of lysine, arginine, proline and threonine residues may also yield carbonyl derivatives. In addition, carbonyl groups may be introduced into proteins by reactions with aldehydes (4- hydroxi-2- nonenal, malondialdehyde) produced during lipid peroxidation or with reactive carbonyl derivatives(ketoamine, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of reducing sugars or their oxidation products with lysine residues of proteins (glycation and glycoxidation reactions). The presence of carbonyl groups in proteins has therefore been used as a marker of ROS-mediated protein oxidation [134].

There are not many data regarding the effect of the thyroid state on protein oxidation. In experimental hyperthyroidism increased protein oxidation was demonstrated in different tissues [146,147]. Enhanced myocardial protein oxidation was also shown in the study of [148] by means of carbonyl group measurement. An elevation of this protein oxidation marker was demonstrated in the plasma of hyperthyroid patients [149,147].

In our study, the increased levels of protein-bound carbonyls in serum of L-thyroxine-treated rats is in agreement with the earlier reports [150,151] suggesting the role of free radicals in the pathogenesis, which demand the need for studies assessing the therapeutic role of antioxidants in hyperthyroidism.

A recent study[152] found a positive association between thyroid hormones in excess 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 [153,141].

In the thyroid homogenates of the L-thyroxine administered 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 [134] 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 [154]. Because of its great toxicity, H_2O_2 synthesis must always remain in adequation 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 [155,156,157].

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 [158]. It is therefore possible that decreased oxidative stress observed in thyrocytes, is due in part to the absence of H_2O_2.

6. Antioxidant status

Substances that neutralize the potential ill effects of free radicals are generally grouped in the so-called antioxidant defence system. Such a system includes both low molecular weight free-radical scavengers and a complex enzyme array involved in scavenging free radicals, terminating chain reactions, and removing or repairing damaged cell constituents. To provide maximum protection, these substances are strategically compartmentalized in subcellular organelles within the cell and act in concert. In examining antioxidant changes found in hyperthyroid tissues, it needs to be underscored that although thyroid hormone can directly control levels of enzymes with antioxidant activity or regulate scavenger content, antioxidant depletion could not be the cause, but the consequence of the oxidative stress. The effects of thyroid hormone on antioxidant status have been extensively investigated in rat tissues, while a few data concerning other species are available [159].

Several antioxidant enzymes exist that convert ROS into less noxious compounds, for example, superoxide dismutase (SOD), catalase, thioredoxin reductase, peroxiredoxin and glutathione peroxidase (GPx) [160,161,162,163,164]. Collectively, these enzymes provide a first line of defense against superoxide and hydrogen peroxides. They are of enormous importance in limiting ROS-mediated damages to biological macromolecules, but they are not able to be 100% effective because certain compounds generated by the interaction of ROS with macromolecules are highly reactive. It is then mandatory to detoxify these secondary products in order to prevent further intracellular damage, degradation of cell components and eventual cell death. This second line of defense against ROS is provided by enzymes such as GPx, glutathione S-transferase (GST), aldo-keto reductase and aldheyde dehydrogenase [165,166,167]. Thus, the central role of reduced GSH appears clear in intracellular endogenous antioxidant defenses as it is involved in all the lines of protection against ROS [35].

The tripeptide γ-glutamylcysteinylglycine or GSH is the major nonenzymatic regulator of intracellular redox homeostasis, ubiquitously present in all cell types at millimolar concentration [168]. This cysteine-containing tripeptide exists either in reduced (GSH) or oxidized (GSSG) form, better referred to as glutathione disulfide, and participates in redox reactions by the reversible oxidation of its active thiol [169]. Under normal cellular redox conditions, the major portion of this regulator is in its reduced form and is distributed in nucleus, endoplasmic reticulum and mitochondria. In addition, GSH may be covalently bound to proteins through a process called glutathionylation and acts as a coenzyme of numerous enzymes involved in cell defense [170]. Glutathione can thus directly scavenge free radicals or act as a substrate for GPx and GST during the detoxification of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds. Glutathione peroxidases constitute a family of enzymes, which are capable of reducing a variety of organic and inorganic hydroperoxides to the corresponding hydroxy compounds, utilizing GSH and/or other reducing equivalents. There are several tissue-specific GPx's that exhibit also tissue-specific functions [171]. All of them are selenoproteins and their primary function is to counteract oxidative attack. During the catalytic cycle, selenium is oxidized by the hydroperoxide to a selenic acid derivative. This intermediate is subsequently reduced by the electron donor. When GSH is used, a seleno-disulfide is formed, which is cleaved by a second GSH molecule to yield the reduced GPx. During catalysis the oxidation state of the enzyme depends on the relative concentration of the reducing (GSH) and oxidized (hydroperoxides) substrates. The phospholipid hydroperoxide GPx — discovered as a factor preventing lipid peroxidation — is considered to be involved in the protection of biomembranes against oxidative stress. In general, these isoenzymes may have a role in the regulation of the delicate regional redox balance, in particular the regulation of the appropriate tone of hydroperoxides known to be involved in cellular signaling, and to evoke several cellular responses, for example, programmed cell death, proliferation, cytokine production, and so on [172]. Glutathione S-transferases are three enzyme families — cytosolic, mitochondrial and microsomal — that detoxify noxious electrophilic xenobiotics, such as chemical carcinogens, environmental pollutants and antitumor agents. Moreover, they protect against reactive compounds produced in vivo during oxidative stress by inactivating endogenous unsaturated aldehydes, quinones, epoxides and hydroperoxides, all of which are produced intracellularly after the exposure to pollutants, or consumption of overcooked or mycotoxin-contaminated food, or polluted water [173]. Glutathione S-transferases exert those protective effects because they are able to catalyze the conjugation of GSH with oxidation end products and represent a second line of defense against the highly toxic spectrum of substances produced by ROS-mediated reaction. Both GPx and GST activities can eventually lower the level of total intracellular GSH. During the course of the reaction catalyzed by GPx, the exaggerated production of GSSG can lead to the formation of mixed disulfides in cellular proteins, or to the release of GSSG excess by the cell, to maintain the intracellular GSH/GSSG ratio. During the GST-mediated reactions, GSH is conjugated with various electrophiles and the GSH adducts are actively secreted by the cell. Mixed disulfide formation together with GSSG or GS-conjugated efflux can result in the depletion of cellular GSH, which can be opposed by a de novo synthesis or by reducing the formed GSSG. In the presence of oxidative stress, GSH concentration rapidly decreases while GSSG — potentially highly cytotoxic — increases because of the reduction of peroxides or as a result of free radical scavenging. This has two important consequences: (1) the thiol redox status of the cell will shift and activate certain oxidant response transcriptional elements, and (2) GSSG may be preferentially secreted from the cell and degraded extracellularly, increasing the cellular requirement for de novo GSH synthesis. Glutathione disulfide can also be reduced back to GSH by the action of glutathione reductase (GRed) utilizing NADPH as a reductant [174]. Glutathione reductase is a flavoenzyme and is represented by a single-copy gene in humans. It has been observed that exposure to agents that lead to increased oxidative stress also leads to an increase in its mRNA content. Further experimental data have shown the importance of GRed activity in GSH metabolism, demonstrating that the enzymatic activity is regulated in response to stress, and that mutations affecting GRed activity would have deleterious consequences. The recycling pathway for GSH formation is thus fundamental in the metabolism of GSH-dependent defense reactions [175]. In conclusion, the presence of GSH is essential, but not in itself sufficient, to prevent the cytotoxicity of ROS, being of fundamental importance the functionality of the glutathione-dependent enzymes, which participate in the first and second lines of defense.

Thyroid hormones increase oxygen consumption via a thermogenetic effect. In hyperthyroidism caused by thyroxine or triiodothyronine administration, the increase in metabolic rate together with the increase in oxygen consumption enhances microsomal oxidative capacity and free radical formation. There are conflicting results about an increase or decrease in the activities of antioxidant enzymes in hyperthyroidism [12, 16; 176-182]. In some studies, it has been reported that SOD activity was significantly increased [12,179,181]. On the contrary, several authors reported that SOD activity were reduced in patients with hyperthyroidism [180,182].

Superoxide dismutase is an important intracellular oxygen radical-scavenging enzyme. It has been demonstrated that hyperthyroidism leads to accelerated free radical formation [183]. Conversely, increased free radical formation enhances intracellular scavenging enzymes, like SOD, in experimentally induced hyperthyroidism [141].

Regarding the way in which thyroid gland hyperfunction influences antioxidant defense capacity, the results are different from one study to another. The organism can defend itself against the effects of oxidative stress by increasing SOD activity as a protection mechanism, but we observed a decreased SOD activity in our study. The observed diminution of SOD activity in rats, following L-thyroxine treatment can be correlated with the observations of [184]. However, our results are not in good agreement with the findings of [141] and [185], who noticed that hyperthyroidism induced in rats by T_3 caused an elevation of SOD activity in liver. Such a discrepancy between our and their results may be due to different experimental conditions and different methods used to assay SOD activities.

There is no difference in SOD activity between hyperthyroid patients and controls or between hypothyroid patients and controls in the studies of both [6] and [186]. Effects of thyroid hormones on SOD activity have been evaluated by others, but results are rather contradictory. The increase of SOD has been shown in the blood of patients with hyperthyroidism [6]. On the contrary, Erdamar et al. [187] found decreased SOD activity in the blood samples of patients with hyperthyroidism.

Varying forms of SOD (Mn-SOD, CuZn-SOD) present in the thyroid are the first line of defense in neutralizing ROS [188]. One study correlates several thyroid disorders to levels of CuZn-SOD and Mn-SOD, which are very high in malignant tumors [189]. This is a natural occurrence in the body to prevent and eliminate excess ROS that might result from, or have caused, these diseases. Therefore, SOD in the thyroid may involve two roles: (i) to serve as an antioxidant enzyme to protect the thyroid from oxidative stress, and (ii) to provide H2O2 for hormone synthesis [190].

There are two types of SOD enzymes reported in higher vertebrates. One is Cu-Zn SOD, mainly found in the cytoplasm of cells, while the other one is mitochondrial in nature and is known as Mn-SOD[191,192]. Mn–SOD activity in cardiac tissue was reported to both increase [11,120,179] and remain unchanged [118,193], even though in all cases hyperthyroidism was elicited by long-term treatment with T_4. Mn–SOD was also found to increase in the soleus and white portion of gastrocnemious muscle from rats made hyperthyroid by combined T_3 and T_4 administration [12] and in soleus[11] and gastrocnemious [194] from T_4- treated rats.

Cu-Zn SOD activity increased in gastrocnemious [194] and in its white portion [12], in agreement with insensitivity of such muscle to thyroid hormone, whereas it was reported to both decrease [179] and remain unchanged [11,120] in cardiac muscle, despite the same prolonged treatment with T_4. Total SOD was found to decrease in liver [180] and increase in heart from young [120,157] but not from old [120] hyperthyroid rats.

The increase in SOD activity in hyperthyroidism indicates the presence of oxidative stress due to the increasing mitochondrial oxidation rate, characterised by an overproduction of superoxide anion. The latter is known for its harmfulness to the cell membrane. The SOD is also known for its role in transforming $O_2^{\bullet-}$ into inorganic hydroperoxide (H_2O_2), which will, in turn, be reduced by both CAT and GPx enzymes [108], [120] and [195]. Accordingly, an increase in CAT activity in the homogenates of hyperthyroid rats is noted. This accelerates the speed of the formation of superoxides and the renewal of H_2O_2 quantity (substrate of CAT), which increases CAT activity until the dismutation of hydrogen peroxide [196], [197] and [147]. Both SOD and CAT function together in a way linked to the dissociation and formation of H_2O_2, and their activities are adjusted by their variation in the thyroid gland's activity.

One enzyme activity leads to the formation of a substrate for another one, whereby the excess of hydrogen peroxide may serve as a factor of SOD inactivation. On the other hand, GPx may be inactivated by the superoxide radical excess. Thus, GPx is protected from its inactivation via superoxide radical just by the enhanced SOD activity [198]. Based on such a sequence of events,it has been postulated that hyperthyroidism might be accompanied by the induction of either SOD or GPx or both [140].

For catalase (CAT) activity an increase in the white portion of gastrocnemious [12] and both decrease [11] and increase [12] were found in soleus from hyperthyroid rats. Decreases in CAT activities were found in brown adipose tissue after T3 or T4 treatment [130] and in liver [11,16], whereas lack of change [120,156, 179] and decrease [11] were found in heart.

The relationship between hyperthyroidism and glutathione peroxidase (GPX) activity also appears not well defined. Indeed, it was reported that cardiac activity decreased after long-term T_4 treatment of both young [11, 156] and old [120] rats, increased [118] and remained unchanged [120] after long-term T4 treatment of young rats, and remained unchanged after short-term T_3 treatment of young rats [110]. Liver GPX activity was found to decrease after T4 treatment [11], but both increased [16] and remain unchanged [110,199] after T3 treatment.

Moreover, it was found that T3 treatment increased GPX activity in gastrocnemious [110], while T_4 and T_3+T_4 treatments decreased such activity in gastrocnemious [194] and in its white portion [12], respectively. T4 administration also decreased GPX activity in both thyroid hormone responsive (soleus) [11] and unresponsive (EDL) [11] muscles. Enzyme activity was found increased in brain from hyperthyroid newborn rats [116].

The changes induced by T3 treatment in both liver [16,110,199] and heart [110], but not in muscle glutathione reductase (GR) activities shown in the various laboratories were consistent with those found for GPX activities. It is interesting that in brain of newborn hyperthyroid rats the activities of antioxidant enzymes (Cu, Zn,-SOD, CAT and GPX) exhibited compensatory increase that did not prevent oxidative stress [116].

Joanta [200] evidenced an increase in the concentrations of total peroxidase and catalase in the liver, thyroid, brain and blood, a decrease in the activity of these enzymes in the myocardium and skeletal muscle. This does not only confirm the main role of the thyroid hormones in regulating the oxidative stress in target cells, but is also in agreement with that of [16]and[6], where an icrease in GPx activity in hyperthyroid rats was observed. In contrast, Asayama et al. [11] found a low glutathione peroxidase concentration in the liver tissue taken from rats with experimental hyperthyroidism. These differences have multiple causes. An explanation could be related to the amount of thyroid hormones administered to the animals. Asayama et al. [11] administered thyroxine in a dose of 0. 0012% in the drinking water, Morini et al. [16] 30 μg T_3/100 g body weight/day and Venditti et al. [110] 10 μg T_3/100 g body weight/day to the rats previously treated with methimazole. The difference in GPx enzyme activity was probably due to the age (eight weeks) of the rats used in the investigation of [6]. The physiological state of the thyroid gland, the dose and the duration of treatment are also of a major influence on antioxidant enzymes. It was reported in previous studies that the level of lipid peroxidation in the heart was affected by both the age and the state of the thyroid gland, in hyperthyroid rats [120]. From another point of view, the above-mentioned effects might involve an accumulation of superoxide anion that inhibits CAT activity, giving rise to hydrogen peroxide (H_2O_2) concentrations [108].

Another explanation could be that at cellular level, there are other antioxidant systems [201], whose activity has not been evaluated by the mentioned investigations.

The decrease in GPx activity could in part be ascriebed to the fact that it is a selenoenzyme-like D1(5′-deiodinase I),which is involved in T_4 transformation into active T_3. As the enhanced hormone production is very pronounced in hyperthyroidism, deiodination of T_4 is also increased. Since the body stores of selenium are limited, deiodination is given preference over GPx in selenium supply. In 1994, Köhrle described GPx as a sort of selenium store easily available for D1 activity [202]. Other selenoproteins such as selenoprotein P mediate the transfer of selenium between the two enzymes. Thus, selenium deficit might be the cause of reduced GPx activity [203].

Function of intracellular GPx is degradation of H_2O_2 and hydroperoxides of free fatty acids, whereas in plasma GPx catalyses degradation of H_2O_2 and hydroperoxides of phospholipids. In addition GPx exert a protective effect on membrane phospholipids by inhibiting their peroxidation processes [204]. According to hypothesis proposed by Seven et al. increased ROS production may lead to elevated GPx activity [205]. Because of the fact that proteins are not synthesized de novo in erythrocytes, it can be suspected that these cells contain high reserves of enzymatic protein levels; therefore on one hand it is possible to activate antioxidant enzymes in response to ROS activity, and on the other hand- correction of losses caused by oxidative stress. Reduction of antioxidant potential of red blood cells occurring in thyrotoxicosis is explained by more rapid degradation of enzymatic proteins [206].

The increase of some antioxidant enzymes activities such as SOD, GPx and CAT, which are the main antioxidants in the body may be indicative of the failure of compensating the induced oxidative stress [207,208]. These enzymes may scavenge excess O_2^- and H_2O_2, and peroxides ROOH produced by free radicals. For example, SOD catalyzes the conversion of superoxide anion radical to H_2O_2. The resulting hydrogen peroxide in turn is decomposed by the enzymes GPx and CAT [209,210]. We suggest that the mentioned alterations are given of functional changes induced by radical over-production and an increase in the biosynthesis of antioxidant enzymes. Thus, the increase of some antioxidant enzymes activities such as SOD, GPx, and CAT may be an indication of the failure of compensating the induced oxidative stress. Also, it has been suggested by[140] that free-radical scavenging enzyme activity can be induced by excessive formation of ROS in experimental hyperthyroidism was previously reported.

Results of the studies analyzing the indicators of SOD, GPx and catalase enzymes in thyroid tissue are quite contradictory [11,12, 211,212]. The discrepancy may be due to variation in the samples analyzed, grade of hyperthyroidism, methods of determination and result expression (enzyme activity or concentration, expression of enzyme concentration or activity per protein or tissue mass).

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 [213] where in T_4 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 present study, in the plasma of hyperthyroidism-induced rats in comparison to the control group ($p<0.\ 001$).

Glutathione is a tripeptide, γ-L-glutamyl-L-cysteinyl-glycine, and is found in all mammalian tissues and it is especially concentrated highly in the liver [214]. GSH is a nucleophilic "scavenger" of numerous compounds and their metabolites, and a cofactor in the GPx-mediated destruction of hydroperoxides, which protects the cell membrane against oxidative damage by regulating the redox status of protein in the cell membrane [215,216]. It is widely distributed and involved in many biological activities including neutralisation of ROS, detoxification of xenobiotics, and maintenance of –SH levels in proteins [108]. In this study, we noted important reduction in GSH levels in hyperthyroid rats, which reflects its consumption through the oxidative stress. This not only confirms the main role of the thyroid hormones in regulating the oxidative stress in target cells, but also is in agreement with previous data. GSH depletion, a major hepatic alteration induced by hyperthyroidism in experimental animals [199] and [180] and man [217], is determined by both loss of tripepetide into the blood and higher intracellular catabolism, despite the enhancement in the rate of GSH synthesis and in the GSH turnover rate triggered in the liver [199,218]. Enhanced production of free radicals and the increase of antioxidant enzymes activities have been suggested as possible mechanisms to explain hyperthyroid-induced oxidative damage [219].

The GSH-dependent defence system plays an important role against lipid peroxidation in cells. Insufficiency of GSH is one of the primary factors that permits lipid peroxidation. It has been reported that GSH plays an important role in the detoxification of hydroperoxides and prevents the effect of lipid peroxidation [220]. Therefore, the decreased level of GSH may be due to the overproduction of free radicals and increased lipid peroxidation in hyperthyroidism [115]. However, lowered blood GSH levels may also be explained by some other possibilities, including: (i) an increased oxidation rate; (ii) increased utilization of GSH during the removal of lipid and other peroxides; and (iii) decreased glucose-6-phosphate dehydrogenase activity, which causes diminished production of GSH.

In contrast with our results, [140],[16] and [221] have demonstrated increased levels of GSH in blood from hyperthyroid rats. Activities of oxygen radical scavenging enzymes are expected to increase in response to sustained oxidative stress such as that in hyperthyroidism [115]. High levels of GSH in the erythrocytes of hyperthyroid rats are open to various interpretations. According to Visser [222], GSH, a required endogenous cofactor in the conversion of T_4 to T_3, is transported in increased amounts from the liver to blood to meet the needs of increased peripheral T_4- T_3 conversion. On the basis of the suggestion by Morini et al. [16] that thyroid hormones alter the membrane fluidity, Seven et al [141] suppose a change in GSH concentration due to altered transport hyperthyroid state.

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 [181, 223]. 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[120].

The great majority of the energy released under basal conditions is used by the cell for the maintenance of the Na^+-K^+ dependent ATP-ase activity. Thyroid hormones enhance the function of this pump by intensifying its activity at cellular level. This increased use of ATP associated with the intensification of oxygen consumption by the oxidative phosphorylation pathway generates reactive oxygen species [224].

At the level of the thyroid follicular cell, inorganic iodine, introduced in the body through diet, is oxidized to the iodinium ion (I^+), extremely reactive, which will bind to tyrosine residues from the structure of thyroglobulin. Iodine is oxidized by an enzymatic complex termed thyroperoxidase (TPO), which requires the presence of oxygenated water (H_2O_2) as an oxidizing agent. Further on, the process of oxidative condensation of iodotyrosines also involves thyroperoxidase (TPO) and oxygenated water (H_2O_2). Although the exact mechanism of the generation of oxygenated water (H_2O_2) is uncertain, it is supposed that NADPH-dependent cytochrome c reductase is involved in the intrafollicular generation of oxygenated water (H_2O_2) [225].

Under normal conditions, TSH stimulates the organification of iodine by the increase in the production of oxygenated water (H_2O_2). In hyperthyroidism, TSH anti-receptor antibodies induce a sustained and continuous secretion of thyroid hormones. The higher the synthesis of thyroid hormones, the higher the production of oxygenated water (H_2O_2) in the thyroid follicle [9].

The activity of some hepatic enzymes, such as NADPH-cytochrome P-450 reductase, is regulated by thyroid hormones. So, the excess of thyroid hormones followed by the intensification of the cytochrome P-450 reductase activity is responsible for the increased production of superoxide and hydroperoxide anion at hepatic level [108].

On the other hand, hypothyroidism is a disease because of a diminished thyroid hormone synthesis, resulting from thyroid gland dysfunction. Physiologic alterations generally occur because of the hypometabolic state induced by hypothyroidism [226].

The depression of basal metabolism is associated with decreased mitochondrial oxygen consumption and less ROS generation, resulting in decreased lipid peroxidation and protein oxidation [210].

Recent studies have shown an increased production of reactive oxygen species in hypothyroidism. There is disagreement on the effect of hypothyroidism on tissue oxidative stress. While Pereira et al. [12] suggested that hypothyroidism tended to diminish lipid peroxidation in lymphoid organs, Dumitriu et al. [113] observed the high levels of blood lipid peroxidation in hypothyroidism. It has been also reported that antioxidant enzyme levels are decreased in hypothyroid stage. These different results were explained in terms of tissue variation in haemoprotein content and/or of antioxidant capacity by Venditti et al. [110].

Hypothyroidism is known to induce metabolic suppression and lower respiration rate, and reduction of free-radical formation, accompanied by a fall in peroxide levels [112]. Our results show a general lack of significant changes in levels of lipid peroxidation (MDA) in serum and thyroid tissue of hypothyroid rats. This is in line with the results of Venditti et al. [110] who showed that in all tissues of hypothyroid rats, the malondialdehyde (MDA) levels did not differ significantly from euthyroid values. Mano et al. [15] found that the concentration of lipid peroxides, determined indirectly by the measurement of thiobarbituric acid reactants, did not change in hypothyroid rats when compared with the euthyroid animals. Dariyerli et al. [227] showed that there is no statistically significant difference found between hypothyroid and control groups in the lipid peroxidation indicator MDA. The results of Yilmaz et al. [228] who reported increased plasma, liver and muscle MDA levels in hypothyroid rats contradict our findings. Sarandol et al. [229] observed increased lipid peroxidation in plasma, liver, heart and muscle of Propylthiouracil treated rats reflecting an enhanced oxidative status in hypothyroidism. On the other hand, Venditti et al. [210] reported significantly decreased levels of hydroperoxides and protein-bound carbonyls in hypothyroid tissues.

This conflicting findings are thought to be due to different study materials in several animal models [110].

In our study we found that carbonyl proteins levels were significantly increased in serum, and the thyroid tissue of the Propylthiouracil treated rats, suggesting the presence of oxidative stress in hypothyroidism. This is in agreement with Nanda et al. [230] who found significantly higher carbonyl proteins levels in plasma of hypothyroid patients compared to their respective controls.

The mechanism of increased oxidative stress in hypothyroidism is controversial. Although most of the studies did not suggest it, an insufficient antioxidant defence system is thought to be a factor.

Antioxidant status parameters, namely thiol groups (SH), superoxide dismutase (SOD) and reduced glutathione (GSH) levels did not differ significantly in serum, and the thyroid tissue of the hypothyroidism-induced rats in comparison to the control group.

GSH is endogenously synthesized in the liver and is the first line of defence against pro-oxidant stress [231]. This antioxidant molecule is one of the main parts of the cellular endogenous antioxidant systems. It exerts its antioxidant function by donating electrons to radicals and changing to its oxidized form, which is subsequently reduced by the enzyme glutathione reductase [232].

In contrast with our results, Das et al. [108] have reported increased GSH levels in the mitochondria of hypothyroid rat liver, while the results of Sarandol et al. [229] who didn't observed any significant changes in GSH levels in the liver and kidney tissues of hypothyroid rats agree with our findings. The increase in GSH content in liver under the hypothyroid state may be an adaptive response to protect the mitochondria from the elevated level of H_2O_2. GSH is reported to be involved in numerous mitochondrial functions including mitochondrial membrane structure and integrity, ion homeostasis and mitochondrial redox state activity of numerous- SH- dependent enzymes [233]. The increase in the GSH level in mitochondria of hypothyroid rats may give protection to –SH-dependent proteins. In fact, the level of the increase in protein-SH groups in the hypothyroid state corroborates the above statement. GSH: GSSG in tissue is now considered one of the important markers of oxidative stress. The decrease in its ratio and the restoration to its normal value by T_3 administration confirms the critical role of thyroid hormone in regulating mitochondrial oxidative stress [13].

The organism can defend itself against the effects of oxidative stress by increasing SOD activity as a protection mechanism, but we did not observe any alteration in the serum and thyroid tissue of the hypothyroid rats. This is in line with the results of Messarah et al. [234] and[235] who observed no difference in SOD levels between hypothyroid rats and controls.

On the contrary, Das et al. [108] found increased SOD activity in the liver of hypothyroid rats which is accompanied with a decrease in catalase activity. SOD activity reduced and CAT activity increased following T_3 administration to PTU-treated rats. It is apparent that SOD and CAT, the two principal enzymes responsible for the metabolism of hydrogen peroxide in liver, are under the regulatory influence of the thyroid status of the body. An increase in SOD activity in the hypothyroid state will accelerate the production of hydrogen peroxide while a decrease in catalase activity will slow down its moval. It is reported that production of superoxide radicals leads to the inactivation of catalase activity and the consequent accumulation of hydrogen peroxide causes inactivation of SOD [236]. In the study of [229] and [11], catalase activity levels were found to be decreased in the liver tissue of hypothyroid rats. In the case of the thyroid gland inhibition, one might expect a fall in cellular respiration and, by analogy, it does not have any effect on the SOD activity, showing the possible effect of thyroid hormones in the determination of the antioxidant enzyme levels. Similar assumptions have already been made by other authors [120,196].

Venditti et al [110] have showed that antioxidants are not affected in the same manner in different tissues of hypothyroid rats; some of them increase, while several decrease or remain unchanged. The physiological state of the thyroid gland, the dose and the duration of treatment are also of a major influence on antioxidants enzymes.

Vitamin E is a potent lipid soluble antioxidant in biological systems with the ability to directly quench free radicals and function as membrane stabilizer [237]. It protects and prohibits the propagation of lipid peroxidation, arising from oxidative stress.

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 FT_4 levels (p=0,000). These results show that Vitamin E has a thyroid function suppressing action. This is in line with the report of Seven et al. [141] who found decreased T_4 and T_3 levels in vitamin E-supplemented euthyroid rats and suggested that vitamin E supplementation in the euthyroid state decreases either T_4/T_3 synthesis or T_4-T_3 conversion. Further studies on deiodinase activity in liver tissue of hyperthyroidism-induced vitamin E-supplemented rats will clarify the crucial impact of vitamin E on T_4-T_3 conversion.

Vitamin E supplementation significantly increased serum 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 serum of the hyperthyroid supplemented rats were also increased compared with the controls (p=0. 0002). Antioxidant capacity markers in serum 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 [141,205] which were not enough to suppress the oxidative stress in hyperthyroidism. Messarah et al [234] 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 which could be the answer to our results.

In our study, vitamin E supplementation significantly increased serum and thyroid tissue protein carbonyls levels and decreased the levels of serum antioxidant markers SH, GSH and SOD in the Propylthiouracil treated group compared with the only Propylthiouracil treated rats. Significantly low levels of the SH groups ($p<0.05$) were found in thyroid homogenates of the Propylthiouracil supplemented group as compared with the only Propylthiouracil treated rats. This could be explained by the relative doses of vitamin E administered, as compared with the study of Sarandol [229] which were not enough to suppress the oxidative stress in hypothyroid rats. For the first time in the literature, Erdamar et al [187] showed that the level of vitamin E was significantly increased in patients with hypothyroidism, which might be due to an adaptation against oxidative stress provoked by hypothyroidism.

Under normal conditions there exists a delicate balance between the rate of formation of ROS and the rate of breakdown of ROS in mitochondria, which is under the subtle control of thyroid hormone. Any alteration in the thyroid state of the body will considerably influence the antioxidative status of mitochondria and can lead to a pathophysiological state.

7. Conclusion

Our results suggest that thyroid hormones in excess are accompanied by increased oxidative stress and impairment of the antioxidant system. Although it has been suggested that the hypometabolic state is associated with a decrease in oxidative stress, literature data are controversial, revealing an individuality of antioxidant status in relation to tissue properties and responsiveness. The present study confirmed an increased oxidative stress in hypothyroid state.

Vitamin E supplementation in hyperthyroidism could exert beneficial effects in favour of the diminution of thyroid hormone levels. Antioxidants treatments might be helpful in reducing the oxidative damage due to hyperthyroidism. Therefore further studies have to be carried out on patients, in order to evaluate its role on antioxidant mechanisms to defend the organism from oxidative stress.

Also, optimal dosage, route of administration and timing of antioxidant therapy should be determined. These findings indicate that thyroid hormones have a strong impact on oxidative stress and the antioxidant system.

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Acknowledgement

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

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