OXIDATIVE STRESS AND SERUM ANTIOXIDANT ENZYME ACTIVITIES IN PTU INDUCED HYPOTHYROID MICE, MUS MUSCULUS

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Abstract: Oxidative stress (OS) is defined as an imbalance between radicals and antioxidant defense. Presently it is the topic of growing interest and known to connect with stress and pathophysiological mechanism of different diseases. Thyroid hormones can target, control and modify the metabolism of almost every cell in the body. Though much work has been done on the oxidative stress (OS) in relation to stress and diseases but very little work has been done to point out the role of thyroid in induction of oxidative stress.
Therefore, the present study is aimed to examine the effect of levothyroxine (L-T_4), vitamin E or both on oxidative stress status in a propylthiouracil (PTU) induced hypothyroid mice. Swiss albino male mice (Mus musculus) of approximately same weight were randomly selected and divided into five different groups. Control, PTU+PTU+L-T_4+PTU+Vit E, PTU+Vit E+L-T_4. After 28 days of experiment animals were killed and serum levels of triiodothyronine (T_3), tetraiodothyronine (T_4), thyroid stimulating hormone (TSH), superoxide dismutase (SOD) and catalase (CAT) were estimated in each group. Results clearly reveal that both antioxidant enzymes SOD and catalase (CAT) levels were significantly increased in all experimental animals treated with PTU (hypothyroidism). However, treatment with L-thyroxine and Vit-E are found to be effective to combat the oxidative stress in all experimental animals. The outcome this experiment concludes that levothyroxine replacement therapy combined with vitamin E reduces cell damage by improving oxidative stress.

Keywords: Oxidative stress, PTU, Replacement therapy, Thyroid hormones

Introduction: Oxidative stress (OS) arises when highly reactive free radicals produce oxidative damages to the DNA, proteins and membrane bound lipids of the cell. Every cell has developed an antioxidant defense mechanism which normally takes care of surplus radicals and prevents subsequent oxidative damage. In the presence of vast radicals if the available antioxidant defense falls short and unscavenged radicals remain free, which ultimately oxidize necessary components of cell and results oxidative stress [1]. This oxidative stress (OS) has been linked with a variety of diseases like Cancer, Diabetes Mellitus, Inflammation, Ageing, Ischemia, Atherosclerosis, Liver Damage, etc.[2][3][4][5][6].
Antioxidant enzymes like Superoxide Dismutase (SOD), Glutathione Peroxides (GPx) and Catalase (CAT) are known as the first lines of defense against O_2 and H_2O_2 mediated injury [7], [8],[9]while Glutathione (GSH), Vitamin C, Vitamin E (mainly a-Tocopherol), Uric acid, Albumin, Carotenoids, and Flavonoids are known as radical scavenging antioxidants and form second line of defense. [8], [9]
The thyroid gland is the largest and most sensitive endocrine gland which secrets triiodothyronine (T_3) and thyroxin (T_4) to regulate the metabolism of the body. The disproportions of thyroid hormones can have an intense effect on an individual's energy levels. Thyroid hormones speed up cellular reactions and increase oxidative metabolism by stimulating enzymes of the active transport. Thyroid hormones also stimulate protein synthesis and increase the rate of lipolysis.
Previous reports suggest that the hyperthyroidism is associated with increases in free radical production and lipid peroxide levels, [10], [11] whereas the hypo metabolic state induced by hypothyroidism is linked with a decrease in free radical production [11], [12] and in lipid peroxidation products. [13] Fernandez *et al* [14] found significant increase of lipid peroxidation in the liver of hyperthyroid rats. Besides, the reaction of the antioxidant systems to both hypothyroidism and hyperthyroidism is still unclear. The changes in the levels of the scavengers a-Tocopherol [15] [16] and activities of antioxidant enzymes [15] in various tissues were found to be improper and often opposite. Conversely, an array of materials capable of scavenging the different species of free radicals hence it is difficult to understand the overall protective effectiveness of the cellular defense system. Earlier reports suggest that hyperthyroidism increases oxidative stress (OS) and causes immune suppression while treatment with L-thyroxine may reverse the effect. [1], [2].All these evidences suggest that oxidative stress (OS) in any diseased condition or stressed condition may be mediated through the thyroid gland. Therefore, it is considered that the thyroid gland plays a vital role in generating oxidative stress in pathological condition. Thus, the present study is aimed to assess the levels of oxidative stress (OS) in the mice treated with PTU and L-T_4 and/or Vit E replacement therapy.

Material and Methods:

Chemicals:

- D1–α-Tocopherol acetate (Vitamin 'E') $C_{31}H_{52}O_3$. Obtained from the Loba Chemicals, private limited Mumbai.
- RIA Kits: The thyroid status of the treated animals was confirmed by serum thyroid hormone levels measured by radioimmuno assay (RIA) KITS supplied by the Board of radiation and isotope technology (BRIT) [Vashi], Mumbai, India.
- Both L-T4 (THYROX 50) and PTU was obtained from *Macleods* Pharmaceuticals Pvt Ltd. Marol Church Road, Andheri (East), Mumbai - 400059, India.

Animal treatment and sample collection: Forty six male swiss albino mice (Mus musculus) weighing 40±2.0g, were procured from Veterinary college of Mhow, Indore (M.P) acclimatized to laboratory conditions for one week. Protocols for animal care, maintenance, and experiments were approved by the Institutional Animal Ethics Committee. Mice of approximately same size, sex and weight were randomly divided into five groups, a control group (n=8) and other four groups (n=32). The control group was fed standard diet and drinking water *ad libitum*. Mice in the experimental groups were treated with 0.05% (w/v) PTU in drinking water for 21 days. Then after mice were randomly divided into four groups and administered PTU.

1. **Group I**: Animals in the PTU group I (n=10) continued to receive PTU for another 21 days;
2. **Group II**: PTU+L-T4 group (n=8). L-T4 (2 µg/100 g per day in 1 mL vehicle) at 8:00 AM.
3. **Group III**: PTU+L-T4+Vit E (n=8) L-T4 (2 µg/100 g per day in 1 mL vehicle) at 8:00 AM and Vit E (20 mg/100 g per day in 1 mL vehicle) at 6:00 PM.
4. **Group IV:** PTU+Vit E (N=8).

Mice of other groups were treated with vegetable oil for 28 days. T4 and Vit E were administered by intra muscular route. By the end of the study period (4 weeks) some rats were died during intra muscular administration. So there were 6 mice in control group, 5 in PTU group, 6 in PTU+L-T4 group, 5 in PTU+Vit E group and 6 rats in PTU+L-T4+Vit E group respectively. After 28 days of the experiment, all animals were carefully weighed in the morning before killed by anesthesia (0.3 mL of chloral hydrate /100 g body weight, i.p.). Blood samples were collected by abdominal aorta puncture and centrifuged at 3000 rpm for 10 min. Serum was separated and stored into EDTA-containing tubes at 4 °C until assessment of T3, T4 and TSH concentrations.

Measurement of serum thyroid hormone levels: Serum T3, T4, and TSH concentrations were measured by radioimmunoassay, using reagents supplied from North Institute of Biological Technology (Beijing, China), as previously described (Yang et al. 2012). The detection ranges of the assay were 0.2–4 nmol/L for T3, 20–320 nmol/L for T4, and 0.15–60 µIU/mL for TSH. Blood samples were obtained between 09:00 and 11:00 AM in a randomized fashion.

Measurement of enzymes: Catalase (CAT) Activity: Serum catalase (CAT) activity was estimated by the method of Aebi [17] by calculating the rate of breakdown of H_2O_2 at 240 nm. An aliquot of blood was homogenized in potassium phosphate buffer, pH 7.0. The spectrophotometric determination was initiated by the addition of sample into an aqueous solution of hydrogen peroxide 0.3mol/L. The change in absorbance at 240nm was measured for 2 min. CAT activity was calculated using the molar extinction coefficient (0.0436 cm2/μmol), and results were expressed as U/g Hb.

Superoxide Dismutase (SOD) Activity: The activity of enzyme superoxide dismutase was measured by the method described by McCord and Fridovich [18]. In this method, SOD present in the sample competes with the detection system for superoxide anion. A unit of SOD is defined as the amount of enzyme that inhibits the rate of adrenalin oxidation by 50%. Adrenalin oxidation leads to the formation of the colored product, adrenochrome, which is detected spectrophotometrically. SOD activity is determined by measuring the rate of adrenochrome formation, observed at 480 nm, in a reaction medium containing glycine-NaOH (50mM, pH 10.0) and adrenalin (1mM). Basal measurements to calibrate the assay were performed in a reaction medium containing 1mL of glycine-NaOH (50mM, pH 10.0) and 17 μL of adrenalin (1 mM). This was used to determine the concentration in samples. The results were expressed as U/mg Hb.

Results: Results are summarized in Table 1. Results clearly shown that mice treated with PTU (hypothyroidism) had significantly higher TSH with normal Free T4 range than the control group (Table 1). Serum T3 and T4 levels were significantly decreased, whereas TSH levels were significantly increased in Group IV treated with L-T4 or/and Vit E. SOD levels in the PTU and PTU+LT4 groups were significantly lower than the control group (p<0.05). However, SOD levels were significantly increased in PTU+Vit E and PTU+L-T4+Vit E group compared to the PTU group (p<0.05).But catalase activity is increased in all experimental groups compared to control.

Discussion: Thyroid hormones are essential for normal growth and development. The condition of hypothyroidism during development alters brain function and impairs behavioral performance.[19] Changes in T3, T4 and TSH level in the different thyroid state of oxidative stress (OS) can induce antioxidant enzymes to fight with the oxidative stress and protect the organism from cellular damage and

apoptosis. Oxidative stress (OS) can put the body in a state of weakness and also enhance the toxic effects of pathogenic factors that can cause occurrence of various diseases and induce point mutations[20] The methods involved in oxidative stress (OS) mediated cell apoptosis might be endoplasmic reticulum stress [21] and mitochondrial pathway. [22] Therefore, thyroid hormones have an important role in general oxidative stress.[1] High metabolic state in hyperthyroidism is along with increasing of free radical production and lipid peroxide levels. [11] Hypothyroidism is distinguished by failure of the redox potential that leads to free radicals chain reaction and metabolic suppression of antioxidant capacity. The antioxidant depletion in hypothyroidism may refer to increased production of free radicals in the inner membrane of mitochondria during electron transport. The cells of experimental animals are damaged due to prolonged oxidative stress that overpasses the animal's organs capacity for antioxidant molecules synthesis or their synthesis from extracellular sources. [23]

Recent reports confirm that thyroid dysfunctions increase lipid peroxidation (LPO) reactions and ROS leading to oxidative destruction of cellular membranes which can lead to cell death.[24] It also cause the production of toxic and reactive aldehyde free reactive oxygen species (ROS) like malondialdehyde (MDA) that would lead to oxidative damage of cellular molecules like lipids, proteins, and DNA. [24] [25] [26]

SOD and CAT are the first line of cellular defense against oxidative injury and known as free radical scavenging enzymes. They decompose O_2 and H_2O_2 before interacting to form a more reactive hydroxyl radical (OH). These enzymes protect the red blood cells against O_2- and H_2O_2-mediated lipid peroxidation. [27] In the present investigation we have detected an increased activity of both SOD and CAT in the mice of all PTU treated groups. But in contrast, few studies have stated no changes in CAT activity in hypothyroid conditions [24], [27]. Pan *et al* [28] also found that PTU-induced hypothyroid rats reveal reduced learning ability and increased oxidative stress. SOD also plays a significant role in antioxidant system and is involved in the protection of organism. [29],[30] The elevated levels of SOD and CAT in the hypothyroid mice indicate that oxidative stress and antioxidant defense system might be involved in neural cell damage caused by hypothyroidism. The fat soluble antioxidant, Vitamin E, can be integrated into cell membranes and can protect the cell membrane against oxidative damage.[31] In the present investigation we found a significant impact of vitamin E on antioxidative stress. [32] Previous studies have also found that vitamin E has inhibitory effect on cell apoptosis and protect the cell from oxidative stress. [33] [34] However, it requires further studies with large sample size and for longer duration to confirm our results. T_4 can prevent the activation of apoptosis pathway, while T_3 is involved in regulating cell proliferation and differentiation. Our study showed that levothyroxine significantly increases the levels of this hormone but decreases the level of SOD. In the present study, we found oxidative damage of mice in hypothyroid condition and showed that the level of antioxidant enzymes was positively related to hypothyroidism.

Moreover, levothyroxine replacement therapy concurrently reduced CAT levels but Vit E supplementation had no direct effect on hypothyroidism though it reduces the level of oxidative stress (OS) in the experimental animal. Thus we advocate that thyroid hormone replacement therapy combined with Vit E supplementation might improve cognitive injury by reducing of oxidative stress.

In conclusion, our research work confirms an increase in the oxidative stress biomarkers in the circulation of experimental animals with subclinical hypothyroidism. The high presence of plasma lipids can be considered as an oxidation substrate for the oxidative stress. However, more investigations are necessary to assess with different animals with a longer duration for the assessment of thyroid hormones role in oxidative stress.

Table 1: Comparison of antioxidant enzyme level and serum thyroid hormone levels in mice treated with PTU, PTU + L-T_4, PTU + Vit E, and PTU + Vit E + L-T_4.

Parameter	Control	PTU treated	PTU + L-T_4,	PTU + Vit E,	Vit E + L-T_4.
Serum TSH (μIU/mL)	2.8	36.2	9.1	34.1	7.5
Serum T_4 (nmol/L)	50.3	17.4	51.2	8.1	52.6
Serum T_3 (nmol/L)	0.95	0.31	1.0	0.32	1.31
CAT, U/g Hb	110.1	172.1	154.1	163.2	108.2
SOD, U/mg Hb	0.8	1.4	1.0	1.1	1.8

Data are expressed as mean ± SD, a $p < 0.05$ compared to the control group; b $p < 0.05$ compared to the PTU group.

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