Original article

Effect of thyroxine replacement therapy on the redox status of primary hypothyroid patients

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ABSTRACT

Background- Oxidative stress has been implicated in various pathological conditions but the available studies concerning oxidant stress in hypothyroidism are controversial and inconclusive. While some authors suggest that tissues may be protected from oxidant damage because of a hypometabolic state in hypothyroidism, others report an increased oxidative stress in hypothyroidism.

Aims and objective- The aim of the study was to determine presence of oxidative stress in patients with primary hypothyroidism and effect of thyroxine supplementation on it.

Materials and Method- 126 patients of primary hypothyroidism (24 males,102 females) based on history and thyroid profile prior to the commencement of therapy were enrolled in the present prospective follow up study (GROUP I). 100 (23 males and 77 females) of these patients were revaluated after three months of levothyroxine replacement therapy in varying doses (50µg-150µg) based on TSH levels (GROUP II). Hundred age and gender matched healthy, euthyroid volunteers were selected as controls for the study (GROUP III). NO was estimated through Greiss reaction, MDA was estimated by thiobarbituric acid method and glutathione through DTNB reagent.

Results- NO levels and MDA levels were significantly higher in pretreatment goup as compared to postreatment and control group($p < .05$). Plasma glutathione levels were significantly lower in pretreatment when compared to controls and posttreatment group ($p < .05$). and there was significant correlation between oxidative stress parameters with thyroid profile.

Conclusions – The present study shows that there is an increased oxidative stress in patients with hypothyroidism as compared to healthy controls and that it decreased on treatment with thyroxine supplementation signifying the role of antioxidant therapy in thyroid diseases.

Key words – hypothyroidism, oxidative stress, Thyroxine.

INTRODUCTION

A disequilibrium between production of reactive oxygen species (ROS) or free radicals and an antioxidant defence mechanism, in favour of former leads to a situation termed as oxidative stress .[1]

Hydrogen peroxide, Superoxide anion, hydroxyl radical and NO (Nitric oxide) are the free radicals[2] most of which are produced under normal endogenous physiological processes, the main source being the mitochondrial and microsomal membrane electron transport chain, reactions involving oxidant enzymes and auto-oxidation processes.[3]

Thyroid hormones have a considerable impact on oxidative stress which can be attributed to their role in cellular metabolism and oxygen consumption. Alteration in their levels could affect redox status

of the body and ROS production by causing changes in the number and activity of mitochondrial respiratory chain components .[4]

NO is a highly reactive molecule generated mainly by neutrophils and macrophages by the action of enzyme NO synthase and can have both anti-inflammatory and pro inflammatory action depending on the site of production and its concentration.[5] Excess NO along with superoxide radicals forms peroxynitrite, a powerful oxidant which can further attack lipids leading to production of toxic and reactive aldehydes of which MDA(Malondialdehyde) is the most important and is used as a measurement of lipid peroxidation.[6,7]

These ROS are kept under cheque by bodies protective mechanisms which 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 defences systems produced in the body.[8] GSH(Glutathione) is one of the main nonenzymatic regulator of redox homeostasis. It directly scavenges free radicals or acts as a substrate for Glutathine peroxidase and Glutathione-S-transferase during the detoxification of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds.[9,10] Thyroid hormones affect the cell antioxidant mechanisms as well in different ways creating a multivariate situation.[11]

Inadequate removal of ROS or their undue overactivity may cause damage to biological macromolecules and severe metabolic dysfunction and therefore has been implicated in pathogenesis of tissue damage in various pathological conditions[12] However in hypothyroidism reports concerning oxidative stress and antioxidant status are controversial. Some reports suggest an increased oxidative stress in hypothyroidism while some suggest hypometabolic state in hypothyroidism protects tissues against oxidative damage.[13-17]

Also there is paucity in information regarding effects of thyroxine supplementation on the oxidative stress parameters in a larger study group. Therefore the present prospective follow-up study integrated to investigate the presence of oxidative stress in hypothyroidism before and after treatment with thyroxine supplementation and their relationship with the severity of disease (based on TSH values).

MATERIAL AND METHOD

126 patients of primary hypothyroidism (24 males,102 females) prior to the commencement of therapy were enrolled in the present prospective follow up study at out-patient department of medicine of a tertiary care hospital of north India (GROUP I). 100 (23 males and 77 females) of these patients were revaluated after three months of levothyroxine replacement therapy in varying doses (50µg-150µg) based on TSH levels (GROUP II). Hundred age and gender matched healthy, euthyroid volunteers were selected as controls for the study (GROUP III). Patients with coronary artery disease, diabetes mellitus, liver and kidney disorders, severe vascular disease, any other endocrine, immunological or inflammatory disorder, on antihypertensives, taking antioxidants, smokers, and pregnant women were excluded from the study. Informed consent was taken from all patients and healthy volunteers. Ethical clearance was received from the ethical committee of the institute.

Measurement:

Overnight fasting blood samples were collected taking aseptic precautions, 3ml in an K_2-EDTA containing vacutainer (MDA, NO and glutathione estimation) and 3ml in plain vacutainer (for thyroid profile and routine investigation). Serum and plasma were separated after centrifugating at 3000g

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for 10 minutes and subjected to routine and specific biochemical investigations.

Thyroid profile:

T_3 and T_4 were assayed by radioimmunoassay and TSH by immunoradiomeric kits procured from the bhabha atomic research centre (BARC ,Mumbai, India)

NO estimation:

Nitric oxide was measured as nitrite (NO2-) which is a stable and non-volatile breakdown product of NO, based on Greiss reaction where Nitrite reacts with sulphanilamide and N-(1-napthyl) ethylenediamine to produce an purple coloured complex (azo dye), the absorbance of which was measured at 546 nm using colorimeter.[108] Sodium nitrite was used as standard and nitrite levels were expressed in micromole/L (µmol/L).[18]

MDA estimation:

Plasma MDA levels were measured on the principle that lipid peroxidation products react with thiobarbituric acid (TBA) to give a red chromogen whose absorbance was read at 546nm and final concentration of MDA was calculated using its molar extinction sufficient and expressed in µmol/L .[19]

Estimation of Glutathione :

The supernatant obtained after addition of metaphosphoric acid, is made to react with 5-5'-Dithiobis-2-nitrobenzoic acid reagent (DTNB) reagent. DTNB combines with sulfhydryl groups to produce yellow colored chromogen whose absorbance is read at 412nm against blank and expressed as µmol/L. [20]

Statistical Analysis

SPSS ver. 18 was used for various statistical analyses. All data was subjected to the Kolmogrov-Smirnov test for normality and presented as mean ± SD. Independent t test and paired t-test were applied for Gaussian distributions and Mann-whitney U (independent sample) and wilcoxan for related samples for non Gaussian distributions. Two sided p value < 0.05 was considered as significant. Correlations between groups were analyzed using Pearson correlation coefficient (r) formula.

RESULTS

Thyroid profile :

TSH values were found higher in pretreatment group compared to both posttreatment and controls. Although mean TSH level was towards the higher side of the normal reference range, it was still significantly higher in posttreatment group than control levels. In patient group pretreatment FreeT 3 , FreeT 4 levels were significantly lower when compared to posttreatment and control levels. However, no significant differences were observed between posttreatment levels and control levels. (**Table 1**).

NO level were significantly higher in pretreatment goup as compared to postreatment and control group. There was still significant difference in NO level after treatment when compared to control levels. MDA levels were found higher in pretreatment hypothyroidism according to both posttreatment hypothyroidisms and controls. MDA levels were also found to be decreased significantly in posttreatment group as compared to pretreatment group, however it was still found higher than controls significantly. Plasma glutathione levels were significantly lower in pretreatment when compared to controls. posttreatment glutathione levels increased as compared to pretreatment group but difference was not significant but the levels were still lower than controls ,the difference being marginally significant. (**Table 1**).

In our study, using Pearsons correlation coefficient analysis, it was found that the plasma MDA levels and NO levels correlated positively with serum TSH levels and negatively with serum T_3 and T_4

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levels in overt hypothyroid individuals. It was found that serum glutathione levels in patients with hypothyroidism correlated negatively with TSH which was not significant and correlation with T_3 and T_4 was also not significant.

After applying Pearsons coefficient we found a significant correlation between nitric oxide and MDA however no significant correlation was found between MDA and glutathione and between NO and glutathione After applying partial correlation analysis keeping TSH as controlling variable, MDA and NO still correlated well **(Table 2).**

DISCUSSION

In the present study, the plasma levels of NO were significantly higher in both pre and posttreatment groups when compared to the control levels.

Recent studies have reported that TSH directly induces TNF-α (Tumour necrosis factor) secretion by bone marrow cells and IL-6 (interleukin-6) by adipocytes. Various other studies have demonstrated that inflammatory cytokines like IL-2, IL-6, IL-15 are increased in hypothyroidism. Elevated TNFα and other cellular cytokine may promote the expression of inducible Nitric oxide synthase enzyme (iNOS) causing prolonged production of NO that lasts for hours, even days.[117-119]In another experimental study on animals it was demonstrated that at low levels of T_3 in hypothyroidism, nNOS mRNA levels increased by three fold and nNOS translocation to mitochondria was favoured with concomitant increase in mtNOS expression and activity.Above mentioned reports support our finding where NO levels were found to be raised in hypothyroid individuals.

Malondialdehyde (MDA) is the breakdown product of the major chain reactions leading to definite oxidation of polyunsaturated fatty acids (linoleic and linolenic acid) and thus serves as a reliable marker of lipid peroxidation.[53]

Several reports have suggested a possible antioxidant effect of L-thyroxine in the literature. Faure et al. demonstrated that free radical mediated oxidation of erythrocyte membrane and autooxidation of brain homogenates is inhibited by supranormal levels of L-thyroxine. Since LDL contains three specific binding sites for T_4, localized on apolipoprotein B-100, it has been suggested that the normal levels of T_4 might protect LDL against oxidation due to its antioxidant effect. It is well known that hypothyroidism leads to decreased activity and number of the LDL receptor, which contributes to hypercholesterolemia. Thus, in hypothyroidism higher cholesterol-to-protein ratio, along with prolonged circulation time of ageing lipoproteins make them more susceptible to oxidation due to repeated exposure to a variety of oxidising species that allows formation and accumulation of lipid peroxidation products.

In another study it was demonstrated that in hypercholesterolemia there is increased production of superoxide ions probably by conversion of xanthine dehydrogenase to xanthine oxidase in endothelial cells. Superoxide (O_2^-) ions have been implicated in the oxidation of LDL. It is interesting to speculate that such increased production of superoxide ions may further enhance LDL modification. Superoxide (O_2^-) ions also provides a source of other oxygen centred radicals such as H_2O_2 and OH^- radicals which may participate in lipid peroxidation and serve to damage cellular membranes. In addition, the Superoxide (O_2^-) ions may react with NO, which is also produced in excess within the endothelium of hypercholesterolemic animals to produce the highly injurious peroxynitrite radicals.

So significant increase in MDA in hypothyroid patients in our study could be due to prolonged circulation time of LDL and increased production of superoxide radical by endothelium and further

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formation of peroxynitrite radicals in hypercholesterolemia a known condition associated with hypothyroidism, leading to formation of lipid peroxidation products.

The difference in antioxidant activity may be caused by various mechanisms. An intensified synthesis of antioxidant and their elevated activity might be an adaptive mechanism in response to oxidative stress whereas decreased activity of enzymatic antioxidants or a decreased non-enzymatic antioxidant concentration may be due to their intensified utilization in combating oxidative stress which may be the case in our study.[128]

The increase of free radicals is not compensated, as one would expect, by a decrease of antioxidants. Thus, it is likely that patient's cells are damaged by prolonged oxidative stress that far exceeds the capacity of the patient's organs to synthesize antioxidant molecules or to synthesize them from extra cellular sources. This may be the reason why plasma glutathione levels did not increase significantly after the treatment in the present study.[12]

NO can react with superoxide ion to form peroxynitrite radical. Peroxynitrite itself is a highly reactive species which can directly react with various biological macromolecules which can decompose into toxic products that include nitrogen dioxide gas, hydroxyl radical and nitronium ion. Further $OH^{\bullet}$ is a powerful oxidizing agent that can react at a high rate with most organic and inorganic molecules in the cell, including DNA, proteins, lipids, amino acids, sugars and metals. Therefore excessive nitric oxide levels with oxidative lipid peroxidation might be a major factor contributing to higher levels of MDA.[37,46]

After three months of treatment with levothyroxine, NO and MDA levels were not completely normalized when compared to healthy controls. Although TSH levels after treatment were within higher normal range, it was still higher than that of healthy controls., In addition we have demonstrated a strong positive correlation between MDA, NO and Thyroid profile levels suggesting that complete normalization of MDA and NO could be possible by further decrease in the levels of TSH. We can speculate that inadequate duration of the treatment or insufficient suppression of TSH levels might be the possible reasons.

CONCLUSIONS

From this large population based follow-up study, it is concluded that hypothyroidism is a state of increased oxidative stress. On treatment with thyroxine, levels of oxidative stress markers NO and MDA decreased significantly whereas plasma glutathione levels increased after treatment. A significant correlation was found between oxidative stress markers and thyroid profile parameters which further suggest to take up studies to delineate the role of antioxidant therapy as adjuvant treatment in management of hypothyroidism.

CONFLICTS OF INTEREST DISCLOSURE

We the author's of the manuscript titled "Effect of thyroxine replacement therapy on the redox status of primary hypothyroid patients" declare that there is no conflict of interest with either pharmaceutical companies or other non-government organization.

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Table 1: Comparison of oxidative stress parameters and thyroid profile in patients with hypothyroidism before and after treatment and in control groups.

Parameters	Controls	Before therapy	After therapy	Group I –II	Group I- III	Group II - III
N	100	126	100	p	p	p
TSH (µIU/ml)	2.37± 1.17	66.01 ± 44.66	3.779 ± 2.27	< 0.001*	< 0.001*	< 0.001*
T_3 (ng/dl)	130.55±32.52	56.22 ± 31.34	123.19± 27.85	< 0.001*	0.087	< 0.001*
T_4 (µg/dl)	8.8± 2.07	3.58 ± 5.75	8.07 ± 1.83	< 0.001*	0.005*	< 0.001*
NO (µmol/L)	35.96 ± 6.14	50.36±9.11	39.55±8.37	< 0.001	< 0.001	< 0.001
GLUTATHIONE (µmol/L)	3.59±1.05	3.28±0.87	3.33±0.82	0.024	0.052	0.270
MDA (µmol/L)	1.54±0.62	4.40±1.42	1.79±0.62	< 0.001	0.004	< 0.001

*p < 0.05 – significant

Table 2: Correlation among parameters in freshly diagnosed patients with hypothyroidism.

PARAMETERES	r	p
MDA-T_3	-0.482	0.000*
MDA-T4	-0.413	0.000*
MDA;TSH	0.676	0.000*
NO- T_3	-0.287	0.001*
NO-T_4	-0.245	0.006*
NO-TSH	0.540	0.000*
NO-MDA	0.551	0.000*
NO-Glutathione	-0.152	0.088
MDA-Glutathione	.001	0.990

*p < 0.05 significant

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