Assessment of Oxidative Stress Index in Sub-Clinical Hypothyroidism

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Subclinical hypothyroidism (SCH) is a mild form of hypothyroidism where the TSH levels are slightly above the normal range and T3 & T4 values are within the normal limits. Oxidative stress caused by reactive oxygen species (ROS) causing an imbalance in the antioxidant/oxidant status of an individual is well recognized in hyperthyroidism & overt hypothyroidism but not in (SCH). Recently much attention is focussed on SCH, as it exhibits the same cardiovascular consequences as overt hypothyroidism. The current study evaluates the oxidative stress index (OSI) and the correlation of OSI with thyroid profile and Paraoxonase -1 (PON-1), the antioxidant enzyme associated with HDL in subjects with subclinical hypothyroidism. OSI was calculated after measuring the total oxidant status (TOS) and total antioxidant status (TAS). This cross-sectional study included 64 subjects with sub-clinical hypothyroidism and 128 subjects with normal thyroid profile. Age of the subjects ranged between 25 and 75 years. Serum levels of TAS, TOS and PON-1 was measured in the subjects whose thyroid profile was determined. OSI was calculated and differences between the variables in the two groups were analysed by the non-parametric tests. Correlation analysis was done by the spearman’s test. The diagnostic ability of OSI was assessed by ROC Curve. Significant increase in the OSI and TOS was observed in subjects with SCH. There was no change in the antioxidant status as well as PON-1 activity. OSI can be considered as an efficient marker in the evaluation of oxidative stress.

Keywords: PON-1, Subclinical hypothyroidism, Oxidative Stress index.
There are only a few studies on SCH reporting oxidative stress. The ubiquity in the population is affected by factors like TSH, cut-off to define SCH, race, sex, age, autoimmune thyroid disorders & intake of iodine. In recent times SCH has attracted more attention as it clearly manifests the similar cardiovascular outcome as in overt hypothyroidism.

Basal metabolic rate and oxidative metabolism are influenced by thyroid hormones and have a vital role in lipid and lipoprotein metabolism. Patients with hypothyroidism are at an increased risk of developing atherosclerosis due to an increase in low density lipoprotein cholesterol (LDL-C), total cholesterol (TC) as well as apolipoprotein B (apo B) and is well accepted. Thyroid hormones have a substantial influence on oxidative stress. The overproduction of ROS disturbs pro-oxidant /antioxidant balance leading to oxidative stress, and resulting in damage to lipids, proteins & DNA. Lipid peroxidation, a free radical chain reaction promotes the generation of (ROS) contributing to oxidative stress. Reports on the effect of lipid peroxidation in hypothyroidism are not equivocal. Kebapcilar et al have reported no significant changes in lipid peroxidation as compared to controls. Study of Dumitriu et al have shown an increase in hypothyroidism. Elevated MDA levels have also been reported in subclinical hypothyroidism. Direct association between hypo and hyperthyroidism complications by reducing the activity of the antioxidant system components have been reported by Erdamar et al. These findings suggest that thyroid hormones have a strong effect on oxidative stress and antioxidant systems. Hence maintenance of adequate antioxidant levels is essential.

Serum paraoxonase 1 (PON1), an HDL associated enzyme capable of hydrolysing paraoxon and preventing lipid peroxidation, protects the LDL against peroxidation and is responsible for most of the antioxidant properties of HDL. Baskol et al found increased plasma levels of malondialdehyde (MDA) and reduced activity of paraoxonase in patients with primary hypothyroidism. Diminished activity of PON1 was also seen in thyroid dysfunction. Amandeep Kaur et al have reported a decrease in the antioxidant status in hypothyroid patients whereas no significant differences were shown in other studies.

Depression of metabolism due to hypothyroidism has been reported to decrease the production of free radicals. The depression of basal metabolism found in hypothyroidism is associated with decreased mitochondrial oxygen consumption and less generation of ROS resulting in decreased lipid peroxidation. However studies have shown an increased production of ROS even in hypothyroidism. Thyroid hormones have a strong impact on the oxidative stress and the antioxidant system. Subclinical hypothyroidism often progresses to overt hypothyroidism which is associated with an increased risk of atherosclerosis.

Data on the overall oxidant antioxidant status of patients with subclinical hypothyroidism are meager. Most of the studies have focussed on the individual antioxidants. Hence we proposed to study the oxidative stress index (OSI) by measuring the total antioxidant status (TAS), total oxidant status (TOS).

**MATERIALS AND METHODS**

This was a cross-sectional study and was carried out at Kasturba Medical College, Mangalore Centre for Basic Sciences, Bejai. Institutional Ethical committee approval (IEC-KMC MLR 12-18/491) was obtained prior to the conduct of the study. The study included subjects visiting the Clinical Biochemistry Laboratory, KMC Hospital, Mangalore for routine check-up. 128 subjects with T3, T4 and TSH within the normal limits (T3-0.8-2ng/dl, T4-5.10-14.1µgm/dl, TSH-0.27-4.2µIU/ml) served as controls. 64 subjects with sub-clinical hypothyroidism (T3, T4 values within the normal range and TSH levels between 4.5 to 10µIU/ml) formed the case group. The subjects were not under any medications. All subjects selected were in the age group of 25-75 years. Subjects with overt hypothyroidism, hyperthyroidism and subclinical hyperthyroidism and those subjects on treatment with thyroxine were excluded.

**Sample collection**

Fasting blood samples obtained from the above mentioned subjects were centrifuged at 3000 rpm for 10 minutes. Separated serum was used for the measurement of thyroid profile (T3, T4...
&TSH) in COBAS 6000 auto analyser and Roche Diagnostic kits were used. The left over samples were stored at -20°C for a week. Assessment of TAS, TOS and paraoxonase were carried out in the stored samples.

Estimation of TOS was done by the method of Ozcan Erel 29. Oxidants present in the sample oxidise the ferrous ion-ortho-dianisidine complex to ferric ion. The ferric ion makes a coloured complex with xylene orange in an acidic medium. The colour intensity is related to the total amount of oxidant molecules present in the sample and was measured by a spectrophotometer. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The assay is calibrated with Hydrogen peroxide and the results are expressed as micromoles of hydrogen peroxide equivalent per litre (µmolH₂O₂Equiv/L). Measurement of Total Anti-oxidant status was done by the method of Oscan Erel 30 which is based on the ability of the antioxidants in the sample to suppress the oxidation reaction and colour formation of standardized solution of Fe²⁺–o- dianisidine.

Oxidative Stress Index (OSI) defined as the Ratio of TOS level to the TAS level was calculated as OSI = TOS (µmolH₂O₂Equiv/L)/TAS (µmoltrolox-Equiv/L). 31

Estimation of Paraoxonase activity was done by the method of Beltowski et al. 32 The activity was estimated spectrophotometrically using 5.5 mM 4-nitrophenyl phosphate as the substrate in 20mM Tris–HCl buffer at a pH of 8.0. The increase in absorbance due to the formation of 4-nitrophenol was monitored at 410 nm for 3 mins. Change in the absorbance per minute was calculated. One IU of paraoxonase activity is defined as µmol of p-nitro phenol formed per minute at 25p C.

**Statistical Analysis**

Statistical Analysis was carried out in SPSS version 20.0. Thyroid profile variables (T₃, T₄, and TSH) were analysed by Unpaired T-test (Mean ±SD) and the other parameters were analysed by Mann-Whitney U test and expressed as Median and inter-quartile range. Categorical variables were computed using chi-square test. Correlation analysis was done by Pearson’s or Spearman’s test. p<0.05 was considered significant.

**RESULTS**

The demographic data of the subjects are shown in Table 1. There was no significant difference in age between the cases and controls. The control group had 40 males and 88 females while the Case group consisted of 15 males and 49 females.

The values of thyroid profile, oxidant antioxidant status and paraoxonase are given in Table 2. It is clearly seen that the levels of TSH are higher in subjects with subclinical hypothyroidism.

**Table 1. Demographic data of the subjects**

| Variable | Controls (n=128) | Cases (n=64) | p value |
|----------|-----------------|--------------|---------|
| Age      | 43.28±15.14     | 42.96±13.42  |         |
| Gender   | 40/88           | 15/49        |         |

Values are shown as Mean ± Standard deviation. n= number of subjects

**Table 2. Thyroid profile and oxidant/antioxidant levels**

| Variables | Control (n=128) | Cases (n=64) | p value |
|-----------|-----------------|--------------|---------|
| T₃(ng/dl) | 1.24±0.33       | 1.14±0.28    | 0.041   |
| T₄(µg/dl) | 8.45±2.08       | 7.33±1.56    | <0.01   |
| TSH(µIU/ml) | 1.92±1.15    | 8.42±2.0     | <0.01   |
| TAS(µmoltrolox-Eq/L) | 1365.90±121.81 | 1309.46±163.13 | 0.645 |
| TOS(µmol H₂O₂Equiv/L) | 46.42±3.82    | 72.21±5.99   | <0.01   |
| PON-1 (IU/L) | 6.19±0.38    | 6.12±0.47    | 0.758   |
| OSI       | 0.06±0.01       | 0.144±0.032  | <0.01   |

Values are expressed as Mean ± Standard deviation, n= number of subjects, p<0.05 was considered significant.

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Though a statistically significant increase in T4 & T3 levels is seen in controls, it is clinically not significant since the values are well within the normal range. Highly significant increase in the TOS was seen while no significant change in the levels of TAS and paraoxonase activity was observed. Significant increase in the OSI was observed in the subjects with subclinical hypothyroidism. Figures 1, 2, 3, 4 depict the mean ± SD values of TAS, TOS, PON-1 and OSI obtained. The values of the above mentioned parameters based on the levels of TSH in the control subjects (0.27- 2.5 & 2.5- 4.2 iIU/ml) and in the cases (4.2-7.0 & 7.1-10 iIU/ml) are presented in Table 3. No significant difference is seen in the values of TAS, TOS, OSI & PON-1 in the controls with TSH values < 2.5 and 2.5- 4.2 iIU/ml. Similarly no significant difference is seen in the subjects with TSH values < 7or 7 – 10 iIU/ml. Table 4 shows the correlation of thyroid profile & PON-1 with OSI in control and cases respectively. A positive correlation of Paraoxonase with TOS and OSI is seen in the control group (r=0.507 & r= 0.175) while a weak negative correlation of PON-1 with TOS and OSI in SCH subjects is seen (r=-0.165 and -0.238).

Table 3. TSH grouping in controls and cases

| Variables       | Controls          | p value | Cases | p value |
|-----------------|-------------------|---------|-------|---------|
| TAS (µmolTroloxEq/L) | 842.8 (979.55) | 0.97 | 1058.8 (1114) | 0.67 |
| TOS (µmolH₂O₂ Eq/L)  | 30.26 (43)       | 0.84 | 48 (79.48) | 0.96 |
| OSI              | 0.03 (0.05)      | 0.90 | 0.05 (0.11) | 0.72 |
| PON-1 (IU/L)     | 5.5 (4.8)        | 0.14 | 5 (5.83) | 0.48 |

All values are expressed as median & inter quartile range; p<0.05 is considered significant.

Table 4. Correlation of clinical parameters with TAS, TOS & OSI in control and cases

| Parameters       | Tf (ng/dl) | Tr (µg/dl) | TSH (µIU/ml) | PON (IU/L) |
|------------------|------------|------------|--------------|------------|
| TAS (µmolTroloxEq/L) | -0.024     | -0.021     | 0.086        | 0.107      |
| TOS (µmolH₂O₂ Eq/L)  | -0.017     | -0.021     | -0.031       | r=0.507**  |
| OSI               | -0.155     | -0.133     | -0.046       | -0.165     |
| PON-1 (IU/L)      | 0.111      | 0.034      | -0.075       | r=0.175*   |

Correlation is significant when p<=0.05. Weak correlation: r=0.3-0.5, moderate correlation r=0.5-0.8, strong correlation r=0.8-1.
DISCUSSION

Oxidative stress has an important role in the development of many chronic diseases. Prospective studies indicate the increased risk for cardiovascular mortality in patients with subclinical hypothyroidism (SCH). According to the present study there was no remarkable change in the total antioxidant status of SCH and control subjects. This finding is in agreement with the report of Torun et al and Cheserek et al. Studies of Lakshmi et al and Joshi et al have reported a significant decrease in Total antioxidant capacity in hypothyroid patients. Bhimte et al have also opined that hypothyroidism resulted in a lowering of TAC. The mean values of TAS observed in the control subjects in the present study is similar to the value reported by Jansen et al in Netherland males and the apparently healthy Chinese adults. However it appears to be lower as compared to

![TAS](image1)

**Fig. 1.** depicts the Mean values of TAS in controls and SCH groups

![TOS](image2)

**Fig. 2.** depicts the Mean values of TOS in controls and SCH groups
the apparently healthy Chinese adults. The values of TAS in SCH subjects were higher when compared to the value reported by Sivakanesan et al in thyroid dysfunction.

The value of TOS observed in the control group is higher than the value obtained by Erel et al in healthy subjects (46,42 ± 3,16 µmol/L). This could be attributed to the quality of the chemicals used in the assay. A 1.5 fold increase in the Total Oxidant levels observed in the SCH subjects is highly significant. Thus measurement of oxidant status (TOS) appears to be a good marker of oxidative stress. The spectrophotometric method of Ozcan Erel used

**Table 5.** Correlation of OSI with TAS, TOS and Paraoxonase (PON-1)

|     | OSI (µmol Trolox Eq/L) | TAS (µmol H' O' Eq/L) | TOS (µmol H', O, Eq/L) | PON-1 (IU/L) |
|-----|------------------------|-----------------------|------------------------|-------------|
| Control | r=(-0.295)** p=0.001 | r=0.514** p=0.000 | r=0.175* p=0.049 |             |
| Test   | r=(-0.356)** p=0.004 | r=0.574** p=0.004 | r=(-0.238) p=0.059 |             |

Correlation is significant when p<0.05. Weak correlation: r=0.3-0.5, moderate correlation r=0.5-0.8, strong correlation r=0.8-1.

**Table 6.** Characteristics of ROC curve

| Variables          | Sensitivity | Specificity | AUC (confidence interval) | p value |
|--------------------|-------------|-------------|---------------------------|---------|
| TAS (µmol Trolox Eq/L) | 70.3%       | 32%         | .520 (.432-.609)          | 0.645   |
| TOS (µmol H', O, Eq/L) | 81.3%       | 54%         | .682 (.605-.759)          | 0.000   |
| OSI                | 51.6%       | 78%         | .640 (.556-.725)          | 0.002   |
| PON-1 (IU/L)       | 42.0%       | 60%         | .514 (.428-.600)          | 0.758   |

p<0.05 was considered significant. AUC-Area under the curve.

**Fig. 3.** depicts the Mean values of Paraoxonase in controls and SCH groups.
to measure TOS in the present study, is stable, reliable, sensitive and inexpensive with a linearity up to 200 µmol H₂O₂/L.

There was no significant change in the activity of HDL associated PON-1 enzyme. The findings of our study agree with the report of Coria et al. who also did not observe any change in the activity of PON1 between overt hypothyroidism, subclinical hypothyroidism and euthyroidism. On the contrary, Azizi et al. have reported a significant reduction in PON1 activity in both hyper and hypothyroid patients. A positive correlation of paraoxonase with total oxidant status and OSI was observed in control subjects while a negative correlation only with OSI seen in subjects with subclinical hypothyroidism is an important finding suggesting the importance of measurement of PON-1 in evaluation of oxidative stress in SCH. A significant correlation of OSI values and severity of depression was found to be present in the study.

**Fig. 4.** depicts the Mean values of OSI in controls and SCH groups

**Fig. 5.** depicts the ROC curve
of Gupta et al. A similar finding was reported by Yanik et al. Increase in the OSI in subjects with SCH indicates that calculation of OSI could be used as a screening test to measure the Oxidant/Anti-oxidant status. From the ROC curve it is evident that the AUC for TOS is the highest followed by OSI. Hence among the two, TOS can be considered as the best indicator for the assessment of oxidant and anti-oxidant status of an individual with subclinical hypothyroidism. Nevertheless it can certainly be used for evaluation of oxidative stress.

To summarize, the thyroid hormones are associated with the oxidative and anti-oxidative status of an individual. Subjects with SCH showed significant increase in oxidant status and oxidative stress index (OSI). There was no significant change in the antioxidant status as well the PON-1 activity in subjects with SCH . The method adapted for measurement of TAS and TOS was sensitive, reliable, and inexpensive which can be readily performed in the laboratory and can also be automated. Though serum concentrations of different oxidant species and antioxidant molecules can be measured in the laboratories they are time consuming, and involve complicated techniques. OSI, a recently investigated parameter is a proportional value between TAS and TOS. It is directly influenced by oxidant and antioxidant status which represents the degree of oxidative stress more clearly.

The result of the study highlights the importance of measurement of TOS as an important marker for the assessment of oxidative stress in subclinical hypothyroidism. In order to obtain a comprehensive understanding of the oxidative stress status of a subject, calculation of OSI is essential. Assessment of oxidant status would certainly aid the physician in the treatment.

**CONCLUSION**

Subclinical hypothyroidism relatively increases the oxidative stress as evidenced by the increase in TOS and Oxidative stress index (OSI). Thus both TOS and OSI can be considered as efficient markers of oxidative stress. TOS can certainly be used as a screening test for assessment of oxidative /antioxidative state of an individual with subclinical hypothyroidism. Nevertheless OSI being a proportional value between TAS & TOS can represent the degree of oxidative stress more clearly and may be put into clinical use. The utility of the test is so great that but it can also be extended to other diseases which involve oxidative stress. Future studies with various cut off values of TSH with the clinical symptoms may improve its diagnostic performance.

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**Conflicts of Interest Statement**

The authors have no conflicts of interest.

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