Chapter 5

To estimate and explore the effect of SCH on Oxidative stress levels and lipid profile, and to find out the correlation of SCH with the oxidative stress levels along with alterations in the lipid profile in primarily infertile female population of Gujarat

5.1 Introduction

Female infertility is the fallout of multiple causes and thus requires a multi dimensional approach in its treatment; looking for every obvious and at the same time many hidden and neglected etiological factors. Even though the clinical relevance of the association between thyroid and reproductive health is very well known, its physio-pathological mechanisms are not fully understood. Among the different mechanisms proposed, a link between oxidative stress (OS) as one of the perpetrators of thyroid disruption is widely accepted. OS is defined as an imbalance between production of free radicals and antioxidant defenses and is now accepted as an important initiator of diseases (Halliwell *et al.*, 1979). Among free radicals the most important and well-studied are reactive oxygen species (ROS), which are produced during oxidative processes in the mitochondria (Littarru*et al.*, 1994; Kang*et al.*, 2003). Reactive oxygen species (ROS) including partially reduced forms of oxygen; i.e. superoxide anion, hydrogen peroxide, and hydroxyl radical, as well as organic counterparts such as lipid peroxides; are produced as natural consequences of oxidative cell metabolism. Earlier studies have shown that hypothyroidism is associated with enhanced oxidative stress involving enzymatic and non-enzymatic antioxidants. In particular, it has been suggested that the increase in reactive oxygen species induced by a deficiency of thyroid hormones can lead to an oxidative stress condition with a consequent lipid peroxidative response (Resch *et al.*, 2003; Erdamar *et al.*, 2003). ROS generation is controlled by a large number of anti free radical systems which act as protective mechanisms under physiological conditions. These systems consist of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase as well as non-enzymatic antioxidants. Variations in the levels of thyroid hormones can be one of the main physiological modulators of in vivo cellular oxidative stress due to their known effects on mitochondrial respiration.

Hypothyroidism-associated oxidative stress is the consequence of both increased production of free radicals and reduced capacity of the antioxidative defense. Hypothyroidism-induced dysfunction of the respiratory chain in the mitochondria leads to accelerated production of free radicals which consequently leads to OS. Metabolic disorder from autoimmune-based hypothyroidism can also increase OS (Pasupathi *et al.*, 2008). The possible role of free radicals, in infertility is evidenced by an imbalance between oxidants and antioxidants which is responsible for tissue injury and affects fertility. Exposures associated with OS like hypothyroidism may induce pregnancy complications (Ruder *et al.*, 2009). Thyroid hormones are associated with the oxidative and antioxidative status of the organism. Data on oxidative

stress due to hypothyroidism are limited and controversial (Dumitriu*et al.,* 1988;Konukoglu*et al.,* 2002; Torun *et al.,* 2009), while only a few studies in the field are reported on SCH, oxidative stress and female infertility in humans (Torun*et al.,* 2009; Baskol*et al.,* 2007).

A correlation of lipid profile alteration among hypothyroid patients is universally accepted. Thyroid hormones are important modulators of intermediary metabolism. They have significant effect on the synthesis, mobilization and metabolism of lipids. During clinical and SCH lipid profile alteration is seen but, relationship between alterations in lipid profile and subclinical hypothyroid condition is still controversial. Thyroid dysfunction particularly hypothyroidism is associated with dyslipidemia which increases the risk of endothelial dysfunction, hypertension and cardiovascular diseases (KGaA*et al.,* 2009). Many evidences suggest that dyslipidemia is a major determinant in the progression of infertility (Broughton*et al.,* 2017; Broughton *et al.*,2017). Hypercholesterolemia has a stronger influence on the development of oxidative stress in overt hypothyroid (OH) patients whereas patients with SCH had altered lipid profiles, increased lipid peroxidation, and induction of enzymatic defense. Oxidative stress biomarkers are seemed to be associated with secondary hypercholesterolemia to hypothyroidism, whereas hypothyroidism per se does not cause oxidative stress in SCH patients. On the other hand, high-plasma lipids can be considered as an oxidation substrate for the oxidative stress (Santi *et al.,* 2010). Studies also suggest that an increased oxidative stress in both hypothyroid and SCH states can be explained by both the insufficient increase in the antioxidant status and the altered lipid metabolism (Torun *et al.,* 2009).

Worldwide, the most rapidly growing and prevailing endocrine diseases are of the thyroid gland, next only to diabetes mellitus (Aryal*et al.,* 2010). SCH is found to be more common than overt hypothyroidism in women of reproductive age, and an early diagnosis and treatment may prevent its conversion to overt hypothyroidism and its associated effects. An increased oxidative stress and lipid profile alterations are reported for hypothyroid infertile female subjects, but adequate data is unavailable for SCH, oxidative stress and alterations in the lipid profile subsequently resulting into the female infertility. Hence the study aims to estimate and explore the effect of SCH on OS and lipid profile and secondly to find out the correlation of SCH with the oxidative stress levels along with alterations in the lipid profile in infertile female population from western India, especially Gujarat region.

5.2 Materials and Methods

5.2.1 Ethical consideration

It was ensured that the study design complies with the ethical standards of the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/BC/PR/1) set up as per guidelines of the Indian Council of Medical Research (ICMR) and with the 1964 Helsinki declaration.

5.2.2 Study Population

The present retrospective study is a matched, case-control study. The study population consists of a total 110 healthy parous control and 270 IF-SCH females with primary infertility as case subjects, (as was screened and reported in the first objective of the present study. Details of the study population is as mentioned in chapter 2[2.2.2.]).

5.2.3 Blood collection and sample preparation

5.2.3.1 Blood collection and hemolysate preparation (for OS level estimation)

A volume of 1 ml venous blood samples was allotted from the total 5 ml of the blood sample taken during the objective-1 study parameters from 110 control and 270 IF-SCH subjects. Samples were collected in heparinized tubes. The blood samples were centrifuged at 2000 rpm for 10 min to separate plasma from RBCs. The RBCs were then re-suspended in saline and washed twice by centrifuging at 2000 rpm for 10 min. The washed RBCs were then re-suspended in chilled distilled water to the original volume of the blood sample.

5.2.3.1.1 Hemoglobin Estimation

Hemoglobin estimation was done by Drabkin's method (Cook JD *et al.,* 1985). Drabkin's reagent (ferricyanide) converts the haemoglobin to cyanmethemoglobin (CMG) and absorbance of CMG is proportional to the haemoglobin concentration. The protocol was followed as per method. The optical density was measured at 540nm against distilled water.

5.2.3.1.2 Estimation of Superoxide dismutase1 (SOD1) activity

The estimation of SOD1 activity in erythrocytes was carried out by the method of Marklund and Marklund (1974) with slight modification utilizing the inhibition of auto-oxidation of pyrogallol by SOD1 enzyme. The protocol was as per the method. Hemolysate was prepared with haemoglobin concentration of about 1g Hb/dl.The auto oxidation of pyrogallol, under alkaline condition generates free oxygen radicals which are used by SOD1 present in hemolysate. Decrease in autooxidation shows indirect evidence of SOD1 activity.The

1gHb/dl of hemolysate was prepared and O.D was adjusted to 0.15 – 0.20 by auto oxidation of pyrogallol at 420 nm. In all tubes, reaction was started by addition of pyrogallol and the change in O.D was recorded for 3 mins at interval of 5 sec at 420nm. The % inhibition of test system was calculated from the standard graph generated from autooxidation og pyrogallol. The SOD1 activity was expressed in units/gHb/min where one unit of SOD1 activity being defined as the amount of enzyme required causing 50% inhibition of pyrogallol autooxdation.

5.2.3.1.3 Estimation of Catalase (CAT) activity

The estimation of catalase activity in erythrocytes was carried out by the method of Hugo Aebi (1984). The protocol was followed as given in the method. Hemolysate was prepared with haemoglobin concentration of about 5g Hb/dl.In the ultraviolet range H_2O_2 shows increase in absorbtion with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time is a measure of the CAT enzyme activity (μmoles of H_2O_2 utilized/g Hb/sec).

5.2.3.1.4 Estimation of Lipid Peroxidation (LPO)

The estimation of LPO level in erythrocytes was carried out by the method of Beuge and Aust (1978). According to the principle of the method, lipid peroxidation leads to the formation of a malondialdehyde (MDA) which reacts with thiobarbituric acid (TBA) and gives thiobarbituric reactive substance (TBARS). TBARS gives a characteristic pink color which can be measured at 532 nm (nmoles of MDA formed/gHb). The protocol was followed as given in the method.

5.2.3.2 Blood collection and serum (sample) preparation (for Lipid profile study)

A volume of 1 ml blood samples aliquots were taken (and stored separately) from the total 5 ml of the blood sample collected during the objective-1 study parameters from 110 control and 270 IF-SCH subjects. The blood samples were collected by venous puncture from overnight fasting individuals serum was separated by centrifugation at 4000 g for 10 minutes at 22^0Cand was collected in Eppendorfand stored until the estimation of lipid profile.Fresh, clear serum under fasting condition with no haemolysis is the specimen of choice. However, plasma collected using heparin as a coagulant may also be used.

5.2.3.2.1 Estimation of Total Cholesterol (TC)

Estimation of Total Cholesterol (TC) was carried out by “Enzymatic” method. The protocol was followed as given in the method. According to the principle of the method, the

cholesterol esters are hydrolyzed to free cholesterol by cholesterol esterase (CE). The free cholesterol is then oxidized by cholesterol oxidase (CO) to cholesten 4-en-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenolic compound in the presence of peroxidase to yield a colored complex which is read at 505 nm. The intensity of colour produced is directly proportional to the concentration of the total cholesterol in the sample.

5.2.3.2.2 Estimation of HDL-Cholesterol

High density lipoproteins (HDL) are separated from other lipid protein fractions by treating the serum with phosphotungstic acid and magnesium chloride. HDL remains in solution while all other lipoprotein fractions are precipitated; cholesterol content of which is estimated by enzymatic method. The protocol followed was as given in the method.

5.2.3.2.3 Estimation of Triglycerides (TG)

Estimation of Triglycerides (TG) was carried out by "Enzymatic" method. Lipases hydrolyses triglycerides sequentially to di and monoglycerides and finally to glycerol. Glycerol kinase (GK) using ATP as PO4 source converts glycerol liberated to glycerol-3-phosphate (G-3-Phosphate). G-3-Phosphate Oxidase (GPO) oxidizes G-3-Phosphate formed to Dihydroxy acetone phosphate and hydrogen peroxide is formed. Peroxidase (POD) use the hydrogen peroxide formed, to oxidize 4-Aminoantipyrine and DHBS (3,5 Dichloro-2-hydroxy benzene sulphate) to a red coloured complex whose absorbance is measured at 520 nm (500-550 nm)or with Green filter which is proportional to Triglyceride concentration. The estimation was done by following the protocol as per the method.

5.2.3.2.4 Estimation of LDL-Cholesterol

Estimation of LDL-Cholesterol was carried out by using Friedwald`s equation; LDL-Cholesterol = Total Cholesterol – [HDL-Cholesterol + (Triglycerides/5)].

5.3 Statistical analysis

All the statistical analysis was done by using Prism 5 software (Graph Pad Software Inc.; 2007). The tests done were Non-parametric unpaired t-test, Fishers exact test for retrospective data and One-way ANOVA test whichever is applicable. The correlation studies were done by using Pearson correlation coefficients. Pearson's correlation coefficient was calculated to determine the relationship. A two–tailed, at minimum 95% confidence intervals and a p-value <0.05 was considered statistically significant.

5.4 Results

5.4.1 Oxidative stress biomarkers status

To check the effects of SCH on the stress levels in infertile females, estimation of oxidative stress biomarkers TBARS (malondialdehyde- MDA), which is a used to measure erythrocyte lipid peroxidation levels, and enzyme activities of the superoxide dismutases 1(SOD1) and catalase (CAT) was carried out. A significant increase (p=0.001) in MDA levels indicating an elevated erythrocyte lipid peroxidation levels in IF-SCH subjects (mean ± SEM; 198.8 ± 5.25 nMole formed/mg Hb, Fig. 5.1A, Table 5.1) as compared to Control group suggesting an increase in stress levels in IF-SCH females.

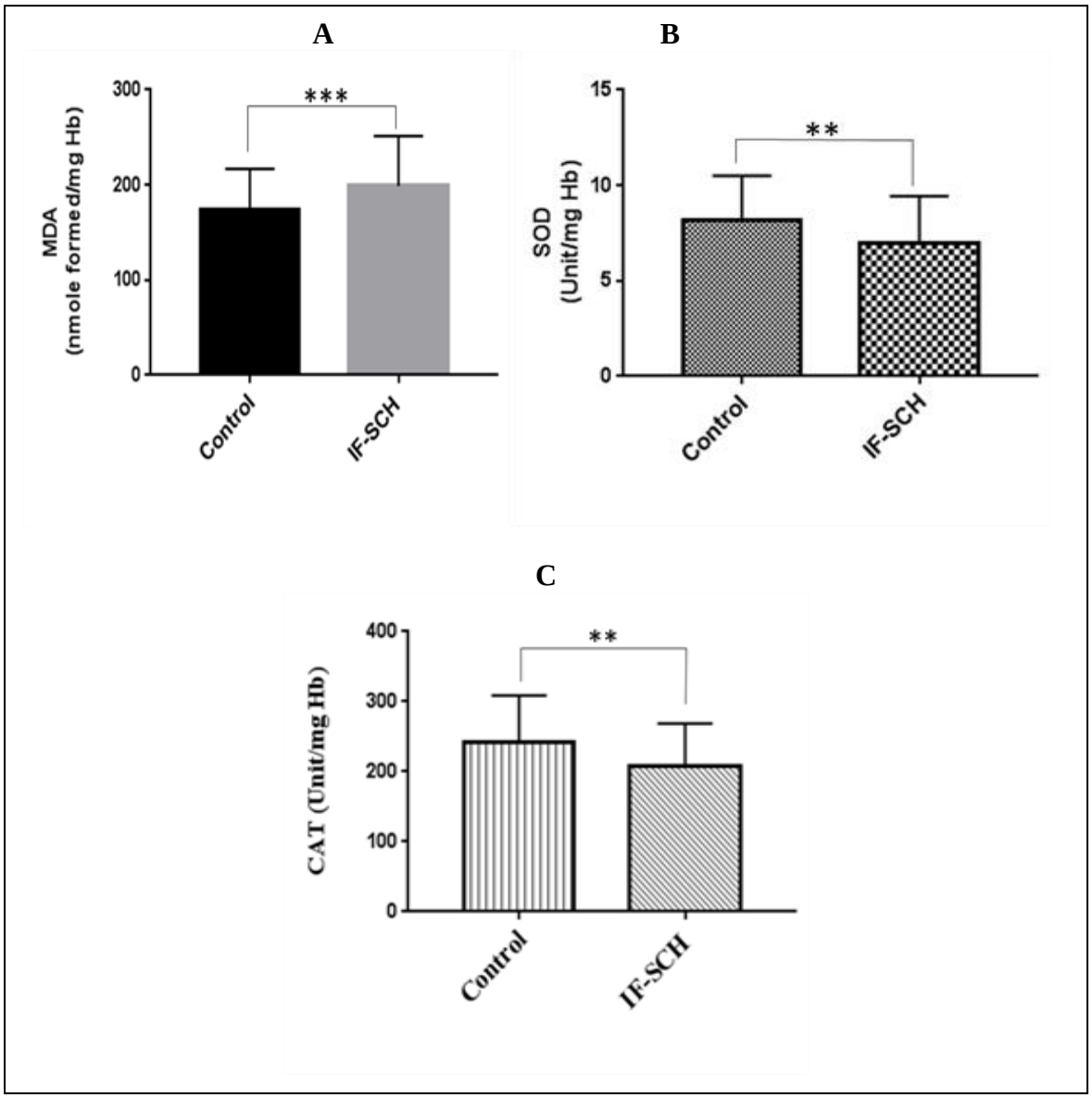

Figure 5.1 Levels of oxidative stress biomarkers in Control and IF-SCH subjects: A. Erythrocyte lipid peroxidation levels- A significant increase (p=0.001) in TBARS (MDA) levels in IF-SCH females indicating an elevated lipid peroxidation. **B.** Superoxide dismutase (SOD) activity is

significantly decreased (p<0.01) in IF-SCH females as compared to control Subjects. **C.** Catalase (CAT) activity is significantly decreased (p<0.01) in IF-SCH subjects as compared to Control.

Superoxide dismutase 1 (SOD) activity was found to be significantly decreased (p<0.01) in IF-SCH females (mean ± SEM; 6.97 ± 0.261 Unit/mg Hb; Fig. 5.1B, Table 5.1) as compared to Control subjects which is an indicator of increased oxidative stress in infertile females. Further another enzyme catalase (CAT) activity was measured which was also found to be decreased (p<0.01) in IF-SCH subjects (mean ± SEM; 207.7 ± 6.44 Unit/mg Hb, Fig. 5.1C, Table 5.1) as against the Control subjects which is also an indicator of increased oxidative stress in infertile females suffering from SCH.

Table 5.1 Levels of oxidative stress biomarkers in Control and IF-SCH subjects

Parameter	**Control subjects**	**IF-SCH subjects**	***p* value summary**
TBARS [MDA] (nmole formed/mg Hb)	173.3 ± 4.352 (n=100)	198.8 ± 5.248 (n=100)	***
SOD (Unit/mg Hb)	8.142 ± 0.2492 (n=90)	6.971 ± 0.2611 (n=90)	**
CAT (Unit/mg Hb)	241.4 ± 8.063 (n=70)	207.7 ± 6.44 (n=90)	**

Data represent mean ± SEM values, IF-SCH; Infertile females with SCH, TBARS; Thiobarbituric acid reactive substance, MDA; Malondialdehyde, SOD; Superoxide dismutase 1, CAT; Catalase enzyme, n; number of study subjects, **p<0.01, ***p=0.001.

5.4.1.1 Correlation between TSH and oxidative stress biomarkers

Correlation studies between TSH and oxidative stress biomarkers were done to find out the correlation between SCH an OS. A positive correlation (r=0.202, p<0.05**)** was obtained between TSH and MDA levels in IF-SCH subjects (Fig. 5.2B, Table 5.2) indicating high levels of erythrocyte lipid peroxidation and consequently an elevated stress levels in infertile females with SCH as compared to Control group (Fig. 5.2A, Table 5.2).

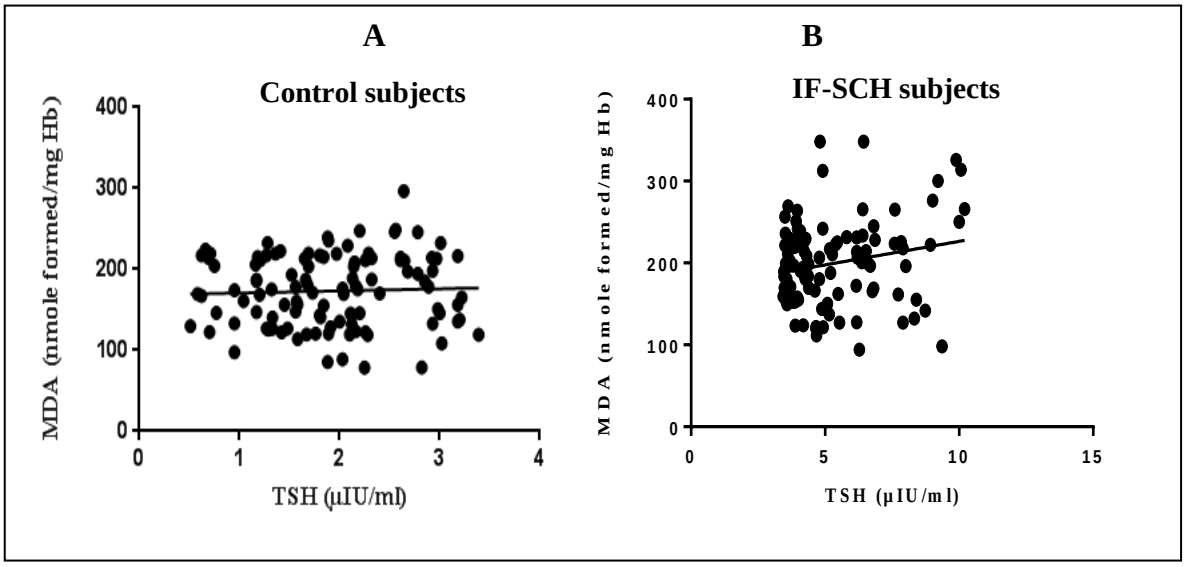

Figure 5.2 Correlation patterns between TSH and MDA levels: A. Control subjects. B. IF-SCH subjects. A significant positive correlation (r=0.202, $p<0.05$**)** was found between TSH and MDA levels in IF-SCH subjects compared to Control subjects.

Further to evaluate the effect of increased TSH levels in IF-SCH females on OS a correlation study between TSH and Superoxide dismutase 1 (SOD1) activity was carried out. The study reports a significant negative correlation (r=-0.213, $p<0.05$) between TSH level and SOD activity in IF-SCH subjects (Fig. 5.3 B, Table 5.2) as compared to Control group (Fig. 5.3A, Table 5.2).

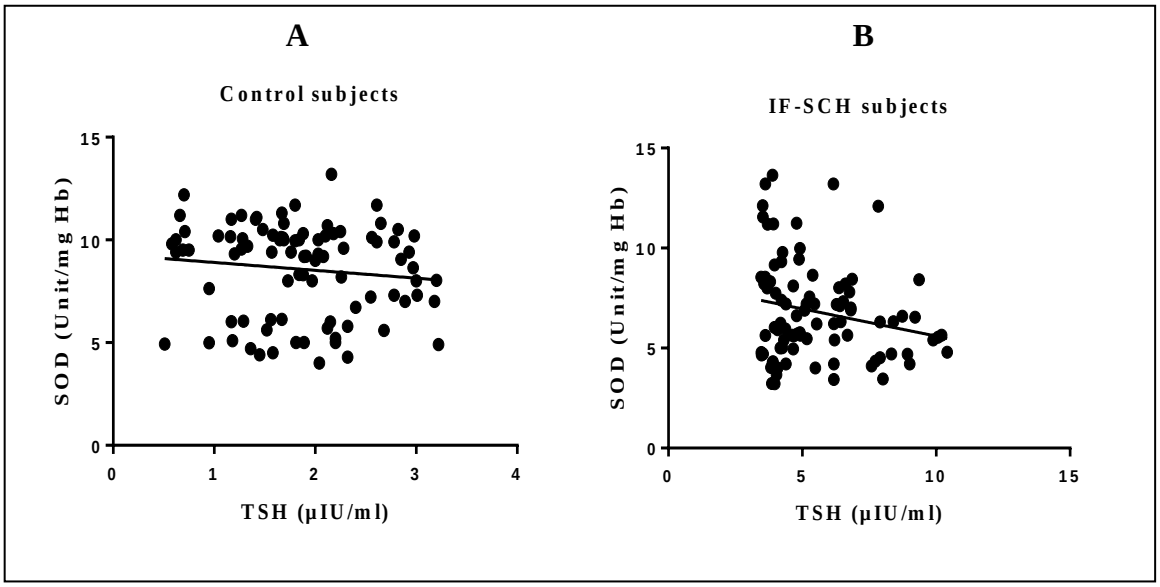

Figure 5.3 Correlation patterns between TSH and SOD levels: A. Control subjects. B. IF-SCH subjects. A significant negative correlation (r=-0.213, $p<0.05$) between TSH and SOD in IF-SCH subjects was reported, while Control group was showing a non significant negative correlation.

A non-significant negative correlation between TSH level and Catalase (CAT) activity was reported in IF-SCH subjects (r= -0.159, *p*=0.134; Fig. 5.4B, Table 5.2) and in Control subjects (r=-0.127, *p*=0.297; Fig. 5.4A, Table 5.2). Though the correlation between TSH levels and CAT activity was statistically not significant, the negative correlation indicates a decrease in CAT activity with an increase in TSH levels which is also an indicator of OS in IF-SCH female subjects

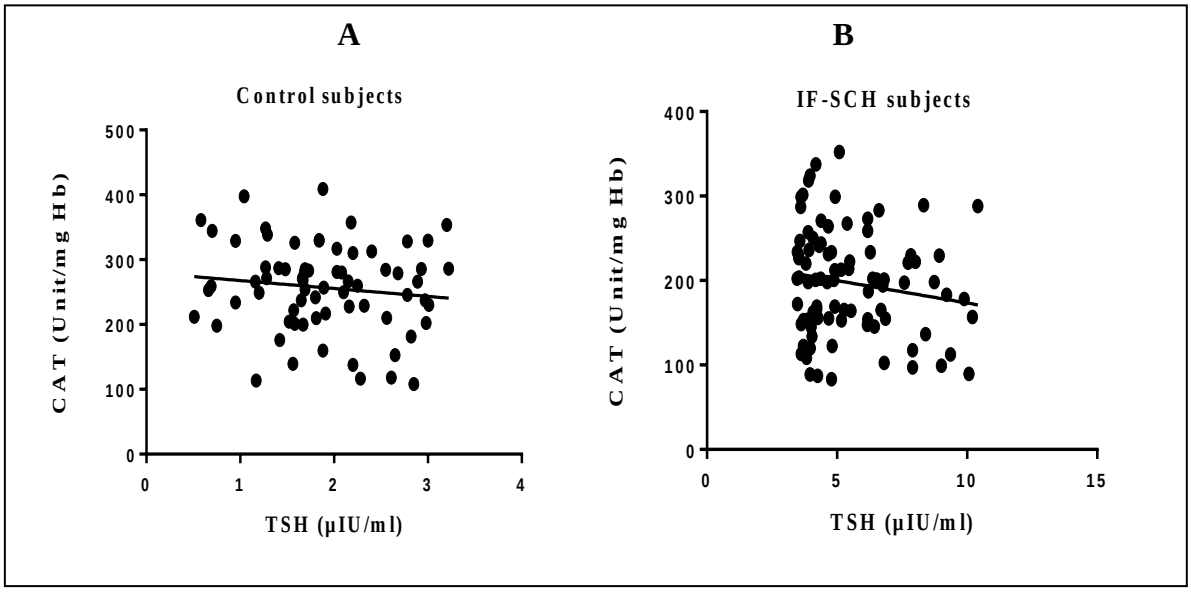

Figure 5.4 Correlation patterns between TSH and CAT levels: A. Control subjects B. IF-SCH subjects. Both the Control (r=-0.127, *p*=0.297) and IF-SCH groups (r= -0.159, *p*=0.134) reported a non-significant negative correlation.

Table 5.2 Correlation status between TSH levels and OS biomarkers

	TSH with MDA		**TSH with SOD**		**TSH with CAT**	
	Control Subjects	**IF-SCH Subjects**	**Control subjects**	**IF-SCH Subjects**	**Control Subjects**	**IF-SCH Subjects**
No. of XY pairs	110	100	90	90	90	70
Pearson r	0.043	0.202	-0.119	-0.213	-0.127	-0.159
95% confidence	-0.146 to 0.228	0.006 to 0.383	-0.318 to 0.091	-0.402 to -0.006	-0.351 to 0.112	-0.355 to 0.050
***p* value**	0.661	0.044	0.266	0.044	0.297	0.134
Significance of correlation	ns	*	Ns	*	ns	Ns

IF-SCH; Infertile females with SCH, MDA; Malondialdehyde, SOD; Superoxide dismutase 1, CAT; Catalase enzyme, n; number of study subjects,*$p<0.05$

5.4.1.2 Correlation pattern between the OS biomarkers

To findout the correlation between the OS biomarkers the correlation studies between MDA, SOD and CAT were carried out. A highly significant (r=-0.345, *p*=0.001) negative

correlation was found between MDA and SOD levels in IF-SCH subjects (Fig. 5.5B, Table 5.3) while Control group did not showed a statistically significant negative (Fig. 5.5A, Table 5.3). This indicates an increased OS in infertile females with SCH.

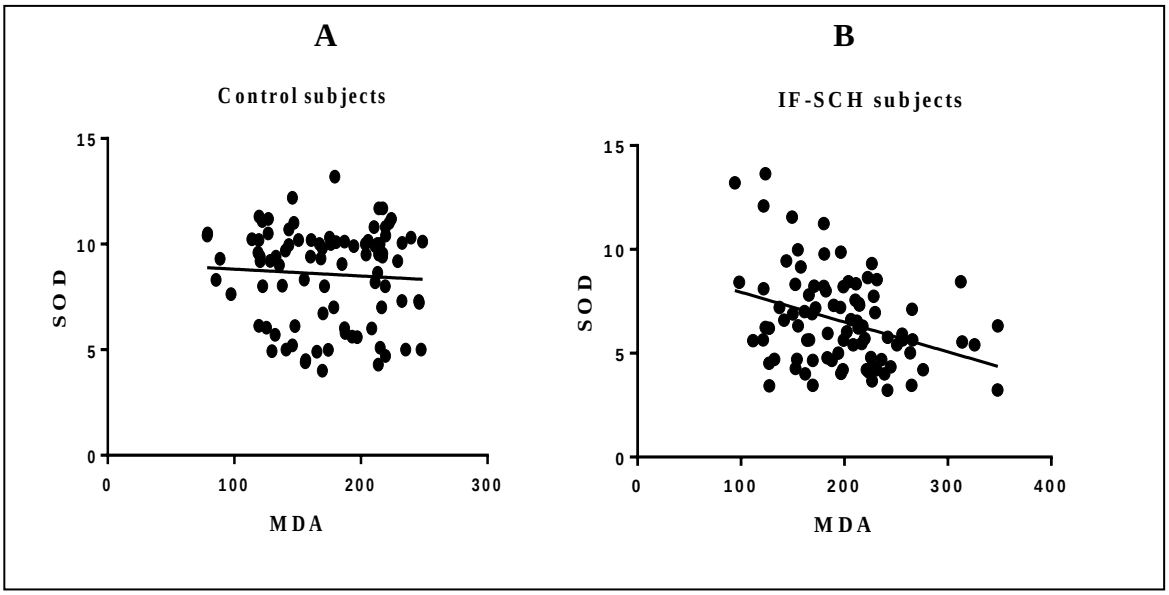

Figure 5.5 Correlation pattern between MDA and SOD levels: A. Control subjects B. IF-SCH subjects. A highly significant negative correlation (r=-0.345, *p*=0.001) was found between MDA and SOD levels in IF-SCH subjects, while Control group also showed a negative correlation but, was not statistically significant.

Correlation study between MDA and CAT also revealed a significant negative correlation (r=-0.234, $p<0.05$) between MDA and CAT in IF-SCH (Fig. 5.6B, Table 5.3), while Control group was showing a non significant negative correlation (Fig. 5.6A, Table 5.3) confirming the increased stress in infertile females.

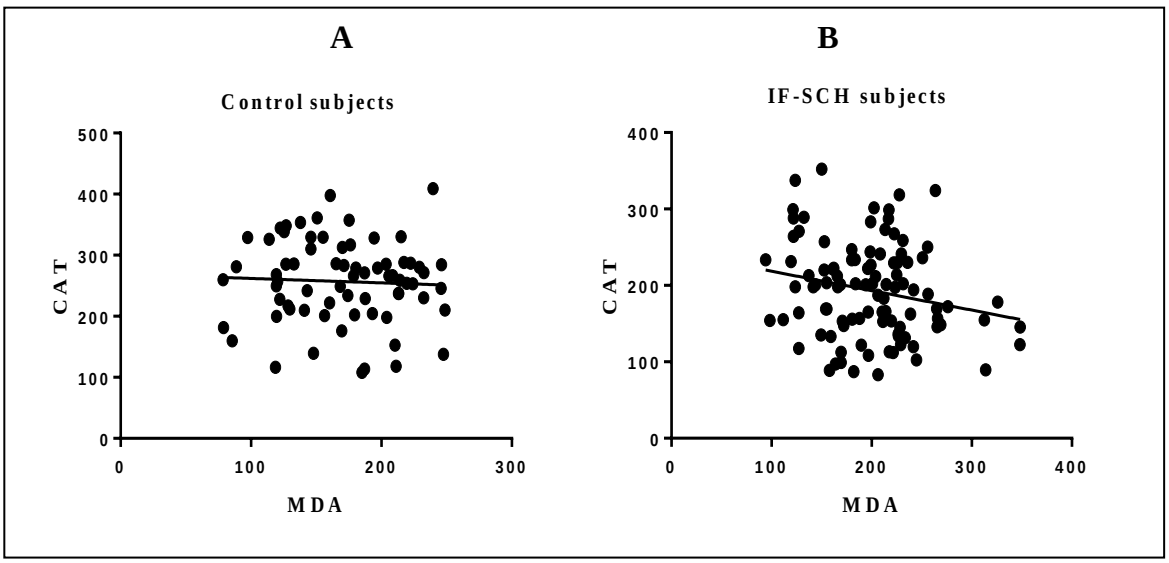

Figure 5.6 Correlation pattern between MDA and CAT levels: A. Control subjects. B. IF-SCH subjects. A significant negative correlation (r=-0.234, p<0.05) between MDA and CAT in IF-SCH subjects was reported, while Control group was showing a non significant negative correlation.

Further to find out the correlation between the enzyme activities a correlation study was carried out between SOD and CAT enzymes, we found a positive correlation in IF-SCH females but the correlation was not statistically significant (r=0.050, p=0.685, Fig. 5.7B, Table 5.3), a positive correlation further reconfirms the increase in OS in IF-SCH subjects.

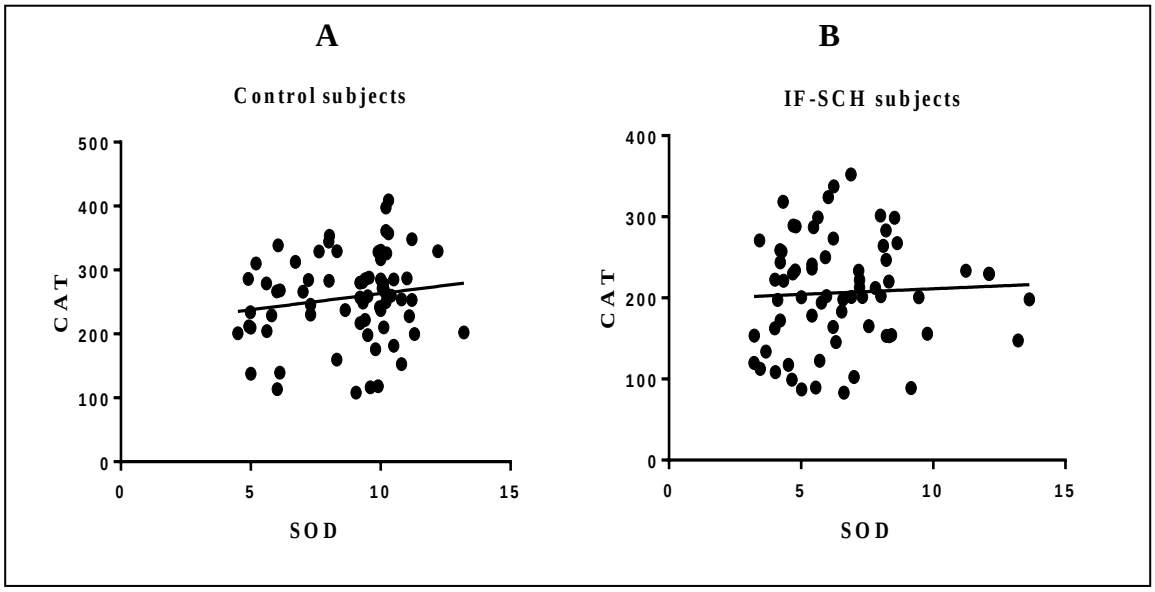

Figure 5.7 Correlation pattern between SOD and CAT levels: A. Control subjects. B. IF-SCH subjects. Both the Control and IF-SCH groups reported a non-significant positive correlation (r=0.050, p=0.685).

Table 5.3 Correlation status between OS biomarkers

	TBARS (MDA) with SOD		**TBARS (MDA) with CAT**		**SOD with CAT**	
	Control Subjects	**IF-SCH Subjects**	**Control subjects**	**IF-SCH Subjects**	**Control subjects**	**IF-SCH Subjects**
No. of XY pairs	90	90	70	90	70	70
Pearson r	-0.050	-0.345	-0.049	-0.234	0.156	0.050
95% confidence	-0.243 to 0.149	-0.515 to -0.148	-0.280 to 0.189	-0.421 to -0.029	-0.082 to 0.378	-0.188 to 0.281
***p* value**	0.628	0.0009	0.691	0.026	0.196	0.685
Significance of correlation	ns	***	ns	*	ns	Ns

IF-SCH; Infertile females with SCH, TBARS; Thiobarbituric acid reactive substance, MDA; Malondialdehyde, SOD; Superoxide dismutase 1, CAT; Catalase enzyme, n; number of study subjects,*p<0.05, ***p=0.001, ns; not significant, *p<0.05, ***p=0.001

5.4.2. Lipid profile alterations in Control and IF-SCH subjects

Alteration in the lipid profile mainly dyslipidemia subsequently causing reproductive disturbance, is very common in hypothyroid patients precipitating with various

morphological and clinical manifestations due to the involvement of the thyroid system in every aspect of fat metabolism. But SCH being an early a symptomatic condition of thyroid dysfunction is difficult to identify and even the management requires an appropriate approach. Hence to find out the alteration in fat/ lipid metabolism in infertile females with SCH, the lipid profile analysis was carried out. A highly significant (p=0.0003, Fig. 5.8A) increased levels of Total cholesterol [TC] was reported in IF-SCH subjects (Table 5.4) as compared to Control subjects. Triglyceride [TG] levels in IF-SCH subjects were also showed a highly significant (p=0.0001, Fig. 5.8B, Table 5.4) increase against the Control group. A significantly (p=0.0001, Fig. 5.8C) increased Low density lipoprotein-Cholesterol [LDL-C] levels were reported in IF-SCH female subjects (Table 5.4) as compared to the Control group. While on estimating the High density lipoprotein-Cholesterol [HDL-C] levels we reported a significant decreased (p=0.021, Fig. 5.8D) in HDL-C levels in IF-SCH subjects (Table 5.4) as compared to the Control group.

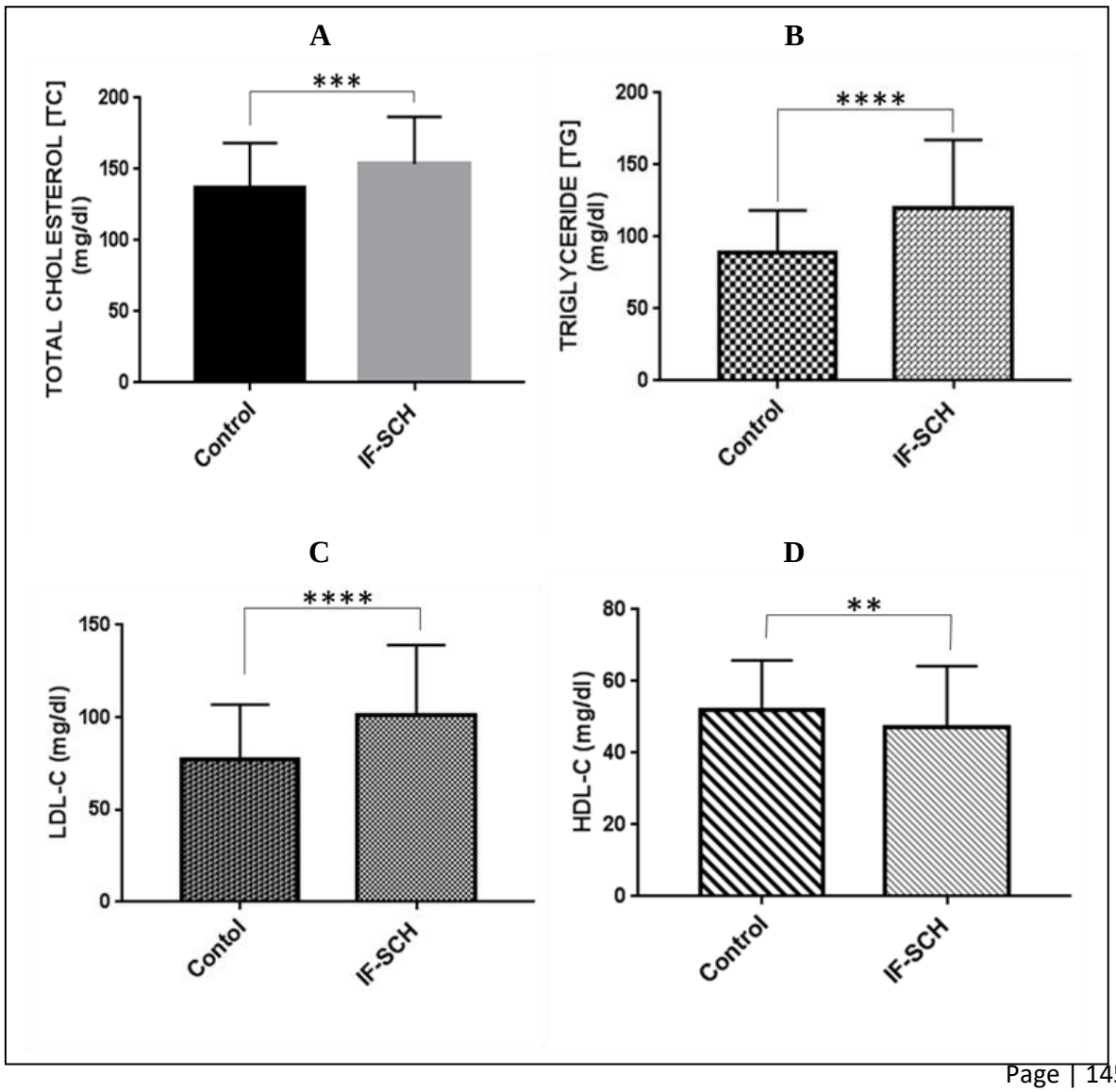

Figure 5.8 Lipid profile alterations in Control and IF-SCH subjects: A. Total Cholesterol [TC] levels- A highly significant increased levels of TC was reported in IF-SCH subjects as compared to Control subjects. **B.** Triglyceride [TG] levels- IF-SCH subjects showed presence of very highly significant increased (p=0.0001) TG levels against the Control group. **C.** Low density lipoprotein-Cholesterol [LDL-C] levels- A highly significant increased (p=0.0001) LDL-C levels were reported in IF-SCH subjects as compared to the Control group. **D.** High density lipoprotein-Cholesterol [HDL-C] levels- A significant decreased ((p=0.021) in HDL-C levels were reported in IF-SCH subjects as compared to the Control group.

Table 5.4 Lipid profile in Control and IF-SCH subjects

	TC (mg/dl)	**TG (mg/dl)**	**LDL-C (mg/dl)**	**HDL-C (mg/dl)**
Normal Values	140-250	45-170	58-100	45-65
Control subjects	136.7±3.175, (n=100)	89.13 ± 2.917, (n=100)	77.31±2.963, (n=100)	52.17±1.366, (n=100)
IF-SCH subjects	153.6±3.328, (n=100)	120.1 ± 4.722, (n=100)	101.4±3.784, (n=100)	47.05±1.717, (n=100)
***p* Value**	0.0003 (***)	<0.0001 (****)	<0.0001 (****)	0.021 (*)

Data represent mean ± SEM values, IF-SCH; Infertile females with SCH, TC; Total Cholesterol, TG; Triglycerides, LDL-C; Low density lipoprotein-Cholesterol, HDL-C; High density lipoprotein-Cholesterol; *p<0.05, ***p=0.001, ****p<0.0001.

It should be taken into an account that, though the study reports an alteration in the lipid profile in the infertile females with SCH, all the values which the study reported are within the normal reference ranges provided by the method used, apart from the values of only LDL-C which was showing a slightly increase value than that of the normal range, in IF-SCH females. All the parameter values of IF-SCH subjects are compared with that of the values of the Control group subjects, and thus the significance is presented with respect to the Control group values. So it must be noted that though the study report an alteration in the lipid profile but it is within the normal range.

5.4.2.1 Correlation between SCH and alteration in Lipid profile

To find out the effect of SCH on lipid profile in infertile females, correlation studies were carried out. The study reports an insignificant (r=0.137, p=0.173) positive correlation between TSH and total cholesterol [TC] levels in IF-SCH subjects (Fig. 5.9A and B, Table 5.5) and the Control subjects.

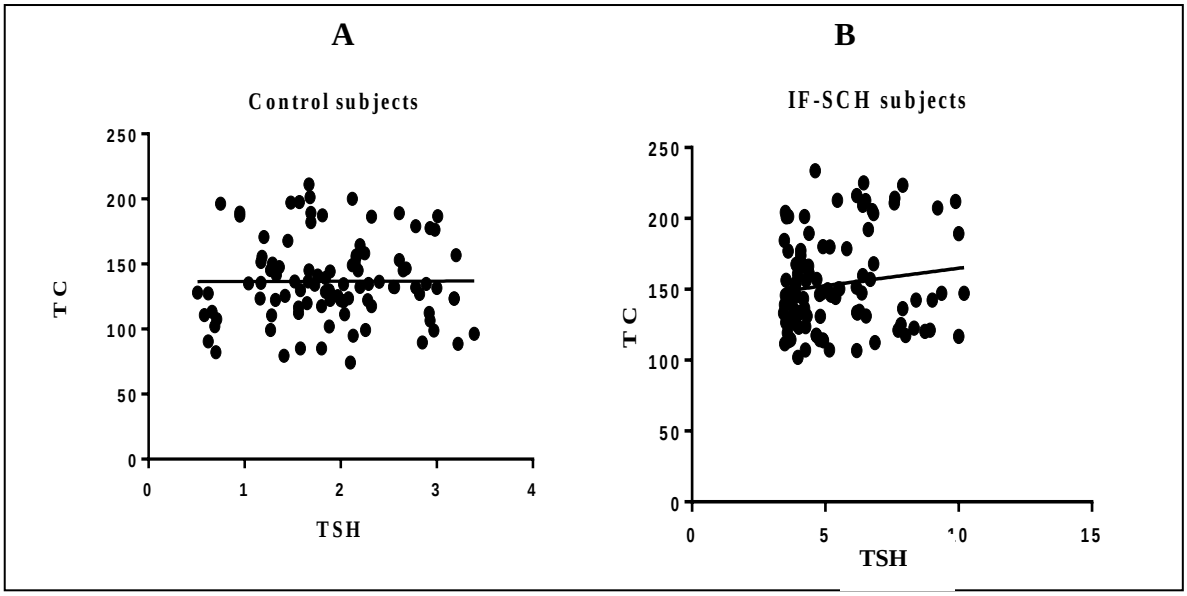

Figure 5.9 Correlation patterns between TSH and TC levels: A. Control subjects. B. IF-SCH subjects. An insignificant positive correlation was found between TSH and TC levels in IF-SCH and also in Control group (r=0.137, *p*=0.173)**.**

Further a statistically significant (r=0.204, *p*=0.042) positive correlation between TSH and triglycerides [TG] in IF-SCH subjects was reported, while Control group was showing an insignificant positive correlation (Fig. 5.10 A and B, Table 5.5).

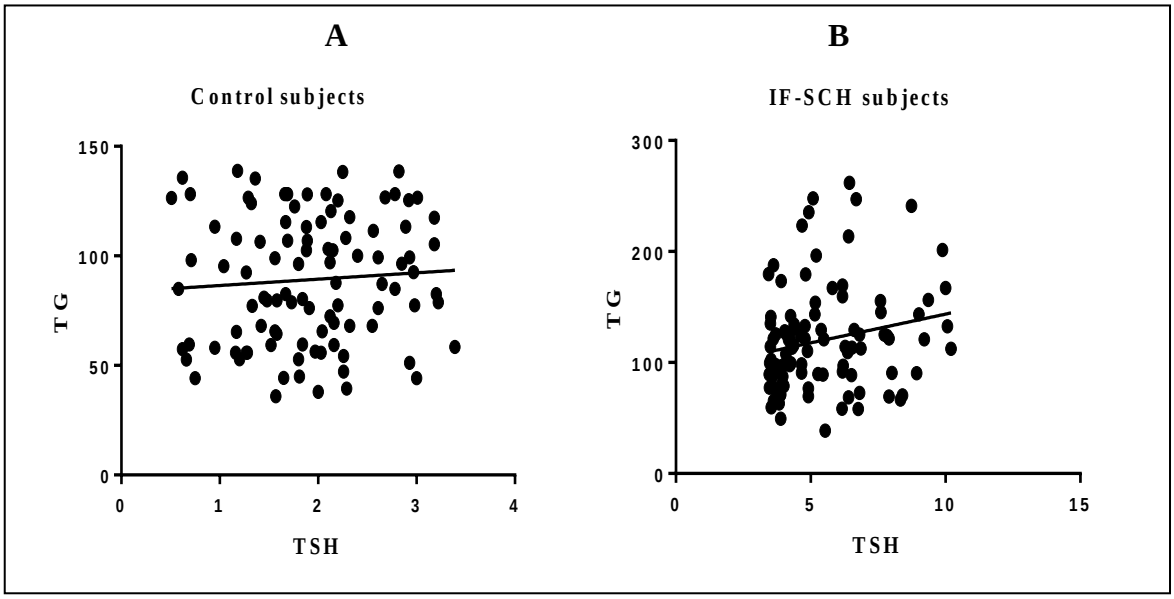

Figure 5.10 Correlation patterns between TSH and TG levels: A. Control subjects. B. IF-SCH subjects. A significant positive correlation (r=0.204, *p*=0.042) between TSH and TG in IF-SCH subjects was reported, while Control group was showing an insignificant positive correlation

While a statistically insignificant positive correlation between TSH and LDL-C levels was reported for both the Control (r=0.097, *p*=0.335, Fig. 5.11A and B, Table 5.6), and IF-SCH subjects.

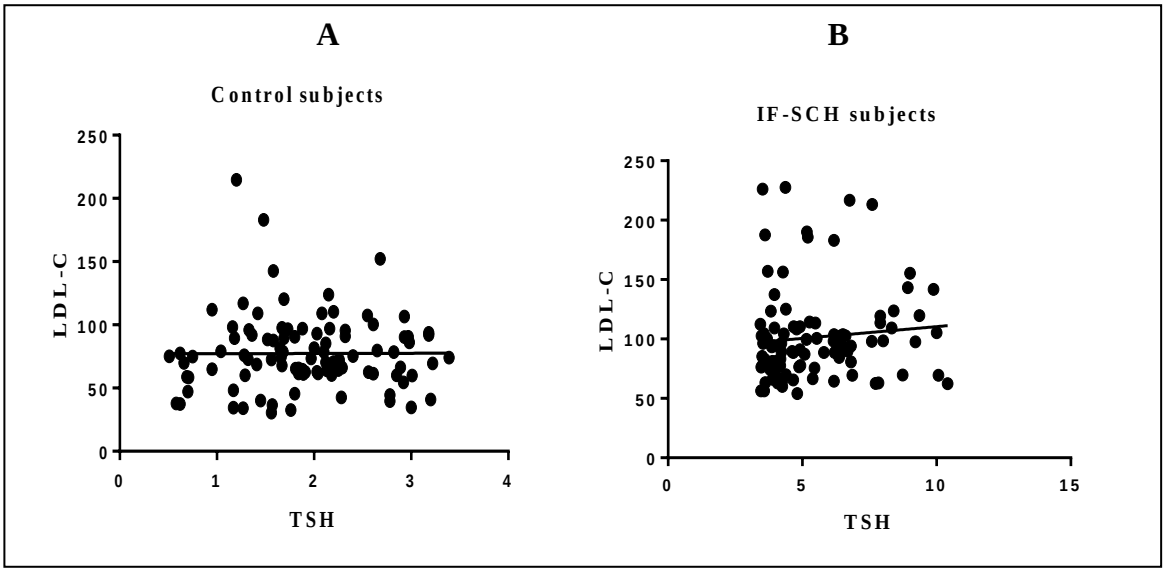

Figure 5.11 Correlation patterns between TSH and LDL-C levels: A. Control subjects. B. IF-SCH subjects. Both the Control and IF-SCH groups reported insignificant positive correlation between TSH and LDL-C levels (r=0.097, *p*=0.335).

Correlation between TSH and HDL-C levels revealed an insignificant (r=-0.158, *p*=0.116, Fig. 5.12A and B, Table 5.6) negative correlation in IF-SCH subjects as well as in the Control group.

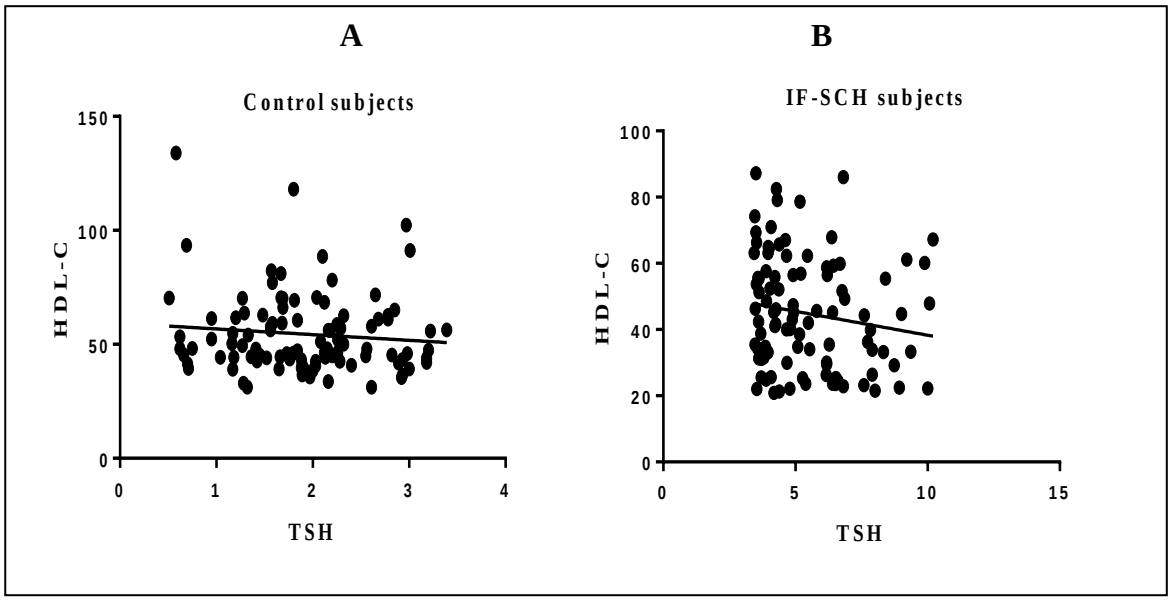

Figure 5.12 Correlation patterns between TSH and HDL-C levels: A. Control subjects. B. IF-SCH subjects. An insignificant negative correlation (r=-0.158, *p*=0.116) was found between TSH levels and HDL-C levels in IF-SCH subjects and also in the Control group.

Increased TC, TG and LDL-C levels as well as decreased HDL-C levels with increase in TSH levels in IF-SCH females indicate an alteration in the lipid profile in females having

subclinical hypothyroid condition. This alteration can be due to SCH and might be a precipitating factor for fem5ale infertility in the selected study population.

Table 5.5 Correlation statuses between TSH, TC and TG levels in Control and IF-SCH subjects

	TSH with TC		**TSH with TG**	
	Control Subjects	**IF-SCH Subjects**	**Control subjects**	**IF-SCH Subjects**
No. of XY pairs	100	100	100	100
Pearson r	0.004	0.137	0.070	0.204
95% confidence	-0.193 to 0.199	-0.061 to 0.325	-0.128 to 0.263	0.008 to 0.385
***p* value**	0.972	0.173	0.486	0.042
Significance of correlation	NS	NS	NS	*

IF-SCH; Infertile females with SCH, TC; Total Cholesterol, TG; Triglycerides, LDL-C; Low density lipoprotein-Cholesterol, HDL-C; High density lipoprotein-Cholesterol, NS; not significant; *p<0.05.

Table 5.6 Correlation status between TSH and Lipid profile in Control and IF-SCH subjects

	TSH with LDL-C		**TSH with HDL-C**	
	Control subjects	**IF-SCH Subjects**	**Control Subjects**	**IF-SCH Subjects**
No. of XY pairs	100	100	100	100
Pearson r	0.005	0.097	-0.101	-0.158
95% confidence	-0.193 to 0.200	-0.101 to 0.288	-0.291 to 0.098	-0.344 to 0.040
***p* value**	0.969	0.335	0.319	0.116
Significance of correlation	NS	NS	NS	NS

IF-SCH; Infertile females with SCH, TC; Total Cholesterol, TG; Triglycerides, LDL-C; Low density lipoprotein-Cholesterol, HDL-C; High density lipoprotein-Cholesterol, ns; not significant; *p<0.05.

5.4.2.2 Correlation between increased OS and altered lipid profile in infertile females with SCH

To find out the correlation between increased OS levels and an altered lipid profile correlation studies between MDA levels and various parameters of lipid profile were carried out in the IF-SCH subjects and compared with the Control subjects. Correlation between MDA and TC levels revealed a significant positive correlation (r=0.232, p=0.020, Fig. 5.13Aand B, Table 5.6) in IF-SCH indicating a correlation between increased OS and altered lipid profile in infertile females with SCH, but not in Control group.

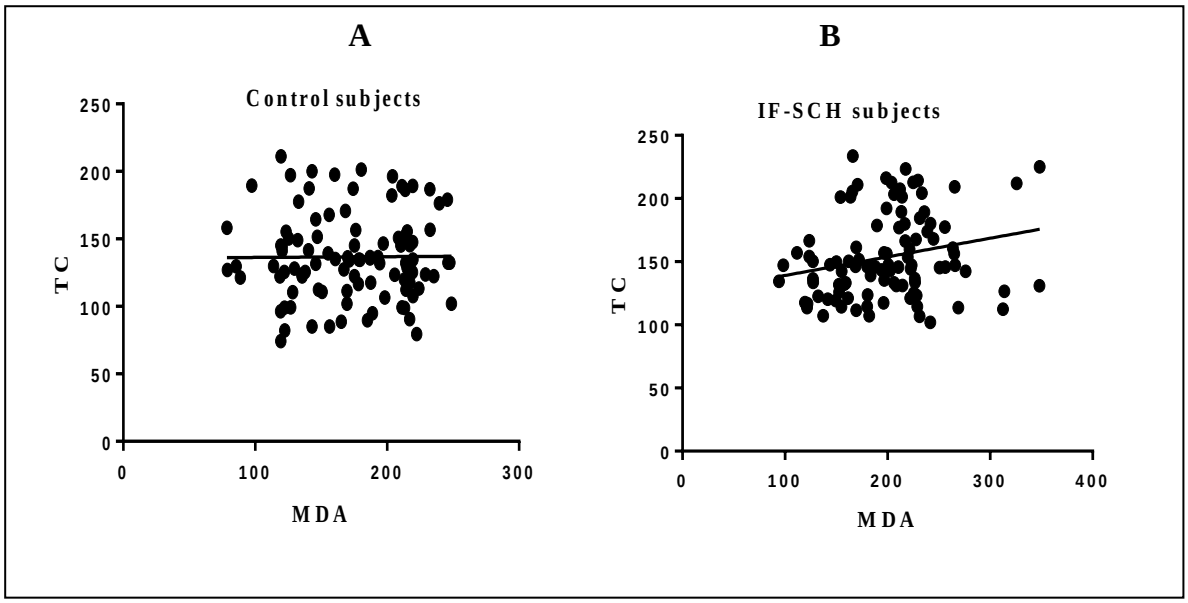

Figure 5.13 Correlation patterns between MDA and TC levels: A. Control subjects B. IF-SCH subjects. A significant positive correlation was found between MDA and TC levels in IF-SCH but not in Control group (r=0.232, *p*=0.020).

The present study reports an insignificant (r=0.101, *p*=0.317, Fig. 5.14B, Table 5.6) positive correlation between MDA and TG in IF-SCH subjects, while Control group was also showing an insignificant positive correlation (Fig. 5.14A, Table 5.6).

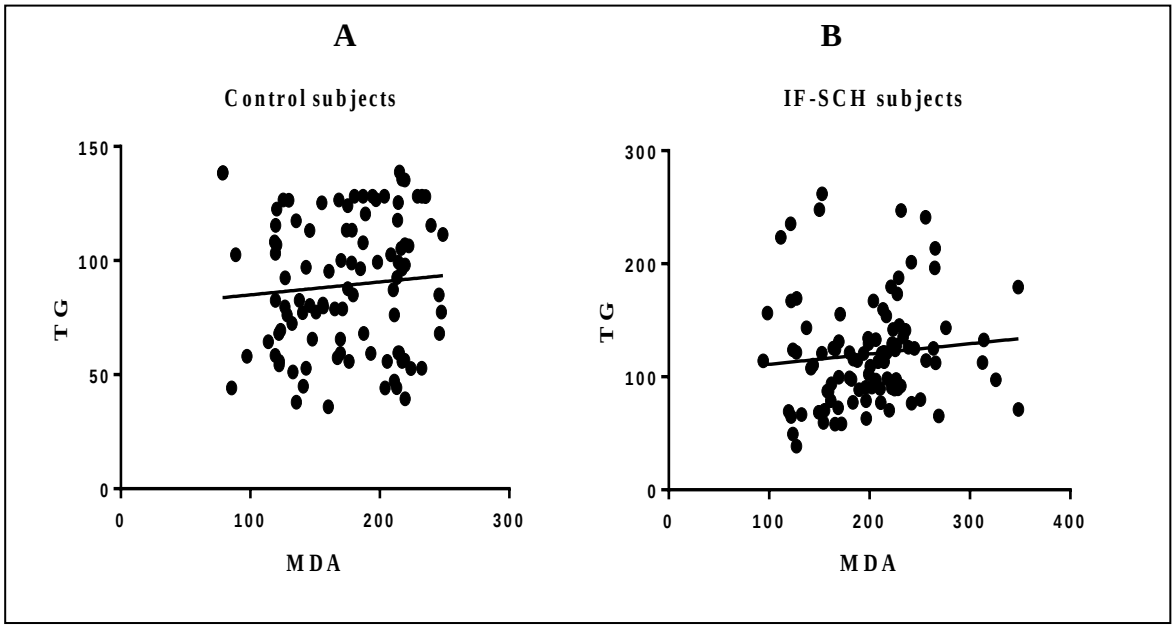

Figure 5.14 Correlation patterns between MDA and TG levels: A. Control subjects. B. IF-SCH subjects. An insignificant positive correlation between MDA and TG in IF-SCH subjects was reported, while Control group was also showing an insignificant positive correlation (r=0.101, *p*=0.317).

Table 5.7 Correlation statuses between MDA, TC and TG levels in Control and IF-SCH subjects

	MDA with TC		MDA with TG	
	Control Subjects	**IF-SCH Subjects**	**Control Subjects**	**IF-SCH Subjects**
No. of XY pairs	100	100	100	100
Pearson r	0.008	0.232	0.084	0.101
95% confidence	-0.189 to 0.204	0.038 to 0.500	-0.114 to 0.276	-0.097 to 0.292
***p* value**	0.938	0.020	0.404	0.317
Significance of correlation	ns	*	ns	Ns

IF-SCH; Infertile females with SCH, MDA; Malondialdehyde, TC; Total Cholesterol, TG; Triglycerides, LDL-C; Low density lipoprotein-Cholesterol, HDL-C; High density lipoprotein-Cholesterol, ns; not significant; *p<0.05.

Further, the IF-SCH groups reported insignificant (r=0.057p=0.577, Fig. 5.15A and B, Table 5.7) positive correlation between MDA and LDL-C levels, and the Control group also reported an insignificant positive correlation.

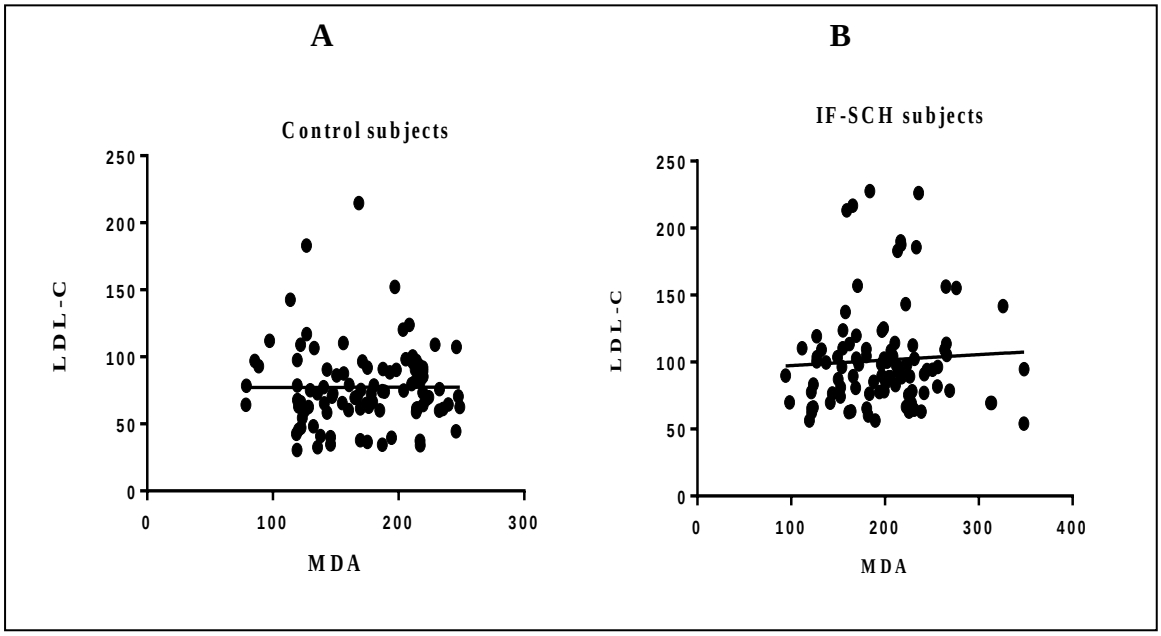

Figure 5.15 Correlation patterns between MDA and LDL-C levels: A. Control subjects. B. IF-SCH subjects. Correlation between MDA and LDL-C levels-Both the Control and IF-SCH groups reported insignificant positive correlation between MDA and LDL-C levels (r=0.057p=0.577).

An insignificant (r=-0.040, p=0.700) negative correlation was found between MDA levels and HDL-C levels in IF-SCH subjects (Fig. 5.16 A and B, Table 5.7) and the Control group also reported a negative correlation which was statistically insignificant. Though the

correlation was statistically not significant, an increase in TG and LDL-C and decreased HDL-C levels, with an increase in MDA levels are the indicators of correlation of increased OS altered lipid profile in the females with SCH which together thus can be a precipitating factor for infertility in the selected population.

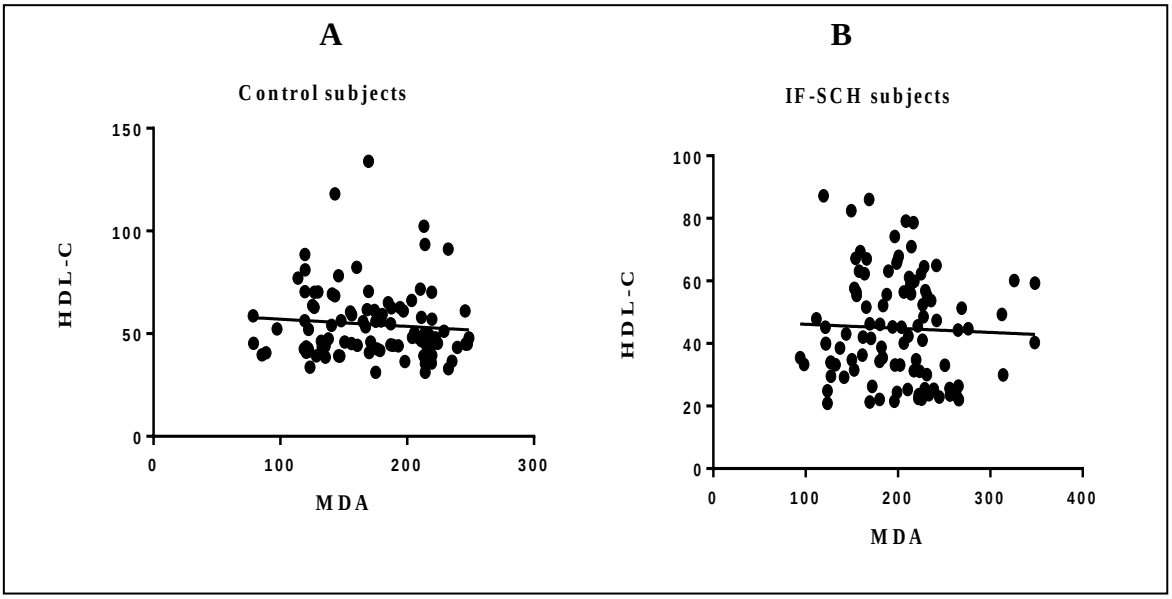

Figure 5.16 Correlation patterns between MDA and HDL-C levels: A. Control subjects B. IF-SCH subjects. An insignificant (p=0.700) negative correlation was found between MDA levels and HDL-C levels in IF-SCH subjects and also in the Control group (r=-0.040, p=0.700).

Table 5.8 Correlation status between MDA, LDL-C and HDL-C in Control and IF-SCH subjects

	MDA with LDL-C		**MDA with HDL-C**	
	Control subjects	**IF-SCH Subjects**	**Control Subjects**	**IF-SCH Subjects**
No. of XY pairs	100	100	100	100
Pearson r	0.002	0.057	-0.087	-0.040
95% confidence	-0.195 to 0.198	-0.142 to 0.250	-0.278 to 0.112	-0.235 to 0.157
***p* value**	0.984	0.577	0.392	0.700
Significance of correlation	ns	ns	Ns	Ns

IF-SCH; Infertile females with SCH, MDA; Malondialdehyde, TC; Total Cholesterol, TG; Triglycerides, LDL-C; Low density lipoprotein-Cholesterol, HDL-C; High density lipoprotein-Cholesterol, ns; not significant; *p<0.05.

5.5 Discussion

In the present study we demonstrate that infertile female subjects with SCH have elevated oxidative stress (OS) and an altered lipid profile (LP), when compared with control subjects.

During last few decades, worldwide increase in endocrine diseases and thyroid gland disorders rank amongst the most abundant endocrine disorders. In spite of being much more common than overt hypothyroidism, subclinical hypothyroidism mostly goes undiagnosed due to its asymptomatic nature thus; an early diagnosis and treatment must be addressed to prevent the conversion of subclinical hypothyroidism to onset of overt hypothyroidism and its associated medical manifestations. Effects of overt hypothyroidism on female fertility has been studied extensively and its adverse outcomes resulting in female infertility is well known, but studies on SCH and its subsequent consequences such as increased oxidative stress (OS) and alterations in the lipid profile (LP) which in turn precipitate into female infertility are scarce and not well documented. The present study aimed to find out and correlate the effect of SCH on oxidative stress levels along with the alterations in lipid profile in primarily infertile female population of Gujarat.

An increased MDA level along with a decrease in the activities of CAT and SOD enzymes resulting into elevated oxidative stress (OS) was observed in primarily infertile females with SCH. The study reports a positive correlation between TSH and MDA levels in subclinical hypothyroid infrtile subjects indicating high levels of erythrocyte lipid peroxidation and consequently an elevated stress levels. The study further reports a negative correlation between TSH levels and SOD and CAT activities which confirms OS in infertile females with SCH. While a positive correlation, though statistically insignificant between SOD and CAT enzymes reveals elevated OS in IF-SCH females in present study. Secondly, in the present study we report an altered lipid profile (LP) with an increase in TC, TG and LDL-C levels along with a decrease in HDL-C levels. In addition, the present study reports a positive correlation between TSH and TC, TG and LDL-C, as well as a negative correlation between TSH and HDL-C in IF-SCH females, which indicates alterations in LP in infertile females with SCH. While correlating OS with LP, we report a positive correlation between TBARS (MDA) and TC levels suggesting a correlation of OS and LP with SCH precipitating to female infertility. In addition, positive (insignificant) correlation between (TBARS) MDA and TG, LDL-C, and a negative (insignificant) correlation of MDA with HDL-C in IF-SCH indicating a correlation between increased OS and altered lipid profile in infertile females with SCH. It should be considered that even though the correlation was statistically not significant, an increase in TG and LDL-C and decreased HDL-C levels, with an increase in MDA levels are the indicators OS with an altered LP and wise versa in the females with SCH

which together might be the cause of infertility in primarily infertile females with SCH from Gujarat.

An elevated oxidative stress is commonly associated with female infertility (Halliwell*et al.,* 2015) and on the other hand overt hypothyroidism also subsequently causes an increase in the OS levels which further may be the cause of female infertility (Pasupathi*et al.,* 2008). Hence, to extrapolate the consequences of overt hypothyroidism to that of SCH, estimation of OS biomarkers was carried out in Control and IF-SCH female subjects. The study reported an increased TBARS (MDA) level in infertile females with SCH as compared to healthy, parous Control females. MDA is a measure of erythrocyte lipid peroxidation (LPO) and thus OS. We reported a significant decrease in superoxide dismutase 1 (SOD) as well as catalase (CAT) activities in IF-SCH females as compared to Control subjects which is an indicator of increased oxidative stress in infertile females with SCH. LPO is an autocatalytic mechanism leading to oxidative destruction of cellular membranes. Studies have shown that, thyroid dysfunctions increase LPO reactions and ROS (Asayama*et al.,* 1990; Venditti *et al.,* 2006; Messarah*et al.,* 2007). Lipid peroxidation (LPO) is reported to be high in hyperlipidaemia, which is very common in hypothyroidism as reported by the studies (Nanda*et al.,* 2008; Konukoglu *et al.,* 2002). Free radical-scavenging enzymes such as SOD and CAT are the first line of cellular defense and protect the red cells against O_2- andH_2O_2-mediated lipid peroxidation (Senthil *et al.,* 2002). SOD and CAT provides enzymatic protection against reactive oxygen species (ROS) and the breakdown products of peroxidized lipids and oxidized protein and DNA (Serdar*et al.,* 2006). Such destruction can lead to cell death and to the production of toxic free radicals such as MDA which is the most important (Messarah *et al.,* 2010). Adriana *et al* observed an increased activity of CAT in the subclinical hypothyroid group. In addition, our study shows an association between lipid parameters and CAT or SOD activities (Santi *et al.,* 2012). Santi *et al*. (2010) showed hypercholesterolemia has a stronger influence on the development of oxidative stress in overt hypothyroid (OH) patients and increased levels of thiobarbituric acid reactive substances (TBARS), SOD, catalase (CAT) and Vitamin E. Torun *et al*. (2009) reported elevated MDA levels in patients with subclinical hypothyroidism.. Baskol*et al*. (2007) showed patients with primary hypothyroidism elevated malondialdehyde (MDA) andnitric oxide (NO) levels while superoxide dismutase (SOD) was not different from controls. Mancini *et al*. (2010) showed low Total Antioxidant Capacity (TAC) levels in hypothyroid patients.

Lipid profile (LP) is influenced by many factors including thyroid hormones. This study has

shown that SCH condition alters the lipid profile and thus might be contributing to infertility in females. Since, thyroid hormones upregulate LDL-receptor expression; hypercholesterolemia is a common feature in hypothyroidism (Huesca-G*et al.,* 2002). Studies have reported an elevated TC and/or LDL-C in SCH compared with controls (Santi *et al.,* 2012; Efstathiadou *et al.,* 2001; Yildirimkaya *et al.,* 1996; Miura *et al.,* 1994). Our results showed that subjects with SCH had significantly higher levels of TC, LDL-C, TG, thus displaying an altered lipid profile when compared with healthy individuals. The present research has shown that increased thyroid stimulating hormone (TSH) levels changes the lipid profiles. Thyroid hormones may stimulate hydroxyl methylglutaryl coenzyme A (HMG CoA), the key enzyme of cholesterol biosynthesis, and induce an increased synthesis of cholesterol. Additionally, the LDL-C receptor gene contains a thyroid hormone responsive element (TRE) that could allow triiodothyronine (T3) to modulate the gene expression of the LDL-C receptor resulting in an increase of LDL-C receptor synthesis. Thyroid hormones and their function are low in target tissue in SH, and researchers conjectured that SCH influences lipid profiles by the abovementioned mechanism (Turhan *et al.,* 2008; Lu *et al.,* 2011).The relationship between SCH and lipid profile is still controversial, but it is known that during clinical and SCH the lipid profile alteration is seen. Some investigators have reported alterations in LP with changes in TSH levels (Iqbal *et al.,* 2006; Efstathiadou *et al.,* 2001; Taddei *et al.,* 2003). We report an association of TSH levels with deleterious changes in serum lipids along with a positive correlation between TSH and total cholesterol, triglycerides levels and LDL fraction as well as a negative correlation between TSH levels and HDL-C fraction in infertile females with SCH. Santi *et al* reported that hypothyroidism directly do not cause OS, but it is seen as a result of hypercholesterolemia caused by hypothyroidism. In subclinical hypothyroid condition too, a high level of lipid can serve as a substrate for OS (Santi *et al.,* 2010). High MDA levels with decreased CAT and SOD activities are reported in infertile females by Torun and also by Agrawal *et al* in their studies (Torun *et al*., 2008; Agarwal *et al.,* 2005; Duntas *et al.,* 2002 and Duntas *et al.,* 2012).

5.6 Conclusions

The present study reports the following findings in Gujarat primarily infertile females with subclinical hypothyroidism:

[1] An increase in the OS biomarkers with increased MDA levels and decreased SOD and CAT activities along with a positive correlation between TSH and MDA as well as between

SOD and CAT levels and a negative correlation between TSH and SOD and CAT levels and between MDA and SOD and CAT levels.

[2] An altered lipid profile with an increase in TC, TG and LDL-C levels along with a decrease in HDL-C levels along with positive correlation between TSH and TC, TG and LDL-C, as well as between MDA and TC, TG and LDL-C levels and a negative correlation between TSH and HDL-C and between MDA and HDL-C levels.

In conclusion, we suggest that primary infertile females should be evaluated for their thyroid status and females with SCH should not be overlooked but monitored for markers of oxidative stress and alteration in lipid profile. If an alteration is found with subclinicalhypothyroidism the initial treatment could be an advice to alleviate stress and multivitamin supplementations, thus avoiding direct use of steroids to target reproductive system to correct the female infertility.

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