Synopsis of the thesis on

Subclinical Hypothyroidism as a cause of Infertility in Female population

Submitted to

The Maharaja Sayajirao University of Baroda

The Department of Biochemistry,
The Maharaja Sayajirao University of Baroda

For the degree of

Ph. D. (Doctor of Philosophy) in Biochemistry

By

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Introduction:

Infertility apart from being a major psychological burden is also considered as a social stigma in Indian culture. Proper evaluation of this disorder should involve a multidimensional diagnostic approach. Amongst different causes of infertility the female factor is the most common. Female infertility can be due to the factors among these all thyroid gland related endocrine disorders are seems to be the most common**.** Thyroid hormones (THs) regulate several essential physiological processes, one of which is reproduction. Apart from overt hypothyroidism, Sub Clinical Hypothyroidism (SCH) is also seen and acts as a silent perpetrator of infertility. SCH is defined as elevated serum TSH in the presence of normal free thyroxin (fT4) levels. Large cohort studies have shown 1-4% prevalence of SCH in infertility with associated ovarian dysfunction. However, Indian studies correlating SCH and infertility are few and inadequate. Female infertility could also be due to Auto immune thyroid disorder (AITD). An increased prevalence of AITD has been found among women attending referral infertility clinics. The role of AITD in causing infertility in Indian population is not been studied. Though the potential consequences of SCH can lead to infertility standardized treatment to correct these are uncommon. (Acharya N; 2011, Krassas G; 2010, Akhter N; 2009, Poppe K; 2002).

One of the precipating factors of thyroid disorder are the environmental pollutants. Endocrine disrupting chemicals (EDCs) disturbing the thyroid system is termed as Thyroid disruptors. Thyroid disruptors such as PCBs may interfere with thyroid homeostasis through several mechanisms of actions in turn leading to subclinical hypothyroidism (SCH). Treatment of infertility is usually done by targeting the reproductive system directly, instead of looking to other possible factors (e.g. SCH) responsible for infertility. And the medications given to alter the levels of reproductive hormones have serious repercussions (Boas M; 2012, Zoeller; RT; 2005).

Genetic variations due to SNPs are frequent in PDE8B gene. One of the candidate gene for the increase in TSH is PDE8B (PhoshpoDiEstrase8B) gene. Many SNPs have been identified for higher TSH levels and a positive correlation between them and various diseases have been established. However such a link with infertility is lacking both for world and Indian populations. (Anna G; 2012, Beverley M; 2009).

The study have proposed a cross talk between HPT and HPO axis showing the possible involvement of three different factors, i.e.an immunological factor, an environmental factor and a genetic factor giving signals to increase TSH levels when thyroid hormones are still within the normal reference range. To address this question we have hypothesized either an independent or a cumulative involvement of all three factors causing SCH and as a consequence female infertility in the population. This study has evaluated the selected infertile female population for the prevalence of thyroid dysfunction and female infertility as a result of presence of endocrine disrupters, by assaying the levels of thyroid hormones, by estimating the alteration in the reproductive hormones, oxidative stress, lipid profile and looking for AITD and establishing a correlation between known SNPs for hypothyroidism

with infertility. This could then be used to treat infertility with greater success and less side effects without disturbing the reproductive system.

PROPOSED OBJECTIVES:

1. To screen local infertile female population of Vadodara and its suburbs for the prevalence of SCH related infertility.
   (A): To evaluate the Thyroid Function test to be performed are;
      (i) TSH estimation.
      (ii) fT_3 estimation
      (iii) fT_4 estimation.
      (iv) Anti TPO-Ab estimation.
   (B): To determine the levels of reproductive hormones:
      (i) Prolactine (PRL) estimation.
      (ii) Luteinizing Hormone (LH) estimation.
      (iii) Follicle Stimulating Hormone (FSH) estimation.
2. To estimate Oxidative stress and to analyze the lipid profile:
   (A): To analyze the changes in oxidative stress parameters to be assayed are
      (i) Malondialdehyde (MDA).
      (ii) Catalase (CAT) enzyme.
      (iii) Superoxide dismutase (SOD) enzyme.
   (B): To assay Lipid profile:
      (i) Estimation of Total Cholesterol (TC).
      (ii) Estimation of Triglyceride (TG).
      (iii) Estimation of HDL-Cholesterol (HDL-C).
      (iv) Estimation of LDL-Cholesterol 9(LDL-C).
3. To estimate the levels of PCBs levels in blood of the selected population.
   Cleanup, extraction and detection of PCBs by Gas Chromatography.
4. To correlate SNPs associated with hypothyroidism & prevalence of infertility.
   Amplification of candidate gene PDE8B for three SNPs:
      (i) rs4704397. (ii) rs6885099.

Study Subjects:

The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Controls are selected from general populations from clinics, by arranging camps and from field work in different areas of the city.

Patients are taken from Ghanshyam Clinic, Vadodara of Late Dr. Mahesh Pandya.

Age Group: 20 – 40 yrs (Female).

Control: - Non pregnant, age matched patients, parous, without any history of reproductive problem.

Patient:-

Inclusion criteria:

a. Primary Infertility diagnosis.
b. Duration: More than 1yr.of unprotected intercourse without pregnancy.

Exclusion criteria:

a. Male Factor Infertility.
b. Any Tubal Anomaly.
c. Congenital or Urogenital Tract Anomaly.
d. History of Thyroid disease/Medication/Surgery.

Objective 1:

To Screen the local infertile female population of Vadodara and its suburbs for the prevalence of SCH related infertility.

As the data on the prevalence of SCH and female infertility for the selected population is nil, the aim of the first objective was to find out the prevalence rate for the same. Thus levels of PRL, LH and FSH were estimated along with TSH estimation.

Strategy for screening SCH patients:

Counseling and screening of infertile patients for SCH was performed after filling prepared consent form. Total 700 subjects including both control and infertile patients were counselled for this research study. From this 70 control subjects and 230 infertile patients were fulfilled the selection criteria and were enrolled for the study.12 hr fasting venous Blood on day 3 of menstrual cycle was collected. Serum separation for Thyroid Function Test (TFT).TFT was carried out to check levels of hormones and was compared with normal range.

Thyroid Function Test:

Thyroid function test was performed by the estimation of TSH, fT_4, fT_3 and anti TPO-Abs in the blood (serum) of patients (n=73) and control (n=52) subjects by Chemiluminescence assay on Minividas. Patients with TSH value between 3.5 to 10μIU/ml were considered as subclinical hypothyroid. Controls with value higher than 3.5μl were not included in this study.

Estimation of Anti TPO antibodies:

Estimation of Anti TPO antibodies in patients (n=45) and control (n=40) was done by kit based ELISA method. Anti TPO-Ab were determined from serum of patients. TPO values smaller than 70 IU/ml was judged as negative (Cut off = 70 IU/ml).

Estimation of Reproductive Hormones:

Luetinizing hormone (LH) levels in patients (n=55) and control (n=55), follical stimulating hormone (FSH) levels in patients (n=57) and control (n=55) as well as prolactin (PRL) levels in patients (n=50) and control (n=50) were estimated using ELISA kits in the serum. Hormone levels of patients and control were then compared with normal values. The estimation of reproductive hormones was done on the third day of menstruation as the levels of these hormones are at their base line during 3^{rd} to 5^{th} day of menstruation cycle and thus can be compared with the normal levels of healthy subjects.

As shown in the table below the study reports for the first time a high prevalence rate of 31.73% of SCH in infertile female population as compared to control group which is an

alarming situation. A Mean TSH values were reported ~5.4 µIU/ml which falls under the reference value for the SCH (i.e. TSH levels 4 – 10 µIU/ml) with p value <0.0001 and normal fT_4 levels in SCH patients as compared to control group having TSH values of 1.9 µIU/ml which falls under the reference range for the normal euthyroid condition (TSH levels 0.35-4.00 µIU/ml). The prevalence of anti TPO-Abs was ~27% in patients as compared to control which was 10%.

Table: I-Total subjects screened for the study:

Subjects	**Control** **N (%)**	**Infertile subjects** **N (%)**
Total	70	230
Euthyroid	52 (74.28)	132 (57.39)
Subclinical Hypothyroid(SCH)	5 (7.14)	**73 (31.73)**
Others	12 (17.14)	25 (10.86)

Mean PRL levels in SCH patients were 26.05 ng /ml with p value <0.0001 which was almost double the values of mean PRL levels of control group which was 13.59 ng /ml having values within the normal reference levels (i.e. 1.2 – 19.5 ng /ml). As reported in the literature we also reports an increase in the PRL levels along with the increase in the TSH levels in SCH infertile patients as compared to healthy euthyroid control group. Mean LH levels for control was 11.51 m IU/ml showing normal reference values (i.e. 4.11 – 20.01 m IU/ml) where as SCH infertile patients were having the mean LH levels 7.331 m IU/ml with p value ≤ 0.01 showing the same trend of decrease in LH levels in SCH patients as in literature stating towards the alteration in the reproductive pathway in SCH condition. Mean FSH levels were also decreased in SCH infertile patients with the value 9.58 m IU/ml with p value ≤ 0.05 as compared to control subjects which was 12.54 m IU/ml falling under normal reference values (i.e. 8 – 22 m IU/ml). Our study also reports the same finding for FSH levels for the selected population was similar as shown in the literature for the other populations.

Objective 2:

To estimate Oxidative stress and to analyze the lipid profile:

Superoxide dismutase 1 (SOD1) estimation: 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. One unit of SOD1 activity being defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxdation

Catalase estimation: The estimation of catalase activity in erythrocytes was carried out by the method of Hugo Aebi (1984). Hemolysate was prepared with haemoglobin concentration of about 5g Hb/dl. Unit: µmoles of H_2O_2 utilized/g Hb/sec

Lipid Peroxidation (LPO):The estimation of LPO level in erythrocytes was carried out by the method of Beuge and Aust (1978).Calculation was done according to the slope calculated from the standard graph of TMP. Units: nmoles of MDA formed/gHb

The study reports an increased level of oxidative stress i.e. MDA levels in SCH infertile patients (n=56) with a p value ≤ 0.01 as compared to control (n=56) subjects. The enzymes which take part in control of oxidative stress like superoxide dismutase (SOD) and catalase (CAT) activities were measured for further quantification of the extent of oxidative stress levels. The activity of the enzyme SOD was found to be decreased in the SCH patients (n=40) with the p value of ≤ 0.01 as compared to control (n=40) subjects. CAT enzyme levels were also decreased in the patients (n=55) with the p value of ≤ 0.001 as against the control (n=52) subjects suggesting an association of SCH and oxidative stress.

Lipid profile alteration in SCH infertile patients:

Estimation of total cholesterol (TC), HDL-cholesterol, Triglycerides (TG) LDL-cholesterol (Enzymatic method) was done by using commercially available kits.

Total cholesterol (TC) concentration was found to be increased in SCH patients (n=52) having p value ≤ 0.05 as compared to control group (n=50). Triglycerides (TG) levels were also going high in SCH group (n=52) having p value ≤ 0.01 as compared to control subjects (n=31). LDL concentration was also found to be showing the trend of increasing concentration in SCH patients (n=55) with p value ≤ 0.01 as against the control subjects (n=50). HDL cholesterol levels were high in control group (n=51) as compared to SCH infertile patients (n=52) with p value ≤ 0.01. The increased LDL concentration in patients was the only one which was above normal reference range, where as TC, TG, and HDL concentration were increasing but within the normal reference levels.

Objective 3:

To estimate the levels of PCBs in blood of the selected population.

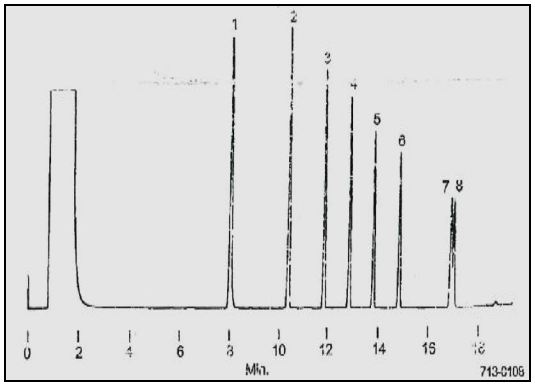
Strategy for PCB detection

Strategy for PCB detection was followed by the reference method as given by Roya Rozati *et al*., 2002. The experimental set up was designed in the lab for carrying out the step for drying the sample with nitrogen gas. The sample was injection into the GC unit and detection was done by Mass Spectroscopy.

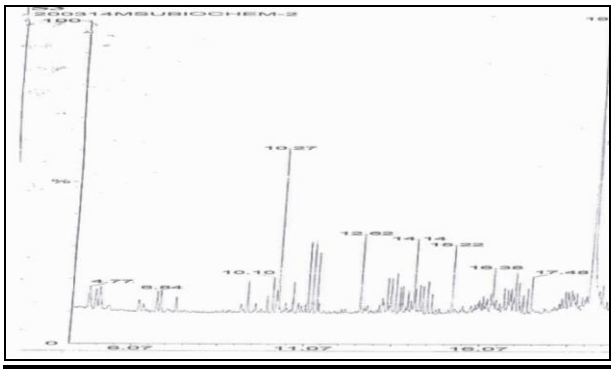
Presence of PCBs was detected in the blood of study subjects (n=5) indicting the involvement of these compounds in causing SCH and finally infertility in female population.

The peaks of samples were compared with the PCBs standard's peaks for the confirmation of results obtained are shown in the figure below.

PCBs standard peaks:



Patient Sample peacks for PCBs:



The presence of PCBs in the blood samples of the study population might be disturbing the thyroid system either by mimicking the thyroid hormones, limiting the transport of thyroid hormones to the brain or by decreasing the thyroid hormones or any other unknown mechanism and thus can cause an increase in TSH levels which can then be result into infertility in female population.

Objective 4:

To correlate SNPs associated with hypothyroidism and prevalence of infertility in infertile SCH patients and controls.

Genotyping of *PDE8B/5* (rs4704397) A/G and *PDE8B/5*(rs6885099) G/A intron variants in SCH patients and controls:

PDE8B is an important gene involved in controlling serum TSH concentration in normal individuals. As no literature were available correlating polymorphism, SCH and infertility. The aim of the present study was to look for the same in the local population.

Genomic DNA isolation:

Blood was collected from patients and controls after written consent was obtained. Exclusion and inclusion criteria to suit our study were used in recruiting patients and controls.

Genomic DNA was isolated from venous blood of patients and controls.

a) Genotyping of *PDE8B* rs4704397 A/G by PCR-RFLP:

The aim of the present study was to investigate the association between *PDE8B* Intron variant (A/G) polymorphism (rs4704397) and to perform possible genotype phenotype correlation in SCH infertile patients and healthy controls from local population. 55 SCH infertile patients and 66 controls were enrolled; genotyping was performed using polymerase chain reaction (PCR)-Restriction Fragment Length Polymorphism-RFLP method. *BslI* restriction enzyme was used for amplicon digestion. *BslI* cleaves if A allele is present in the amplicon and undigested product remains if G allele is present Our results showed that genotype and allele frequencies of rs4704397 A/G was not consistent with Hardy-Weinberg expectations in both patient and control population (p<0.0001; p=0.0002 respectively), suggesting the significant association of allele with SCH susceptibility.

b) Genotyping of *PDE8B* rs6885099 G/A by ARMS-PCR:

The aim of the present study was to investigate the association between *PDE8B* intron variant (G/A) polymorphism (rs6885099) in SCH infertile patients and healthy controls from local population to understand a possible genotype phenotype correlation.

Genotyping of *PDE8B* rs6885099 (G/A) SNP was performed by Amplification-refractory mutation system (ARMS) PCR method in 59 SCH infertile patients and 76 controls. Where, human growth hormone (HGH) gene fragment was used as PCR positive control. Our results showed that genotype and allele frequencies of rs6885099 G/A (*p=0.111*; *p*=0.0820 respectively), were not significantly different between SCH infertile patients and unaffected controls suggesting there is no association of allele with SCH susceptibility indicating no risk for the disease.

Linkage Disequilibrium (LD) Analysis:

LD analysis revealed that both SNPs studied are in low LD association (rs4704397 A/G: rs6885099 G/A; (D'= 0.060).

Haplotype analysis of *PDE8B/5* (rs4704397) A/G: *PDE8B/5*(rs6885099) G/A single nucleotide polymorphisms in SCH infertile patients against controls in the female population:

The haplotype GG (*p*=0.002) and GA (*p*=0.087) were found to be less frequent in SCH infertile patients, suggesting its crucial role in disease protection. Whereas, AG (*p*=0.043) and AA (*p*=0.0001) haplotypes were more found to be significantly associated with patients, suggesting their importance in SCH susceptibility.

Genotype-Phenotype Correlation Analysis:

TSH levels were analyzed with respect to *PDE8B* rs4704397 A/G and rs6885099 G/A polymorphisms in patients with SCH by applying unpaired t-test. TSH levels were significantly higher in patients carrying GA genotype as compared to those carrying AA genotype of *PDE8B* rs4704397 A/G polymorphism. However, no significant difference in TSH levels was observed with respect to PDE8B rs6885099 G/A polymorphism.

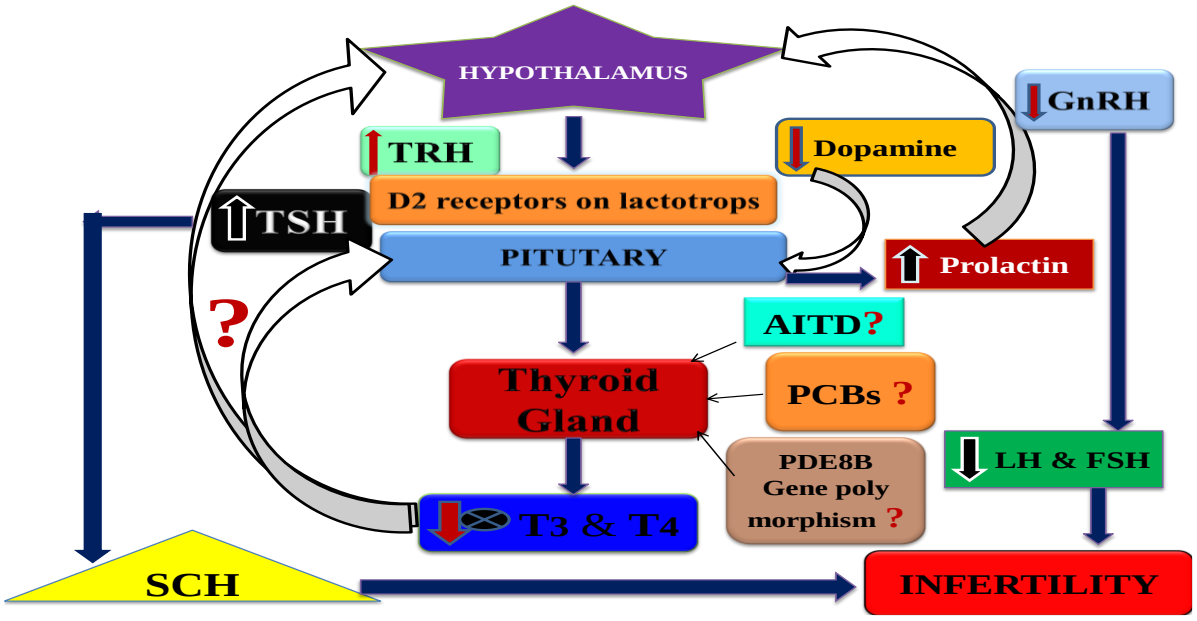
The present study demonstrates a significant association of *PDE8B* rs4704397 A/G polymorphism genotype as well as allele frequency between SCH patients and controls. GA genotype showed increased TSH levels as compared to AA genotype for *PDE8B* rs4704397 A/G polymorphism, suggesting it could act as a risk factor for SCH in female population. Larger studies with different ethnicities are required to find out the impact of this polymorphism as a risk factor for SCH and infertility. No association was observed for *PDE8B* rs6885099 G/A polymorphism genotype as well as allele frequency between SCH patients and controls. Both the SNPs exhibited low LD association.

Conclusion:

There is a close relationship between the Hypothalamic-Pituitary-Thyroid axis (HPT) and the Hypothalamic-Pituitary Ovarian axis (HPO). In hypothyroid condition when the levels of thyroid hormones decrease it gives signal to increase TSH which in turn can lead to female infertility. In case of SCH condition even though the thyroid hormone levels are within the normal range, TSH levels increases above the upper limit of the normal reference range, in this condition a question arises if not the thyroid hormones, then what is that gives the signal to increase the TSH levels which in turn can be a cause of female infertility. We proposed the following crosstalk as an explanation to this phenomenon. The coming figure shows the

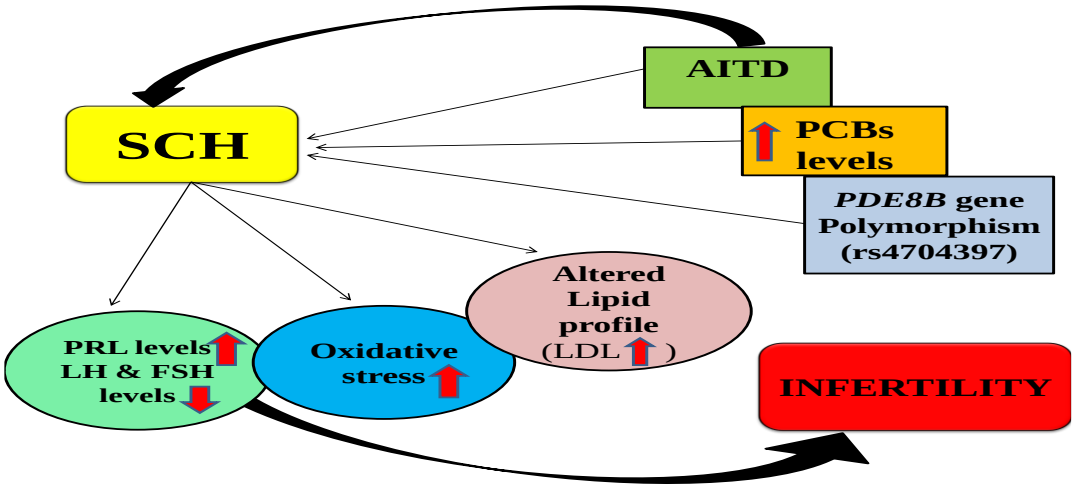
proposed a cross talk between HPT and HPO axis, which demonstrates the possible involvement of three different factors, i.e.an immunological factor, an environmental factor and a genetic factor giving signals to increase TSH levels when thyroid hormones are still within the normal reference range. The study proposes either an independent or a cumulative involvement of all three factors causing SCH and as a consequence female infertility in the population. In the present study we have focused on the nearly an untouched area correlating SCH and female infertility in a local population. Concrete data is unavailable in the field of SCH and female infertility as far the selected population is concerned and even the Indian studies correlating SCH and infertility are few and inadequate. Though the potential consequences of SCH can lead to infertility standardized treatment to correct these are uncommon.

Proposed Cross talk between HPT & HPO axis:



Hence in this primary study we are reporting for the first time an involvement of autoimmune, endocrine and environmental factors in causing the subclinical hypothyroidism leading to female infertility. The study was designed to screen the infertile female population for the prevalence of thyroid dysfunction and female infertility as a result of presence of endocrine disrupters, by assaying the levels of thyroid hormones, by estimating the alteration in the reproductive hormones, oxidative stress, lipid profile and looking for AITD and establishing a correlation between known SNPs for hypothyroidism with infertility. The fruitful outcomes of the proposed study increases the state of knowledge in the subject with respect to the correlation of SCH and all of the above mentioned factors with infertility as far as the Indian population is concerned.

Summary of results reported in this study:



As shown in the diagrammatic presentation of the study we found the presence of anti TPO-Ab in the SCH infertile patients which is due to Auto Immune Thyroid Disease (AITD) causes
infertility. We also report the increase in PRL hormone and a decrease in LH and FSH hormone levels in the study group along with an increase in oxidative stress as well as an alteration in the lipid profile for the same. We also found the presence of PCBs in the blood of the population indicating the involvement of thyroid disruptors responsible for infertility. Genetic variations due to SNPs are frequent in PDE8B gene. Many SNPs have been identified for higher TSH levels and a positive correlation between them and various diseases have been established. However such a link with infertility is lacking both for world and Indian populations but our study is the first to report this link as we found the association of rs4704397 polymorphism with TSH levels and thus infertility.

Treatment of infertility is usually done by targeting the reproductive system directly, instead of looking to other possible factors such as SCH responsible for infertility. And even the medications given to alter the levels of reproductive hormones have serious repercussions on the female with long term implications. Hence we are proposing the assessment of thyroid function test as a first check up in fertility centers. The doctors and medical practitioners should go for this as a initial diagnosis step instead of prescribing the wrong medications. This could then be used to treat infertility with greater success and less side effects without disturbing the reproductive system.

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POSTER PRESENTATIONS

1. Title : Subclinical hypothyroidism and female infertility. At National Symposium on Changing Environment and lifestyle: Impact on Reproductive Health. November 19-20, 2013 at AIIMS, New Delhi.

2. Title : Subclinical hypothyroidism and female infertility at International Conference on Reproductive Health with Emphasis on Occupational, Environmental and Lifestyle Factors & 26th Annual Meeting of the Indian Society for the Study of Reproduction & Fertility :February 18-20, 2016.

PARTICIPATION IN SYMPOSIA & SEMINARS

1. Two days National Symposium on "Omics.. to Structural Basis of Diseases" from September 30 to October 1, 2016 at Department of Biochemistry, Faculty of Science, The M. S. University of Baroda, Sayajigunj, Vadodara, Gujarat.

2. Three days "International Conference on Reproductive Health & 26th Annual Meeting of Indian Society for the study of Reproduction and Fertility", on February 18-20, 2016 organized by National Institute of Occupational Health (NIOH; ICMR), Ahmedabad.

3. One day workshop organized by The Department of Science & Technology, Government of India on 17th July, 2015 at the University of Chennai, Chennai.

4. Participated in UGC-DRS Sponsored Two Day Seminar on "Molecular Basis Of Diseases" under 'Diamond Jubilee Celebrations' held at The Department of Biochemistry, The M.S. University of Baroda, Vadodara in Association with Alumni Association of Biochemistry Department (ABCD), from 1st to 2nd August, 2014.

5. Two days International Conference on ''Integrating Basic And Translational Research In Modern Biology" held on 27th & 28th December 2013 at the department of Microbiology, Faculty of Science, The M. S. University of Baroda, Sayajigunj, Vadodara, Gujarat ;on the occasion 50th Anniversary of the department.

6. National Symposium on Changing Environment and lifestyle: Impact on Reproductive Health. November, 19-20, 2013 at AIIMS, New Delhi.

Acknowledgement

Awarded fellowship under women scientist scheme to carry out the research work as a Principal Investigator by DST; G.O.I.; WOS-A.

Manuscripts communicated

Nil

Note: Three manuscripts are in the process for communication.

I) Subclinical hypothyroidism and female infertility-An Indian study

II) *PDE8β* gene polymorphism in infertile SCH female population.

III) Role of PCBs in subclinical hypothyroid infertile female population.

Date: 29/12/2017

Signature of the Candidate
(Mansuri Tabassum)

Guide
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