CHAPTER 4

MATERIALS AND METHODS

4.1 Collection of serum sample

Total of 48 blood samples were collected from out-patients with Gestational diabetes mellitus, as well as control samples visiting at several reputed Maternity Hospitals in Chennai, TAMILNADU. The blood was centrifuged after collection to obtain serum. The serum samples were analysed for the levels of lipid profile, minerals, enzymic and non enzymic antioxidants, hematology, insulin hormone, insulin receptor and LDL structural changes.

4.2 Determination of Lipids

Serum sample was added to dissolve the organic phase of chloroform/methanol (1:1) mixture. To the mixture, 10% methanol was added and incubated at 80°C for 90 minutes and cooled for 5-10 minutes and then distilled water and hexane were added and vortex-mixed. Two layers were formed from which the organic upper layer was transferred into a 2ml GC vial avoiding any particulate matter and evaporate to dryness in the fume hood overnight. The aqueous lower layer was discarded and the solution was reconstituted in hexane prior to GC-FID analysis. After sample preparation the processed samples were subjected to lipid analysis at 300°C, Triglycerides at 380°C and cholesterol at 260°C. Statistical analysis of the present data was performed by using ANOVA.

4.3 Determination of Minerals

Sample preparation.

To 1.0 ml of serum sample was added with 1.0ml of nitric acid, 1.0ml of perchloric acid, and heated in a boiling sand bath upto the sample turns into colourless solution. The solution was made upto 10 in 100ml dilution.

After sample preparation, the processed samples were subjected to minerals analysis in Atomic Absorption Spectrophotometer equipped with a burner for air/acetylene flame, hallow cathode lamps for Copper, Zinc, Magnesium, Iron and except calcium with addition of nitrous gas with Deuterium lamp background.
4.4 Determination of Antioxidant status

The serum SOD (Kakkar et al., 1974), Catalase (Sinha, 1972), GPx (Rotruck et al., 1973) and GST (Habig et al., 1974) were analysed spectrophotometrically as enzymic antioxidants.

The levels of non enzymic antioxidants such as vitamin C (Jacob, 1990) was measured spectrophotometrically and vitamin E was measured by Bipyridyl method (Baker et al., 1980). Reduced glutathione was measured spectrophotometrically (Moron et al., 1979). The levels of prooxidants such as LHP was quantified using 1.8ml of Fox reagent and analysed spectrophotometrically (Miyazawa, 1980), MDA by Draper and Haley (1980) method and NO by Griess (1879) modified by Fiddla (1977) method.

4.5 Haematology

Complete blood count

The blood samples obtained from control and test subjects were analyzed for the levels of Hb, RBC, ESR, MCV, MCHC, mixed with EDTA by subjected the blood sample in a cuvette analyzer. “ABX micros 60”.

Ten µl of serum was added to 1ml of cholesterol reagent and mixed well. The mixture was allowed to stand for 10min incubation and the sample was analyzed using “ABX micros 60”.

Determination of HbA1c

The blood samples obtained from control and test subjects were analyzed for the level of HbA1c in “Micromat II HbA1c” which work on the principle of Affinity Chromatography. The glycated Hb fraction is separated from non-glycated Hb fraction and percentage of HbA1c in the sample was determined.

To ten µl of blood lysing agent was added. The mixture was allowed to stand for 60 seconds incubation. After lysed sample is added into the tube, with the tube rotate one time. In 50sec after reaction buffer was added for washing purpose, and another 40sec for filtration again one time rotate, then 3rd buffer was added, then 80sec for filtration. The samples were analysed after 80 seconds.
4.6 Determination of Insulin Hormone

The blood samples obtained from control and test subjects were analyzed for the level of Insulin, which work on the principle of Relative light unit and the percentage of Insulin in the sample was determined.

To two µl serum sample was added into a light reaction vessels. To this 50µl insulin trace reagent was added in the vessels and the plate was stirred for 60sec. And the vessels were allowed to incubation at 1hr at room temperature. It was washed for five times using 350µl of buffer. A total of Fifty µl of A&B signal reagent were added, and mixture was allowed to stand for 2-5 min incubation.

4.7 Study of gene variation of Insulin Receptor

To five milliliters of blood was diluted with a 2x Guanidine thiocyanate solution to final concentration, homogenized, and precipitated with Lithiumchloride as described (Ullrich et al., 1977) for cDNA Synthesis and PCR. Five to ten g of total RNA was used for cDNA synthesis. The cDNA was specifically primed with an oligonucleotide spanning nucleotides 2858-2879 of the human insulin receptor (Ullrich et al., 1985).

The strand of cDNA synthesis was performed with 50 mM KCl, 10 mM Tris-HCl, (pH 8.3), 4 mM MgCl2,1 mM dNTPs, 10 µg of bovine serum albumin per ml, 50 pmol of primer. The reaction product was directly subjected to 40 cycles of PCR amplification without further purification.

PCR was performed as described earlier using oligonucleotides flanking the 12-amino acid insertion site. The oligonucleotides used represented nucleotides 2136-2257 and 2327-2348 of the human insulin receptor sequence (Ullrich et al., 1985) and gave rise to specific fragments of 112 (HIR-A) and 148 (HIR-B) base pairs (bp), respectively. PCR products were analyzed after Electrophoresis in 5% Polyacrylamide gels.

4.8 Study of LDL structure with FTIR

The samples then ground in an agate mortar and pestle in order to obtain powdered form of the tissue. The powder was mixed with completely dried potassium bromide and then the mixture was subjected to a pressure of 5 X 10⁶ Pa in an evacuated die to produce a pellet for use in a FTIR spectrometer installed at Sophisticated Analytical Instrumentation facility, IIT Madras. Pellets were scanned at room temperature (25±1°C) in the 4000 – 400 cm⁻¹ spectra range. The intensity of the
absorption bands directly relate to the concentration of the corresponding functional groups.

Figure 4.1 FTIR Instrument