MATERIAL AND METHODS

PROCUREMENT & MAINTENANCE OF ANIMALS:

Male and female albino rats of Charles foster strain weighing around 200 – 250 grams were obtained from Sun Pharmaceuticals Ltd. (Baroda) and were maintained in a photo schedule of 8:16 in a well ventilated, temperature controlled room in the animal house of Sarabhai Research Center (Baroda). After acclimatization the animals were bred and male pups from the same litter size of the mothers were used for experimental purpose. The animals were given pure drinking water and were fed *ad libitum* with rat feed obtained from Pranav Agro Industries (Baroda).

EXPERIMENTAL SET UP:

Male pups were divided into various groups and treatment schedules. Six pups were used per treatment group.

- **CONTROL (C):** Rat neonates were injected with 0.9% saline intraperitoneally from day I to day 21 at 16:00 hrs.
- **HYPERMELATONEMIC (M):** Rat neonates were injected with melatonin (N-acetyl, 5 methoxy tryptamine) intraperitoneally from day 1 to day 21 in a graded dose of 200 µg (day 1 to day 7), 400 µg (day 8 to day 14) and 600 µg (day 15 to day 21) per animal per day at 16·00 hrs.

- **CONTROL NON-ALLOXANISED:** Rat neonates were injected with 0.9% saline intraperitonially from day 1 to day 21 at 16:00 Hrs. and a single injection of vehicle was given on the 22nd day. Note: The data of this group showed no significant difference with that of the control group, the data represented is that of control group only (**C**)
- **CONTROL ALLOXANISED (CA100):** Rat neonates were injected with 0.9% saline intraperitoneally from day 1 to day 21 at 16:00 hrs and a single injection of alloxan (2, 4, 5, 6-tetraoxypyrimidine; 5, 6-dioxyuracil) was given on the 22nd day intraperitoneally with a dose of 100 mg/kg body weight
- **HYPERMELATONEMIC ALLOXANISED (MA100):** Rat neonates were injected with melatonin (N-acetyl, 5 methoxy tryptamine) intraperitoneally from day 1 to day 21 in a graded dose of 200 µg (day 1 to day 7), 400 µg (day 8 to day 14) and 600 µg (day 15 to day 21) per animal per day at 16.00 hrs and a single injection of alloxan (2, 4, 5, 6-tetraoxypyrimidine; 5, 6-dioxyuracil) was given intraperitoneally on the 22nd day with a dose of 100 mg/kg body weight.
- **HYPERMELATONEMIC ALLOXANISED (MA150):** Rat neonates were injected with melatonin (N-acetyl, 5 methoxy tryptamine) intraperitoneally from day 1 to day 21 in a graded dose of 200 µg (day 1 to day 7), 400 µg (day 8 to day 14) and 600 µg (day 15 to day 21) per animal per day at 16:00 hrs and a single injection of alloxan (2, 4, 5, 6-tetraoxypyrimidine; 5, 6-

dioxyuracil) was given intraperitoneally on the 22nd day with a dose of 150 mg/kg body weight.

After respective treatment, the overnight fasted animals were given mild anesthesia and sacrificed on 22nd(weaning stage), 45th (pubertal stage) and 60th (adult stage) days Blood was collected from the jugular vein and allowed to clot for an hour. It was then centrifuged at 3 - 4°C to obtain clear serum which was used for estimating glucose, insulin, cholesterol, triglyceride, total lipids, phospholipids and free fatty acids. The animals were weighed daily from birth till the day of sacrifice. The viscera was cut open and liver, pancreas, spleen, kidneys, adrenals, testes, adipose tissue and femoralis muscles were excised out. The liver, femoralis muscle and adipose tissue were blotted free of blood, weighed and used for making homogenate. The pancreas, spleen, kidneys, adrenals and testes were blotted free of blood and weighed and were then fixed in Bouins fluid to be processed for histology.

PREPARATION OF CHEMICALS:

- **Saline Solution:** 900 mg of sodium chloride (obtained from SRL chemicals) was dissolved in 100 ml of redistilled water.
- **Melatonin Solution:**
  - **Solution A:** 1.2 mg of melatonin (obtained from Sigma Chemical Co. U.S.A) was weighed and dissolved in few drops of 70% ethanol and diluted to 0.6 ml with 0.9% saline to get the concentration of 200 µg/0.1 ml to be injected per animal from day 1 to day 7.

- **Solution B:** 2.4 mg of melatonin (obtained from Sigma Chemical Co. U.S.A.) was weighed and dissolved in few drops of 70% ethanol and diluted to 0.6 ml with 0.9% saline to get the concentration of 400 µg/0 1 ml to be injected per animal from day 8 to day 14.
- **Solution C:** 3.6 mg of melatonin (obtained from Sigma Chemical Co. U.S.A.) was weighed and dissolved in few drops of 70% ethanol and diluted to 0.6 ml with 0 9% saline to get the concentration of 600 µg/0.1 ml to be injected per animal from day 15 to day 21.

Alloxan Solution: Alloxan (obtained from SRL Chemicals) was dissolved in appropriate volume of redistilled water to get concentrations of 100 mg/kg and 150 mg/kg body weight. The required concentration was adjusted in 0.1 ml to be injected.

ANALYTICAL METHODS:

HISTOLOGY: Pancreas was excised out from the animals sacrificed under mild anesthesia and was blotted free of blood and tissue fluids. After taking the weight the pancreas was fixed in Bouin's fluid. Blocks were prepared by following the procedure of dehydrating in ethanol grades, clearing the tissue in toluene and embedding in paraffin wax. Sections of 5µ were cut on a microtome and stained with Heamatoxylin – Eosin. The stained sections were mounted in DPX. The islet cell count was done by taking 4-5 serial sections from the pancreas of 4 different animals.

🕮 BIOCHEMICAL ASSAYS:

🗁 **Protein:** The protein content of the tissue extracts was estimated by the method of Lowry *et al*, (1951).

- 🗎 **Principle:** In alkaline solution, copper ions and protein molecules in the sample form a complex with the amino acids containing phenolic hydroxyl group, viz., (tyrosine and tryptophan) and react's with Folin-Ciocalteau reagent to give a blue color due to the reduction of phosphomolybdate. The intensity of the color is proportional to the concentration of proteins.
- 🗎 **Procedure:** In the sample tube, 0 1 ml of homogenate (20 mg/ml) was taken and diluted to 1 ml with redistilled water. In the standard tube, 0.1 ml of Bovine Serum Albumin (20µg/100 ml) was added and in the blank tube 1.0 ml of redistilled water was added. To all the three tubes 5.0 ml of alkaline copper reagent was added, mixed and allowed to stand for 10 minutes at room temperature. 0.5 ml of Folin-Ciocalteau phenol was then added to each tube and shaken well. The blue color developed was read at 660 nm after half an hour against a reagent blank in a spectrophotometer. The protein concentration is expressed as mg/100 mg of tissue.

🗁 **Glycogen:** The glycogen content of the tissue extracts was estimated by the method of Seifter *et al*, (1950).

- **Principle:** The tissue sample containing glycogen was digested with 30% potassium hydroxide, and then precipitated with ethanol. The precipitate was then treated with anthrone reagent and the glucose in the hydrolysate was determined colorimetrically as reducing sugar at 620 nm.
- **Procedure:** Pre-weighed pieces of tissue were digested with 2 ml of 30% potassium hydroxide for 20 minutes in a boiling water bath. The contents were cooled in an ice bath and 2.5 ml of 95% ethanol was added, thoroughly mixed and glycogen was precipitated by bringing the contents to boiling in a water bath. The supernatant was decanted and the tubes were allowed to drain on a filter paper for few minutes. The procedure was repeated to get complete precipitation of glycogen. The precipitates were dissolved in known amount of redistilled water and aliquots (1 ml) of different dilutions were taken as samples. 4 ml of anthrone reagent was added under ice cold conditions and the contents were allowed to cool. The test tubes were covered with glass marbles and heated for 4 minutes in a boiling water bath. The contents were cooled for half an hour and the color was read at 620 nm against a reagent blank on a spectrophotometer. The amount of glycogen is expressed as mg/100 mg of tissue.

- **LIPID:** The total lipid content of the tissue extracts was estimated by the method of Folch *et al*, (1957).
  - **Principle:** Lipids are soluble in some organic solvents. This property of specific solubility in non-polar solvents is utilized for extracting lipids from tissues. In biological materials the lipids are generally bound to proteins and they are therefore extracted with a mixture of methanol and chloroform. Inclusion of methanol in the extraction medium helps in breaking the bonds between the lipids and proteins.
  - **Procedure:** Pre-weighed tissue was crushed along with fine and clean sand particles in a test tube with a clean glass rod. 5 ml of chloroform-methanol mixture (2:1 v/v) and 2 ml of calcium chloride (0.2%) was added to the lysate and kept over night. The upper layer was removed with a syringe and the remaining solution was filtered through whatman filter paper in a graduated tube and the volume was made up to 4 ml with chloroform-methanol mixture. 2 ml of this content was added to pre-weighed lipid tubes, which were kept in an oven at 60°C for drying. After the tubes were dried completely, they were weighed again to get the difference in weight, which was taken as the amount of total lipid. The amount of total lipid is expressed as mg/100 mg of tissue.

- **CHOLESTEROL:** The total cholesterol content of the tissue extract was estimated by the method of Crawford (1958).
  - **Principle:** The method depends on the interaction of ferric chloride and concentrated sulphuric acid with cholesterol in glacial acetic acid. The resulting stable reddish purple color is compared photometrically with the color produced by treating a cholesterol standard at 540 nm.
  - **Procedure:** The remaining 2 ml solution from lipid extraction (see total lipids) was added to a test tube and dried similarly in oven at 60°C. After the tubes were dried completely, 3 ml of working ferric chloride reagent was added to the tubes and the tubes were heated in a water bath to boiling for 5 minutes. The tubes were cooled and 2 ml of concentrated sulphuric acid was added to each tube in ice bath and were allowed to cool for half an hour. In the blank tube, 3 ml of ferric chloride reagent and 2 ml of concentrated sulphuric acid were added in a similar manner. In the standard tube, 3 ml of cholesterol standard (75µg) and 2 ml of concentrated sulphuric acid were added. The color developed was read at 540 nm against the blank reagent in a spectrophotometer. The amount of cholesterol is expressed as mg/100 mg of tissue.

- **Glycogen Synthetase:** The enzyme was assayed by the method of Leloir and Goldemberg (1962).
  - **Principle:** In the presence of glycogen primer, glycogen synthetase forms the glucose of uridine diphosphoglucose (UDPG) and C_4 of the terminal glucose residue of glycogen liberating uridine diphosphate (UDP). The assay is based on the measurement of the amount of UDP formed from UDPG in the presence glycogen and glucose-6-phosphate. The UDP estimation is carried out by using a preparation of pyruvate kinase (PK) which catalyses the transfer of phosphate from phosphoenolpyruvate (PEP) to UDP. The pyruvate is estimated colorimetrically.
  - **Procedure:** The following were added to the 'test' and 'blank' tubes. 0.01 ml of glycogen (40 mg/ml), 0.01 ml of glycine buffer (0.75M - pH 8.5) and glucose-6-phosphate (0.05M) were added and mixed. 0.1 ml of homogenate (10 mg/ml) and 0.01 ml of UDPG (25µm/ml) were then added to the test alone. The reaction was started by the addition of UDPG and incubated at 37°C for 10 minutes. The blank tube contained all components except UDPG. The tubes were kept in boiling water bath for 1 minute. After incubation0.01 ml of UDPG was added in the blank tube and 0.025 ml of PEP (0.01M) and 0.025 ml of PK (8 IU) were added and the tubes were incubated at 37°C for

15 minutes. At the end of the incubation, the reaction was arrested by adding 0.15 ml of dinitrophenyl hydrazine (0.1%). The contents of the tube were mixed and allowed to stand for 5 minutes at room temperature and 0.2 ml of 10N sodium hydroxide was added for the maximum development of color. 1.1 ml of ethanol (95%) was then added and the tubes were centrifuged for 15 minutes at 3000 rpm. The optical density of the supernatant fluid was measured at 520 nm. Appropriate standards were run along with each assay. Protein was estimated according to the method of Lowry *et al*, (1951). The enzyme activity is expressed as µ moles of UDP formed/ mg protein/ 10 minutes.

- **Glycogen Phosphorylase:** The enzyme was assayed by the modified version of the method of Cori *et al*, (1943) by Cahill *et al*, (1957).
  - **Principle:** Glycogen phosphorylase cleaves the phosphoric bond of A – 1,4 linkages between glucose molecules, to yield glucose -1-phosphate. The property of synthesizing glycogen from glucose-1-phosphate by liberating inorganic phosphorus is made use of in this procedure.
  - **Procedure:** In the sample tube were added 0.2 ml of sodium citrate buffer (0.1M pH5.9), 0.3 ml of potassium fluoride (0.154M), 0.05 ml of glucose-1-phosphate (0.2M)

and homogenate (20 mg/ml). The incubation was carried out at 37°C for 30 minutes. The reaction was terminated by adding 1 ml of trichloroacetic acid (10%). In the control tubes all the contents were added along with trichloroacetic acid prior to incubation. The tubes were centrifuged at 3000 rpm for 30 minutes. The supernatant fluid was analyzed for phosphorus content according to the method of Fiske and Subbarow (1925) as described below.

To the supernatant fluid, 0.4 ml of sulphuric acid (10N) and 0.8 ml of ammonium molybdate (2.5%) were added and the tubes were allowed to stand for 10 minutes. After 10 minutes 0.4 ml of ANSA was added. After 10 minutes, the intensity of the blue color developed was read at 660 nm against the reagent blank. Appropriate standards were run along with each assay. The enzyme activity is expressed as μ moles of Pi released/ mg protein/ 15 minutes.

- **Glucose – 6 – Phosphatase:** The enzyme was assayed by the method of Harper (1963) using disodium salt of glucose-6-phosphate as substrate.
  - **Principle:** Glucose-6-phosphatase catalyses the reaction Glucose-6-phosphate + H_2O----------> glucose + phosphate The rate of the reaction is measured by the increase of inorganic phosphate with time.

- **Procedure:** In the sample tube were added homogenate (25 mg/ml in citrate buffer pH 6.5), 0.1 ml of glucose-6-phosphate (0.08M). The incubation was carried out at 37°C for 15 minutes. The reaction was terminated by adding 2 ml of trichloroacetic acid. In the control tubes, all the reagents were added as above except for glucose-6-phosphate. The tubes were centrifuged at 3000 rpm for 30 minutes The supernatant fluid was analyzed for phosphorus content according to the method of Fiske and Subbarow (1925) as described below.

  To the supernatant fluid, 5 ml of ammonium molybdate (2.5%) and 1 ml of ANSA were added. After 10 minutes, the intensity of the blue color developed was read at 660 nm against the reagent blank. Appropriate standards were run along with each assay. The enzyme activity is expressed as μ moles of Pi released/ mg protein/ 15 minutes.

- **Serum Glucose:** The serum glucose content was estimated by the method of Trinder (1969) using glucose kit (GOD/POD method) from Qualigens diagnostics a division of Glaxo India.

  - **Principle:** Glucose is oxidized by the enzyme glucose oxidase (GOD) to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of the enzyme peroxidase (POD) oxidizes phenol which combines with

4-aminoantipyrine to produce a red colored quinoneimine dye. The intensity of the color developed is proportional to glucose concentration in the sample.

- **Procedure:** In the sample tube, 10 µl of serum was added, in the blank tube 10 µl of redistilled water was added and in the standard tube 10 µl of glucose standard (100 mg/dl) was added. 1 ml of working enzyme reagent was added in all the tubes. The contents were thoroughly mixed and incubated at 37°C for 15 minutes. The optical density of the sample and the standard were measured against blank in a spectrophotometer at 505 nm. The amount of glucose is expressed as mg/dl of serum.

- **Serum Cholesterol:** The serum cholesterol content was estimated by the method of Allain *et al*, (1974) using cholesterol kit from Chema diagnostics Italy.

  - **Principle:** All cholesterol esters present in plasma are hydrolyzed quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, free cholesterol is then oxidized by cholesterol oxidase to cholesten-4-ene-3-one and hydrogen peroxide. The hydrogen peroxide reacts with p-chlorophenol and 4-aminoantipyrine in the presence of peroxidase to form a quinoneimine dye. The intensity of color formed is proportional to the cholesterol concentration and can be measured photometrically at 510 nm.

- **Procedure:** In the sample tube, 20 µl of serum was added, in the blank tube, 20 µl of redistilled water was added and in the standard tube, 20 µl of cholesterol standard (200 mg/dl) was added. 2 ml of working enzyme reagent was added in all the tubes. The contents were thoroughly mixed and incubated at 37°C for 5 minutes. The optical density of the sample and the standard were measured against blank in a spectrophotometer at 510 nm. The amount of cholesterol is expressed as mg/dl of serum.

- **Serum Triglyceride:** The serum triglyceride content was estimated by the method of Bucolo *et al*, (1973) using triglyceride kit from Chema diagnostics Italy.

  - **Principle:** Triglycerides are hydrolyzed by lipoprotein lipase to produce glycerol and free fatty acids. The glycerol participates in a series of coupled enzymatic reactions, in which glycerol kinase/ glycerol phosphate oxidase are involved and hydrogen peroxide is generated. The hydrogen peroxide reacts with p-chlorophenol and 4-aminoantipyrine in the presence of peroxidase to form a quinoeimine dye. The intensity of color formed is proportional to the triglyceride concentration and can be measured photometrically at 510 nm.

- **Procedure:** In the sample tube, 20 µl of serum was added, in the blank tube, 20 µl of redistilled water was added and in the standard tube, 20 µl of triglyceride standard (200 mg/dl) was added. 2 ml of working enzyme reagent was added in all the tubes. The contents were thoroughly mixed and incubated at 37°C for 5 minutes. The optical density of the sample and the standard were measured against blank in a spectrophotometer at 510 nm. The amount of triglyceride is expressed as mg/dl of serum.

- **Serum Phospholipid:** The serum phospholipid content was estimated by using phospholipid kit from Labkit Italy.

  - **Principle:** Phospholipids are hydrolyzed to produce choline and phosphatadic acid. In presence of oxygen, choline is then oxidized by choline oxidase to betaine and hydrogen peroxide. The hydrogen peroxide reacts with dichlorophenol and 4-aminoantipyrine in the presence of peroxidase to form a quinoeimine dye. The intensity of color formed is proportional to the phospholipid concentration and can be measured photometrically at 505 nm.

  - **Procedure:** In the sample tube 10 µl of serum was added and in the standard tube 10 µl of phospholipid standard (100 mg/dl) was added. 1 ml of working enzyme reagent was added in all the tubes. The blank

tube contained only 1 ml of working enzyme reagent. The contents were thoroughly mixed and incubated at 37°C for 10 minutes. The optical density of the sample and the standard were measured against blank in a spectrophotometer at 505 nm. The amount of phospholipid is expressed as mg/dl of serum.

- **Serum Total Lipids:** The serum total lipids content was estimated by using total lipids kit from Labkit Italy.
  - **Principle:** Total lipids form a colored complex when treated with phosphovainilline in sulphuric acid solution. The intensity of the color is proportional to the total lipids concentration and can be measured photometrically at 520 nm.
  - In the sample tube, 100 µl of serum was added and in the standard tube, 100 µl of total lipids standard, (750 mg/dl) was added. 2.5 ml of sulphuric acid was added in all the tubes. The blank tube contained only 2.5 ml of sulphuric acid. The contents were thoroughly mixed and incubated at 100°C for 10 minutes. 200 µl of supernatant was collected on cooling from each tube and 4.5 ml of phosphovainilline was added to each tube. The contents were thoroughly mixed and incubated at 37°C for 15 minutes. The optical density of the sample and the standard were measured against blank in a

spectrophotometer at 520 nm. The amount of total lipids is expressed as mg/dl of serum.

- **Serum Free Fatty Acid:** The free fatty acids (FFA) were estimated in serum by the method of Horn and Menahan. (1981).
  - **Principle:** FFA are first extracted with a chloroform:heptane:methanol mixture, with silicic acid being added to eliminate the interfering phospholipids. FFA is then complexed with copper by the addition of copper nitrate-triethanolamine reagent. This FFA-Cu complex serves to stabilize the chromophore that is formed by the addition of diethyl dithiocarbamate, which is used as the colour developing reagent.
  - **Procedure:** To about 200 mg of activated silicic acid was added 6.0 ml of CHM solvent (chloroform, heptane and methanol mixed in the ratio 250: 150: 7, v/v) and 0.2 ml of serum. The contents were thoroughly mixed, and then shaken intermittently for 30 minutes. The solution was then centrifuged and the supernatant was transferred to tubes containing 2 0 ml of copper nitrate-triethanolamine reagent. The contents were agitated on a mechanical shaker for 20 minutes, after which the mixture was separated into 2 phases by centrifugation. 2.0 ml of upper layer was mixed with 1.0 ml of colour reagent (0.1% solution of diethyl dithiocarbamate in n-

butanol). The yellow colour developed was read photometrically at 430 nm against a blank that had received water instead of the sample. A set of standards containing 10-100 µg of palmitic acid (in CHM solvent) were treated similarly to construct a calibration curve. The amount of free fatty acids in serum is expressed as mg/dl.

🕮 *IN VITRO* STUDIES:

🗁 **Glucose Uptake:** The glucose uptake by liver and muscle slices was done by the method of Patel and Ramachandran, (1992).

🗎 **Procedure:** Melatonin treated animals and controls were sacrificed on 22^{nd}, 45^{th} and 60^{th} day under mild anesthesia. Liver and femoralis muscle were excised out and pieces weighing about 30 – 50 mg were cut and washed in chilled Krebs-Ringer Bicarbonate medium (KRB). The slices were incubated in the KRB medium (previously gased with carbogen for 15 minutes) containing 0.2 mg of bovine serum albumin/ ml. The pH was adjusted to 7.4 and incubation carried out for 90 minutes at 37°C in a shaker incubator. The liver and muscle slices of all the experimental groups were incubated in media containing:

☞ 5 ml KRB medium + Glucose (2 mg/ ml) + Insulin (I) (1 IU/ ml).

- 5 ml KRB medium + Glucose + Acetylcholine chloride (**Ac**) (1.5 mg/ml).
- 5 ml KRB medium + Glucose + Insulin + Acetylcholine chloride.
- 5 ml KRB medium + Glucose + Melatonin (**M**) (1 mM/5ml).
- 5 ml KRB medium + Glucose + Melatonin + Insulin.
- 5 ml KRB medium + Glucose + Melatonin + Acetylcholine chloride.
- 5 ml KRB medium + Glucose + Melatonin + Insulin + Acetylcholine chloride.
- 5 ml KRB medium + Glucose + Luzindole (**L**) (1 µM/ ml).
- 5 ml KRB medium + Glucose + Luzindole + Insulin.
- 5 ml KRB medium + Glucose + Luzindole + Acetylcholine chloride.
- 5 ml KRB medium + Glucose + Luzindole + Insulin + Acetylcholine chloride.
- 5 ml KRB medium + Glucose

The glucose concentration in the medium at 10 minutes and 90 minutes of incubation was estimated using glucose kit (GOD/POD method) from Qualigens diagnostics a division of Glaxo India.

Determination of ^{14}C – Glucose Oxidation:

gastrocnemius and liver ^{14}C – glucose oxidation was

estimated by the method of Johnson and Turner, (1971) and Kraft and Johnson, (1972).

- **Procedure:** 10 mg of femoralis muscle or liver were weighed and placed in 2 ml ampulae containing the following: 170 µl medium (pH 7.4), 10 IU penicillin in 10 µl of medium and 0.5 µCi of ^{14}C – glucose (the specific activity of the substrate is 310 mCi/ mmole). After the tissue was aerated with a gas mixture (3% CO_2, 20% O_2, 77% N_2) for 30 seconds, the ampulae was tightly closed with rubber cork containing CO_2 trap. The CO_2 trap (a glass bulb) was made by breaking 3 mm diameter glass rod into 20 mm segment and heat sealing one end. A piece of filter paper was inserted into the glass bulb before insertion into the rubber cork and 0.1 ml of diethanolamine buffer was applied to the filter paper. The closed system with CO_2 trap was placed in an incubator at 37°C. The CO_2 traps were replaced every two and a half hours for five hours. Upon removal of second trap, 0.01 ml of 1N sulphuric acid was added to the ampulae to halt further metabolism and release of any residual CO_2 from the sample. The system was again closed for 1 hour before the 3rd and final trap was removed and they were placed in the scintillation vials containing 10 ml of scintillation fluid and the samples were counted in LKB

wallac scintillation counter. Results are expressed as CPM of $^{14}CO_2$ released/ mg tissue.

HORMONE ASSAY:

RIA FOR INSULIN: Insulin was measured in serum by using radioimmuno assay kit INSIK-5 from DiaSorin s.r.l. (Saluggia, Italy) (Product Code: P2796).

- **Principle:** The principle of the assay is based on the competition between labeled insulin and insulin contained in standards or specimens to be assayed for a fixed and limited number of antibody binding sites. After the incubation, the amount of labeled insulin bound to the antibody is inversely related to the amount of unlabelled insulin present in the sample.
- **Procedure:** In the sample tube 100 µl of serum was added and in the standard tube 100 µl of standard was added in a third tube 100 µl of tracer was added. To all the three tubes 100 µl of antiserum was added and the contents were mixed with a vortex and incubated for 1.5 hours at room temperature. To sample and standard tubes 1 0 ml of precipitating reagent was added and the contents were mixed with a vortex and allowed to stand for 15 minutes at room temperature. The tubes were then centrifuged for 15 minutes at 1500-2000 g. The supernatant was decanted carefully and the radioactivity

was measured of the precipitates. The amount of insulin is expressed as µMol/dl of serum.

- **STATISTICAL ANALYSIS:** The results are expressed as mean ± standard error of mean (SEM). The data was subjected to student's t-test with a 95% confidence limit and Oneway ANOVA with Bonferroni's Multiple Comparison Post test was performed using GraphPad Prism version 3.00.