MATERIALS AND METHODS

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1. EXPERIMENTAL ANIMALS

The experimental animals were broadly classified in two groups:

1.1 Adult animals:

Female albino rats of *Wistar* strain (200-250 g) of 180 days old were obtained from Sun Pharmaceuticals Ltd., Vadodara and maintained in the departmental animal house at 21-23° C and light and dark cycles of 12:12 h respectively. Animals were provided with standard rodent pellet diet purchased from M/S Pranav Agro Industries limited, Vadodara. Food and water were provided *ad libitum*. (Chapters 1 to 4)

1.2 Neonates:

Albino *Wistar* rats of both sexes weighing 200-250g were used for the study. Animals were maintained under 12:12 light and dark schedule and 21-23° C temperature regimen. Throughout the experimental period, animals were provided with standard rat chow and water *ad libitum*. The rat chow was purchased from M/S Pranav Agro limited, Baroda. When the mated females delivered pups, males and females were separated and equal number of pups mixed from different litters was assigned to lactating mothers. The treatment was started on day 2 post partum and continued until postnatal day (PND) 14. The control and treated rats were weaned off on PND 21 and housed in separate cages depending on their treatments and sex and were maintained on standard rat chow and water *ad libitum* until 120 days. (Chapters 5 to 8).

1.3 Animal Experimentation Ethical Guidelines:

Animal experiments were conducted according to the guidelines of CPCSEA. The animal experiments were approved by the animal ethical

committee of the Departments of Biochemistry and Zoology, The M. S. University of Baroda, Vadodara (Approval no 827/ac/04/CPCSEA).

Following the treatment schedule, adult animals were sacrificed and selected tissues were separated and stored at -80 C till biochemical assay. Blood was collected prior to sacrifice by keeping the animals under light ether anesthesia and the separated serum obtained was used for further analysis. During the entire treatment schedule, body weight, food and water were monitored on a daily basis.

2. EXPERIMENTAL GROUPS:

Animals were divided into different groups each consisting of six animals.

2.1 Experimental set up for chapters 1 and 2

- **Sham operated control (SO):**

  Sham operated control animals were treated with saline as vehicle for 15 days.

- **Ovariectomized (OX):**

  Ovariectomized animals were administered saline as a vehicle for 15 days.

- **Ovariectomized + Estrogen supplemented (OX+E2) :**

  Ovariectomized animals were given Estrogen replacement ip at a dose of 30μg/kg body weight for a period of 15 days.

- **Ovariectomized + Progesterone supplemented (OX+P4) :**

  Ovariectomized animals were given Progesterone replacement ip at a dose of 2mg/kg body weight for a period of 15 days.

- **Ovariectomized + Melatonin(Low dose) supplemented (OX +ML):**

Ovariectomized animals were given melatonin supplement (ip) at a dose of 1mg/kg body weight for a period of 15 days.

- **Ovariectomized + Melatonin(High dose) supplemented (OX +MH):**

  Ovariectomized animals were given melatonin supplement (ip) at a dose of 10mg/kg body weight for a period of 15 days.

- **Ovariectomized + Estrogen supplemented + Melatonin(Low dose) supplemented (OX+E2+ML):**

  Ovariectomized animals were given melatonin at a dose of 1mg/kg body weight and estrogen replacement at a dose of 30µg/kg body weight for a period of 15 days.

- **Ovariectomized + Estrogen supplemented + Melatonin(High dose) supplemented (OX+E2+MH):**

  Ovariectomized animals were given melatonin at a dose of 10mg/kg body weight and estrogen replacement at a dose of 30µg/kg body weight for a period of 15 days.

2.2. Experimental set up for chapters 3 and 4

- **Sham operated control (SO):**

  Sham operated control animals injected (ip) with the vehicle (saline) for 15 days.

- **Ovariectomized (OX):**

Ovariectomized animals administered with saline for 15 days.

- **Sham operated diabetic control (D):**

Diabetic sham operated animals administered with saline for 15 days after induction of diabetes.

- **Ovariectomized + Diabetic (OVX+D):**

Ovariectomized diabetic rats administered with saline for 15 days after induction of diabetes.

- **Ovariectomized + Diabetic + Estrogen supplemented (OVX +D+E_2):**

Ovariectomized diabetic animals subjected to estrogen replacement (ERT) administered at a dose of 30µg/kg body weight of 17β estradiol (ip) for a period of 15 days.

- **Ovariectomized + Diabetic + Progesterone supplemented (OVX + D+P_4):**

Ovariectomized diabetic animals subjected to progesterone replacement (ip) at a dose of 20mg/kg body weight for a period of 15 days.

- **Ovariectomized + Diabetic + Melatonin(Low dose) supplemented (OVX + D+ML):**

Ovariectomized diabetic animals subjected to melatonin supplementation (MST_L) as a low dose of 1mg/kg body weight (ip) for a period of 15 days.

- **Ovariectomized + Diabetic + Melatonin(High dose) supplemented (OVX + D+MH):**

Ovariectomized diabetic animals subjected to melatonin supplementation (MST_H) at a dose of 10mg/kg body weight (ip) for a period of 15 days.

- **Ovariectomized + Diabetic + Estrogen supplemented + Melatonin (Low dose) supplemented (OVX + D+E_2+ML):**

Ovariectomized diabetic animals subjected to a combination of MST_L (1mg/kg) and ERT (30µg/kg) for a period of 15 days.

- **Ovariectomized + Diabetic + Estrogen supplemented + Melatonin (High dose) supplemented (OVX +D+E_2+MH):**

Ovariectomized diabetic animals subjected to a combination of MST_H (10mg/kg) and ERT (30µg/kg) for a period of 15 days.

2.3 Experimental set up for chapter 5

- **Group I** : Control neonates divided into two subgroups:
  - Female control animals treated with saline as vehicle for 15 days in the neonatal phase, and maintained without any treatment until 120 days of age **(NF)**.
  - Male control animals treated with saline as vehicle for 15 days in the neonatal phase, and maintained without any treatment until the age of 120 days **(NM).**

- **Group II** : Neonates treated with corticosterone in the neonatal period were divided into two subgroups:
  - Female neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until 120 days **(CF).**
  - Male neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until 120 days of age **(CM)**.

- **Group III:** Neonates treated with melatonin simultaneous to corticosterone administration. These were further divided into two subgroups:

- Female neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) and melatonin (40µg/animal/day) in the evening (16:00 hrs) and maintained thereafter without treatment from PND 2 to PND 14 without treatment until the age of 120 days **(CF.Mel)**.

- Male neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) and melatonin (40µg/animal/day) in the evening at 16:00 hrs from PND 2 to PND 14 and maintained thereafter without treatment until 120 days of age **(CM.Mel)**.

2.4 Experimental set up for chapter 6

- **Group I** : Control neonates divided into two subgroups:
  - Female control animals treated with saline as vehicle for 15 days in the neonatal phase, and maintained without any treatment until 120 days of age **(NF)**.
  - Male control animals treated with saline as vehicle for 15 days in the neonatal phase, and maintained without any treatment until the age of 120 days **(NM)**.

- **Group II** : Neonates treated with corticosterone in the neonatal period were divided into two groups:
  - Female neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until 120 days **(CF)**.

- Male neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until 120 days of age **(CM)**.

- **Group III**: Adult melatonin treatment in neonatal corticosterone exposed rats. These were further divided into two subgroups:

  - Female neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until the age of 120 days and then at 120 days were treated with melatonin (1mg/anima/day) in the evening (18:00 hrs) for a period of 15 days **(CF.Mel)**.

  - Male neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until the age of 120 days and then at 120 days were treated with melatonin (1mg/animal/day) in the evening (18:00 hrs) for a period of 15 days **(CM.Mel).**

2.5 Experimental set up for chapter 7

- **Group I** : Control neonates were divided into two subgroups:
  - Female control animals treated with saline as vehicle for 15 days in the neonatal phase, and maintained without any treatment until 120 days of age **(NF)**.

- Male control animals treated with saline as vehicle for 15 days in the neonatal phase, and maintained without any treatment until the age of 120 days **(NM)**.

- **Group II** : Neonates treated with corticosterone in the neonatal period were divided into two subgroups:
  - Female neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until diabetes induction at 120 days. Animals with glucose levels of 300mg/dl or above were selected and treated with saline as vehicle for a period of 15 days **(CDF)**.
  - Male neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment and diabetes induced at 120 days of age. Animals with glucose levels of 300mg/dl or above were selected and treated with saline as vehicle for a period of 15 days **(CDM)**.
- **Group III**: Neonates treated with melatonin simultaneous to corticosterone administration. These were further divided into two subgroups:
  - Female neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) and melatonin (40µg/animal/day) in the evening (16:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment without treatment until the age of 120 days. Diabetes was induced in these animals and animals with glucose levels of 300mg/dl or above were selected **(CDF.Mel)**.

- Male neonates treated with Corticosterone (1µg/animal/day) in the morning (8:00 hrs) and melatonin (40µg/animal/day) in the evening at 16:00 hrs from PND 2 to PND 14 and maintained thereafter without treatment until 120 days of age. Diabetes was then induced in these animals and animals with glucose levels of 300mg/dl or above were selected **(CDM.Mel)**.

2.6 Experimental set up for chapter 8

- **Group I** : Control neonates were divided into two subgroups:
    - Female control animals treated with saline as vehicle for 15 days in the neonatal phase, and maintained without any treatment until 120 days of age **(NF).**
    - Male control animals treated with saline as vehicle for 15 days in the neonatal phase, and maintained without any treatment until the age of 120 days **(NM).**
- **Group II** : Neonates treated with corticosterone in the neonatal period were divided into two subgroups:
    - Female neonates treated with corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until diabetes induction at 120 days. Animals with glucose levels of 300mg/dl or above were selected and treated with saline as vehicle for a period of 15 days **(CDF)**.
    - Male neonates treated with corticosterone (1µg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment and diabetes induced at 120 days of

age. Animals with glucose levels of 300mg/dl or above were selected and treated with saline as vehicle for a period of 15 days **(CDM)**.

- **Group III**: Neonates treated with corticosterone and treated with melatonin in the adult stage. These consisted of two subgroups:
    - Female neonates treated with Corticosterone (1μg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until the age of 120 days. Diabetes was induced in these animals and animals with glucose levels of 300mg/dl or above were selected for further administration of melatonin at a dosage of 1mg/kg body weight for a period of 15 days **(CDF.Mel)**.
    - Male neonates treated with Corticosterone (1μg/animal/day) in the morning (8:00 hrs) from PND 2 to PND 14 and maintained thereafter without treatment until 120 days of age. Diabetes was then induced in these animals and animals with glucose levels of 300mg/dl or above were selected for further administration of melatonin at a dosage of 1mg/kg body weight for a period of 15 days **(CDM.Mel)**.

3. PREPARATION OF CHEMICALS:

3.1 Melatonin:

Melatonin (N-acetyl 5-methoxytryptamine) was procured from Sigma Co. USA and requisite amount was weighed and dissolved in few drops of alcohol and diluted with 0.9% saline. (All the chapters)

3.2 Corticosterone:

Corticosterone was procured from Sigma Co.USA was weighed in the requisite amount and was first dissolved in a drop of alcohol and then diluted with 0.9% saline. (Chapters 5 to 8)

3.3 Estradiol:

17 β estradiol was procured from Sigma Co.USA and weighed in the requisite amount and was first dissolved in a drop of alcohol and then diluted with 0.9% saline. (Chapters 1 to 4)

3.4 Progesterone:

Progesterone was procured from HIMEDIA chemicals and weighed in the requisite amount and was first dissolved in a drop of alcohol and then diluted with 0.9% saline. (Chapters 1 to 4).

4. EXPERIMENTAL PROCEDURES

4.1 OVARIECTOMY:

For the set of experimentation in adult animals, they were either ovariectomized or sham operated (Waynfort and Flecknell, 1992). Ovariectomy was performed under mild anesthesia and the ovaries were removed bilaterally

using a single ventral incision. In case of sham operation, ovaries were located by inducing a ventral incision but were not removed from the body. Following the surgical procedure, animals were kept in a resting phase for about 20 days to enable them complete recovery from surgical stress and to allow the excess circulating sex steroid levels to diminish. (Chapters 1 to 4)

4.2 INDUCTION OF TYPE I DIABETES

To induce diabetes, Alloxan monohydrate was obtained from Sigma Chemicals, USA. Animals were fasted overnight prior to alloxanization and alloxan was administered intraperitonealy at a dosage of 120mg/kg body weight. The animals were monitored for food and water intake, body weight and mortality thereafter for the next six to seven days before analyzing their blood glucose level. Blood was withdrawn after seven days from the orbital sinus of alloxan treated animals. Animals having a blood glucose level above 300mg/dl were only considered diabetic and were considered for treatment further. (Chapters 1,2,3,4,6,8)

4.3 HISTOLOGY OF PANCREAS

Pancreas from the spleenic region was removed at the time of sacrifice and is fixed in formaline for histological studies. Paraffin sections of 5 micron thickness were cut and staining was done using Haematoxylin Eosin(HE) (Chapters 4 to 8).

4.4 GLUCOSE TOLERENCE TEST

At the end of treatment schedule, animals were fasted overnight and glucose tolerance test was done by feeding them orally with a glucose solution

at a dose of 2g/kg body weight. Blood was collected from the retro orbital sinus at 0min, 30min, 60min, 90min, and 120min after glucose load. Serum was separated and, glucose was estimated in all the collected samples to plot a tolerance curve for all the experimental groups (Singh, 2010; Singh *et al.*, 2010 a, b); (Chapters 1,3,5,6,7,8).

4.5 INSULIN RESPONSE TEST:

Response to insulin was checked by injecting Insulin to the rats at a dose of 1U/kg body weight intraperitoneally (i.p) in the fed state a day following the completion of treatment and blood was collected at 0, 30, 60, 90, and 120 min from the retro orbital sinus under mild ether anesthesia . Serum was separated and used to estimate the glucose level and an insulin response curve was plotted to evaluate the results (Singh, 2010; Singh *et al.*, 2010a, b); (Chapters 1,3,5,6,7,8)

4.6 Area Under Curve (AUC):

During both GTT and IRT, area under curve was calculated by using the Graph Pad Prism Version 3.0 for Windows , Graph Pad software , San Diego CA /USA.

4.7 Fasting insulin resistance index (FIRI) and Insulin sensitivity index (K_{IS})

In order to evaluate the insulin sensitivity for the treatment regimens, FIRI and insulin sensitivity index were calculated in all the experimental groups. FIRI was calculated by the method of Kamgang *et al.* (2008) and insulin sensitivity index was checked by Duncan *et al.* (1995); (Chapters 1,3,5,6,7,8).

5 BIOCHEMICAL ESTIMATIONS:

5.1 SERUM HORMONES:

5.1.1 INSULIN

METHODOLOGY EMPLOYED: Elisa based assay using a Rat Insulin ELISA kit (MERCODIA, Sweden). (Chapters 1,3,5,6,7,8)

PRINCIPLE: Mercodia Rat Insulin ELISA is a solid phase two site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti insulin antibodies and anti insulin antibodies bound to microtitration well. A simple washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3, 3`, 5, 5`-tetramethylbenzidine. The reaction is stopped by adding acid to give colorimetric endpoint that is read spectrophotometrically.

PROCEDURE: To 25 µl calibrator or sample add 50 µl of enzyme conjugate and incubate it for 2hrs at room temperature. Wash about six times and then add 200 µl of TMB substrate and again incubate for 15min before adding the stop solution 50 µl. Read the absorbance at 450nm and evaluate.

Calculations: The concentrations corresponding to the different OD values were calculated by using a standard curve and were expressed in µl.

5.1.2 ESTRADIOL

METHODOLOGY EMPLOYED: ELISA based assay using ELISA kit (BIOCHECK, Inc.CA). (Chapters 2,4,5,6,7,8)

PRINCIPLE: The Biocheck E2 EIA is based on the principle of competitive binding between E2 in the test specimen and E2 HRP conjugate for a constant amount of rabbit anti-estradiol. In incubation, goat anti IgG coated wells are incubated with 25microlit E2 standards and samples. 100microlit Estradiol HRP conjugate and 50 μlit rabbit anti Estradiol reagent at RT for 90min. During incubation, a fixed amount of HRP labeled E2 competes with the endogenous E2 in the standards or sample for a fixed number of binding sites of the specific E2 antibody. Thus, the amount of E2 peroxidase bound in the well progressively decreases as the concentration of E2 in the specimen increases. Unbound E2 peroxidase conjugate is then removed and the wells washed. Next a solution of TMB reagent is added and incubated at RT for 20min resulting in the development of blue color. The color development is stopped with addition of in HCL and the absorbance is measured spectrophotometrically at 450nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabelled E2 in the sample. A standard curve is obtained by plotting the concentration of the standard versus the absorbance. The E2 concentration of the specimen run concurrently with the standard can be calculated from the curve.

PROCEDURE: Secure the desired number of wells in the holder and dispense 25 μl of standard or sample. Next add 100 μl estradiol HRP conjugate reagent into each well. Then add 50 μl of anti rabbit estradiol reagent to each well, mix

well for 30 seconds and incubate at room temperature for 90 minutes. After incubation rinse and flick the wells with distilled water and then dispense 100 µl of TMB reagent. Incubate again for 20 minute at room temperature. Then add 100µlit stop solution and read the absorbance at 450nm within 15minutes.

Calculations: The E_2 concentration of the specimen run concurrently with the standard and can be calculated from the standard curve. It were expressed in pg/ml.

5.1.3 PROGESTERONE

METHODOLOGY EMPLOYED: ELISA based assay method by Shrivastav *et al.*, 2009 using ELISA kit from Immuno-Technology & Steroid Laboratory Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, New Delhi. (Chapters 2,4,5,6,7,8)

PRINCIPLE: The ELISA of progesterone is a competitive solid phase assay. Serum samples or standards are incubated in antibody coated wells with HRP conjugate. After incubation the liquid contents are washed to remove the unbound enzyme conjugate. The bound enzyme activity is measured by developing colored product from colorless substrate after incubation. Quantity of color developed is directly related to the bound enzyme conjugate and is inversely related to the concentration of analyte. Unknown values are determined by interpolation from the standard curve.

PROCEDURE: 50 µl of standard or sample is dispensed in the wells and then 100 µl of enzyme conjugate is added and incubated at RT for 60min. After

thorough washing step 100 µl of substrate is added and incubated for 15min before adding the stop solution and reading is taken at 450nm.

Calculations: The P_4 concentration of the specimen run concurrently with the standard and can be calculated from the standard curve. It were expressed in ng/ml.

5.1.4 CORTICOSTERONE

METHODOLOGY EMPLOYED: ELISA based assay method using ELISA kit from Immuno-Technology & Steroid Laboratory Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, New Delhi. (Chapters 2,4,5,6,7,8)

PRINCIPLE: The ELISA of corticosterone is a competitive solid phase assay. Serum samples or standards are incubated in antibody coated wells with horse radish peroxidase conjugate. After incubation, the liquid contents of the wells are decanted and the wells are washed in running tap water for removing the unbound enzyme conjugate. The bound enzyme activity is measured by developing colored product from colorless substrate after incubation. Quantity of color developed is directly related to the bound enzyme conjugate and is inversely related to the concentration of analyte. Unknown values are determined by interpolation from the standard curve.

PROCEDURE: 50µlit of standard or sample is dispensed in the wells and then 25µl of enzyme conjugate is added and incubated at RT for 60min. After thorough washing step 100 µl of substrate is added and incubated for 15min before adding the stop solution and reading is taken at 450nm.

Calculations: Results are obtained from the standard curve by interpolation. The curve serves for the determination of corticosterone concentration in sample measured at the same time as the standards. The results were expressed in ng/ml.

5.1.5 TESTOSTERONE

METHODOLOGY EMPLOYED: ELISA based assay using ELISA kit (IBL immuno biological limited).

PRINCIPLE: The Testosterone solid phase ELISA Kit is based on the principle of competitive binding. The wells are coated with an antibody directed towards an unique antigenic site on the Testosterone molecule. Endogenous Testosterone of a patient sample competes with a Testosterone horseradish peoxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is reverse proportional to the concentration of Testosterone in the sample. After addition of the substrate solution, the intensity of colour developed is reverse proportional to the concentration of Testosterone in the patient sample.

PROCEDURE: All standards, samples, and controls should be run in duplicate concurrently so that all conditions of testing are the same. 1. Secure the desired number of microtiterwells in the holder. 2. Dispense 25 µl of each standard, controls and samples with new disposable tips into appropriate wells. 3. Dispense 200 µl enzyme conjugate into each well. 4. Thoroughly mix for 10 seconds. It is important to have a complete mixing in this step. 5. Incubate for 60 minutes at room temperature. 6. Briskly shake out the contents of the wells. Rinse the wells 3 times with diluted Wash Solution (400 µl per well). Strike the

wells sharply on absorbent paper to remove residual droplets. 7. Add 200 µl of Substrate Solution to each well. 8. Incubate for 15 minutes at room temperature. 9. Stop the enzymatic reaction by adding 100 µl of Stop Solution to each well. 10. Read the OD at 450±10 nm with a microtiter plate reader within 10 minutes after adding the Stop Solution.

Calculations: The T concentration of the specimen is run concurrently with the standard and calculated from the standard curve. It was expressed in ng/ml.

5.2 SERUM GLUCOSE

METHODOLOGY EMPLOYED: Colorimetric kit based assay using kit (Agappe Diagnostics). (Chapters 1,3,5,6,7,8)

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 min. The OD were read in a spectrophotometer at 505 nm.

Calculations: Sample values were calculated based on the standard observations and the amount of glucose is expressed as mg/dl of serum.

5.2 SERUM LIPID PROFILE:

5.2.1 SERUM CHOLESTEROL

METHODOLOGY EMPLOYED: Colorimetric kit based assay using kit (Accurex biomedical Pvt Ltd, India). (Chapters 1,3,5,6,7,8)

PRINCIPLE: Cholesterol esterase hydrolysis cholesterol esters into free cholesterol and fatty acids . In the second reaction cholesterol oxidase converts cholesterol to cholest 4-en-3-one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidatively couples with 4-aminoantipyrine and phenol to produce red quinoneimine dye which has absorbance maximum at 510 nm (500-530). The intensity of the red colour is proportional to the amount of total cholesterol in the specimen.

PROCEDURE: Take 0.01ml of serum or standard in the tubes and add 1ml reagent to each. Incubate the assay mixture for 10min at room temperature and then read at 510nm against blank.

Calculations: Sample values were calculated based on the standard observations and the amount of total cholesterol was expressed as mg/dl of serum.

5.2.2 TRIGLYCERIDES

METHODOLOGY EMPLOYED: Colorimetric kit based assay using kit (Accurex Biomedical Pvt Ltd, India). (Chapters 1,3,5,6,7,8)

PRINCIPLE: Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol-3-phosphate which is

oxidized by glycerol phosphate oxidase to dihydroacetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidizes phenolic chromogen to a red colored compound.

PROCEDURE: Take 0.01ml of serum or standard in the tubes and add 1ml reagent to each. Incubate the assay mixture for 20minutes at room temperature and then read at 510nm against blank.

Calculations: Sample values were calculated based on the standard observations and the amount of TG was expressed as mg/dl of serum.

5.2.3 HDL CHOLESTEROL

METHODOLOGY EMPLOYED: Colorimetric kit based assay using kit (Nicholas Piramal India Limited). (Chapters 1,3,5,6,7,8)

PRINCIPLE: Chylomirons, VLDL (Very low density lipoproteins), and LDL (Low density lipoproteins) are precipitated by adding phosphotungstic acid and magnesium ions to the sample. Centrifugation leaves only the HDL (High density lipoproteins) in the supernatant. The cholesterol content in it is determined enzymatically.

PROCEDURE: Take 100 µl serum or standard in tubes and add 1ml of reagent to it. Mix it well and incubate for 10minutes at 20 – 30 C and then read against blank at 546nm.

Calculations: Sample values were calculated based on the standard observations and the amount of HDL cholesterol was expressed as mg/dl of serum.

5.3 SERUM MARKERS OF HEPATIC AND RENAL DYSFUNCTION

5.3.1 Serum Glutamate Pyruvate Transaminase (SGPT)

METHODOLOGY EMPLOYED: Colorimetric kit based assay using kit (Agappe Diagnostics Ltd, India). (Chapters 2,4,5,6,7,8)

PRINCIPLE: The rate of NADH consumption is measured photometrically and is directly proportional to the ALAT (Alanine Amino Transferase) concentration in the sample.

PROCEDURE: Take 100 µl of sample. Mix it with Reagent 1 (1000 µl). Mix and incubate for 5 minutes. To this mixture add 250 µl of Reagent 2. Mix well and read the decrease in absorbance after one minute at 340 nm.

Calculations: Sample values were calculated based on the standard observations and the SGPT was expressed as U/L of serum.

5.3.2 Serum Glutamic-Oxaloacetic Transaminase (SGOT)

METHODOLOGY EMPLOYED: Colorimetric kit based assay using kit (Crest Biosystems, a division of coral clinical system). (Chapters 2,4,5,6,7,8)

PRINCIPLE: The rate of NADH consumption is measured photometrically and is directly proportional to the ASAT (Aspartate Amino Transferase) concentration in the sample.

PROCEDURE: Take 100 µl of sample. Mix it with Reagent 1 (1000 µl). Mix and incubate for 5 minutes. To this mixture add 250 µl of Reagent 2. Mix well and read the decrease in absorbance after one minute at 340 nm.

Calculations: Sample values were calculated based on the standard observations and the SGOT activity was expressed as U/l of serum.

5.3.3 ALKALINE PHOSPHATASE (ALP)

METHODOLOGY EMPLOYED: Colorimetric kit (p-NPP method) based assay using kit purchased from Reckon diagnostics Pvt Ltd. (Chapters 2,4,5,6,7,8)

PRINCIPLE: The increase in absorbance due to formation of 4 - nitroophenolate israte of is measured photo metrically and is directly proportional to the ALP (Alkaline Phosphatase) activity in the sample

PROCEDURE: Take 20 µl of sample. Mix it with Reagent 1 (1000 µl). Mix and incubate for 1 minute. To this mixture add 250 µl of Reagent 2. Mix well and read the decrease in absorbance after one minute at 405 nm.

Calculations: Sample values were calculated based on the standard observations and the ALP activity was expressed as U/lof serum.

5.3.4 ACID PHOSPHATASE (ACP)

METHODOLOGY EMPLOYED: Colorimetric kit (Kinetic method) based assay using kit (Aspen Laboratories). (Chapters 2,4,5,6,7,8)

PRINCIPLE: The enzymatic reaction sequence employed in the assay of Acid Phosphatase is as follows:

α - Naphthylphosphate + H2O ⟶ α - Naphthol + Inorganic Phosphate

α - Naphthol + Fast Red TR ⟶ Diazo Dye (CHromophore)

The α - Naphthol released from the substrate α - Naphthylphosphate by acid phosphatase is coupled with Fast Red TR to produce a coloured complex which absorbs light at 405nm. The reaction can be quantitated photo metrically because the coupling reaction is instantaneous.

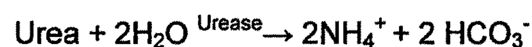
PROCEDURE: Take 20 µl of sample. Mix it with Reagent 1 (1000 µl). Mix and incubate for 1 minute. To this mixture add 250 µl of Reagent 2. Mix well and read the decrease in absorbance after one minute at 405 nm.

Calculations: Sample values were calculated based on the standard observations and the ACP activity was expressed as U/L of serum.

5.3.5 UREA

METHODOLOGY EMPLOYED: Colorimetric kit (Kinetic method) based assay using kit (DiaSys Diagnostic Systems GmbH and Co.KG, Germany). (Chapters 2,4,5,6,7,8)

PRINCIPLE:



Urea + $2H_2O \xrightarrow{Urease} 2NH_4^+ + 2\,HCO_3^-$

2 Oxoglutarate+NH_4^+ + NADH $\xrightarrow{GLDH}$L-Glutamate + NAD^++H_2O

GLDH: Glutamate dehydrogenase

PROCEDURE: The method is optimized for 2 point kinetic measurement. One of it is Substrate start wherein 1000 µl of reagent 1 is mixed with 10 µl of sample or standard. It is mixed well and incubated for 0-5 minutes before adding 250 µl of reagent 2. Next it is incubated for 60 sec at 25°C or 30°C and then read absorbance once and after exactly 60 sec later read the second

absorbance. The second point measurement is the sample start wherein 10 µl of sample or standard is mixed with 1000 µl of monoreagent and follow the similar incubation scheme as for the substrate start and take two point readings at a gap of 60 sec.

Calculations: Sample values were calculated based on the standard observations and urea content was expressed as mg/dl of serum.

5.3.6 CREATININE

METHODOLOGY EMPLOYED: Colorimetric kit (Kinetic method) based assay using kit (Nicholas Piramal) India Limited. (Chapters 2,4,5,6,7,8)

PRINCIPLE: Creatinine forms a coloured complex with picrate in alkaline medium. The rate of formation of the complex is measured.

PROCEDURE: Into a clean and dry cuvette pipette out 1ml of reagent mixture and add 10µl of either sample or standard to it. After exactly 30 seconds read absorbance A1 and after another 120 seconds read A2.

Calculations: Sample values were calculated based on the standard observations and the creatinine measure was expressed as mg/dl of serum.

6 TISSUE PARAMETERS

6.1 CARBOHYDRATE METABOLISM

6.1.1 GLYCOGEN

METHODOLOGY EMPLOYED: The glycogen content in liver and muscle was estimated by the method of Seifter *et al*, 1950. (Chapters 1,3,5,6,7,8)

PRINCIPLE: Glycogen present in the tissue is first hydrolysed to glucose and then estimated as per known weight of tissue by using a conversion factor of 1.11. (1gm glycogen yields 1.11 gm of glucose on complete hydrolysis.) Fresh tissue is digested in hot KOH solution and glycogen is precipitated to glucose using ethyl alcohol. This type of precipitation is critical, glycogen should be entirely precipitated and the ppt is then suspended in water. The suspension is then treated with anthrone reagent prepared in sulphuric acid. H_2So_4 reduces anthrone in presence of glucose to develop a green colour. The colour intensity is directly proportional to the amount of glucose.

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.

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the different standards run and the amount of glycogen was expressed as mg/100mg of tissue.

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6.1.2 GLYCOGEN PHOSPHORYLASE

METHODOLOGY EMPLOYED: The activity of Glycogen phosphorylase in liver and muscle was estimated by the method by Cahill *et al*, (1957). (Chapters 1,3,5,6,7,8)

PRINCIPLE: Glycogen phosphorylase cleaves the phosphoric bond of α– 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.

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the different standards run and the activity of Glycogen phosphorylase was expressed as µM PO4 released /100mg protein/10min.

6.1.3 GLUCOE-6-PHOSPHATASE

METHODOLOGY EMPLOYED: The activity of Glucose-6-phosphatase in liver was estimated by the method by Harper (1965). (Chapters 1,3,5,6,7,8)

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),and 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.

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the different standards run and the activity of Glucose-6-phosphatase was expressed as µM PO4 released /100mg protein/10min.

6.1.4 TOTAL PROTEIN (For calculating the specific activity of enzymes)

METHODOLOGY EMPLOYED: The protein content of the tissue extracts (Liver, Muscle and Kidney) 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 reacts with Folin Ciocalteau reagent to give a blue colour due to the reaction of phosphomolybdate. The intensity of colour is propotional to the concentration of proteins.

PROCEDURE: A set of tubes containing BSA in the concentration range of (0-100µg) was used. The volume in each tube was made to 1 ml with d/w. 5 ml of freshly prepared alkaline copper sulphate solution was added in each of these tubes, mixed thoroughly and were incubated at room temperature for 10 min. In each of these tubes 0.5 ml of folin-ciocalteau reagent was added and the contents were mixed immediately. It was allowed to stand for 30 min at room temperature for the color to develop. The absorbance of each tube at 660 nm was recorded. A standard curve of absorbance at 660 nm versus µg of BSA was plotted to determine the amount of protein in the sample.

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the standards run and the amount of protein was expressed as mg/100mg of tissue.

6.2 TISSUE LIPIDS

6.2.1 TOTAL LIPIDS

METHODOLOGY EMPLOYED: The total lipid content in liver, muscle and kidney was estimated by the method by Folch *et al*, (1957). (Chapters 1,3,5,6,7,8)

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%) were added to the lysate and kept overnight. 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. .

Calculations: Sample values were calculated based on the difference in weight, and the amount of total lipid was expressed as mg/100 mg of tissue.

6.2.2 CHOLESTEROL

METHODOLOGY EMPLOYED: The total tissue cholesterol in liver, muscle and kidney was estimated by the method by Crawford (1958). (Chapters 1,3,5,6,7,8)

PRINCIPLE: The method depends upon interaction of $FeCl_3$ and H_2So_4 with cholesterol in presence of glacial acetic acid solution. The resulting red – purple colour is measured spectrophotometrically. The content of total cholesterol in dry lipid sample determines the intensity of colour. The exact chemical nature of the reaction is however not known.

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.

Calculations: Sample values were calculated based on the standard readings, and the amount of total cholesterol content was expressed as mg/100 mg of tissue.

6.3 <u>PARAMETERS TO MEASURE OXIDATIVE STRESS</u>

6.3.1 LIPID PEROXIDATION

METHODOLOGY EMPLOYED: Lipid peroxidation in liver, muscle and kidney was estimated by the method by Beuge and Aust , 1978. <u>(Chapters 2,4,5,6,7,8)</u>

PRINCIPLE: Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and gives thiobarbituric acid reactive substance. (TBARS). TBARS gives a characteristic pink color that can be measured calorimetrically at 532 nm.

PROCEDURE: Take 1ml of 10% tissue homogenate and mix it with 1ml of TBA reagent. Keep in boiling water bath for 20min, cool the tubes, centrifuge at 30000 rpm for 15 min and read absorbance at 532nm.

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the different standards run and the Lipid peroxidation was expressed as nmoles of MDA/mg protein.

6.3.2 GLUTATHIONE REDUCED

METHODOLOGY EMPLOYED: Glutathione content in liver, muscle and kidney was estimated by the method by Beutler *et al,* (1969). <u>(Chapters 2,4,5,6,7,8)</u>

PRINCIPLE: Glutathione is a mojor non protein thiol present in the same tissue. The sulphahydryl groups in glutathione reduce the 5,5`-dithio bis-2-nitro

benzoic acid to form one mole of 5-thio-2-nitro benzoate per mole of SH , this has an intense yellow color with an absorbance at 412nm and can be used to measure –SH group.

PROCEDURE: The test system contained tissue extract of 100 µl, 1ml precipitating reagent solution, 3ml phosphate buffer and 0.5ml DTNB. Absorbance was recorded against a blank containing precipitating reagent, phosphate buffer and DTNB solution. GSH was taken as standard, mixed well and the absorbance recorded at 412nm within 1 min of adding DTNB.

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the different standards run and the Lipid peroxidation was expressed as µgram of GSH /min/mg protein.

6.3.3 ASCORBIC ACID

METHODOLOGY EMPLOYED: Total Ascorbic acid content in liver, muscle and kidney was estimated by the method by Roe *et al.* (1954). (Chapters 2,4)

PRINCIPLE: Ascorbic acid is oxidized to dehydroascorbic acid in presence of norit (activated animal charcoal). It is then coupled with 2,4 Dinitro phenyl hydrazine (mild reducing agent). H_2SO_4 converts DNPH into a red coloured compound which is assayed colorimetrically.

PROCEDURE: Homogenize the weighed amount of tissue with 6% TCA under cold condition and make upto desired dilution. To the sample of clear extract add animal charcoal (0.5 g/25ml), shake well and let it stand for 15 min. Filter through Wattman filter paper # 42. Take aliquot of extract containing not more than 4ml. Take three tubes and label them as blank, standard and sample.

Pipette out reagent or homogenate extract. Take 4ml homogenate in sample tube, 6% TCA in blank tube and standard ascorbic acid in standard tube. Add about 1ml of 2,4 DNPH in each tube, then add thiourea (about 3 drops) in each tube and incubate at 37°C. for 3 hr or in boiling water bath for 15 min. Transfer to icebath then add 5ml of 85% Sulphuric acid in each tube mix carefully and allow it to stand for 30 mins. Take reading at 540nm.

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the different standards run and the Ascorbic acid content was expressed as mg/100mg of tissue .

6.3.4 GLUTATHIONE PEROXIDASE

METHODOLOGY EMPLOYED: Activity of Glutathione peroxidase in liver, muscle and kidney was estimated by the method by Rotruck *et al* 1973. (Chapters 2,4,5,6,7,8)

PRINCIPLE: Glutathione peroxidase catalysis the reduction of hydrogen peroxide by reduced glutathione resulting in H2O and oxidised glathione which is then instantly and continuously converted in to GSH by and excess of GR use with NADPH providing for a constant level of GSH. Reduced glutathione acts as a reductant. The estimation is based on the oxidation of GSH by 5,5' dithio bis 2, nitro benzoic acid (DTNB) to measure the total glutathione content of biological samples.

PROCEDURE: The assay mixture containing 0.4 ml of phosphate buffer (0.4 M, P^H 7.0), 0.1 ml sodium azide (10 mM), 0.2 ml of reduced glutathione (4mM) and 0.2ml of tissue homogenate was mixed and 0.1 ml of H_2O_2 (30 mM) was

added and made up to 2.0 ml with water. The tubes were incubated at 37°C for 10 min along with control tubes containing all reagents except the enzyme. The reaction was terminated by addition of 0.5 ml of 10% TCA which was then centrifuged at 4000rpm for 10 min at 4°C . 1ml of this supernatant was taken and added to 3.0 ml of disodium hydrogen phosphate and 1.0 ml DTNB solution (40mg/100ml of 1% sodium citrate). The color formed was measured at 412 nm.The blank contained disodium hydrogen phosphate and 1.0 ml of DTNB solution.

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the different standards run and the activity of GPx was expressed as μg of GSH/min/mg protein.

6.3.5 CATALASE

METHODOLOGY EMPLOYED: Activity of Catalase in liver, muscle and kidney was estimated by the method by Sinha *et al.* (1972). (Chapters 2,4,5,6,7,8)

PRINCIPLE: Catalase is a heme containing enzyme, which catalyzes dismutation of hydrogen peroxidase into water and oxygen. Decomposition of hydrogen peroxidase by Catalase is measured spectrophotometrically at 240nm.

PROCEDURE: The assay mixture containing 0.5 ml of 0.2M H_2O_2, 1 ml of sodium phosphate buffer and 0.4 ml distilled water was mixed with 0.1 ml of cell extract was added to initiate the reaction. Then, 2ml dichromate-acetic acid reagent was added after 15, 34, 45, and 60s, to arrest the reaction. To the control tube, the enzyme was added after the addition of the dichromate acetic

acid reagent. The tubes were then heated for 10 min, allowed to cool, and the green color developed was read at 590nm against blank containing all components except the enzyme on a spectrophotometer. The activity of Catalase was expressed as units/mg protein (1 unit is the amount of enzyme that utilizes 1µmol of H_2O_2 consumed/min).

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the different standards run and the activity of Catalase was expressed as mmoles of H_2O_2 decomposed/mg protein/min.

6.3.6 SUPEROXIDE DISMUTASE

METHODOLOGY EMPLOYED: Activity of SOD in liver, muscle and kidney was estimated by the method by Marklund and Marklund, 1974. (Chapters 2,4,5,6,7,8)

PRINCIPLE: Pyrogallol auto-oxidizes at 420nm at pH 8.0. SOD inhibits this auto oxidation of pyragallol in a rate limiting fashion. 50% inhibition of pyragallol auto oxidation is equivalent to 1 I.U. of enzyme.

PROCEDURE: The final assay mixture contained 1 ml of potassium phosphate buffer (0.2 M, pH 8.2), 10µl of homogenate and 50 µl of pyragallol. For control tubes required volume of distilled water was taken in place of tissue homogenate. This was used to determine the uninhibited auto oxidation of pyragallol. The reaction was started by the addition of pyragallol and the change in optical density was recorded for 180 seconds at intervals of 30 seconds. Change in absorbance / minute was calculated from the reading. The SOD activity was expressed as IU per mg protein. One unit of SOD activity

being defined as the amount of enzyme required to cause 50 % inhibition of pyragallol auto-oxidation.

Calculations: Sample values were calculated based on the standard curve plotted from the values obtained for the different standards run and the activity of SOD was expressed as mmoles of U/mg protein.

7. Statistical Analysis

All data were expressed as Mean ± SE and the statistical significance was evaluated using One Way ANOVA followed by Bonferroni`s Multiple Comparisons test using Graph Pad Prism Version 3.0 for Windows , Graph Pad software, San Diego CA /USA .