Chapter - II

Materials and Methods
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MATERIALS AND METHODS

ANIMALS

Albino male rats of *wistar* strain weighing between 200-350g procured from Zydus Research Centre, Ahmadabad, under Registration No.167/1999/CPCSEA from the Ministry of Social Justice and Empowerment, Government of India and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai, India, were used for the study. Animals were housed in plastic cages under a well-regulated light: dark (12h: 12h) schedule at 26±2°C and were fed with standard commercial laboratory chow and tap water *ad libitum*.

Healthy, adult male *wistar* rats (*Rattus norvegicus*) weighing from 250-300g were used for the experiments. The animals were housed in an air-conditioned animal house at a temperature of 26±2°C and exposed to 12-14 hours of day light and were maintained on a standard chow and water *ad libitum*. A maximum number of two animals per cage were maintained. Animals under different groups of experiments were caged separately. The treatment was given daily early evening considering the production of melatonin in the body.
CHEMICALS

Mercuric chloride (HgCl₂; MC; ~98% pure) was obtained from Merck-Schuchardt (Germany). Melatonin (~99% pure), thiobarbituric acid and pyrogallol were obtained from HiMedia laboratories (Mumbai, India). All other chemicals were of analytical grade and purchased from local commercial sources.

Dosage, Duration and mode of treatment.

Mercuric chloride (MC; HgCl₂) was given by oral feeding at a dosage of 2mg and 4mg/kg body weight daily for 60 days. The doses were selected based on the LD₅₀ values i.e. 1/40th and 1/20th of LD₅₀ respectively. The control animals were provided with only distilled water, as the HgCl₂ was dissolved in it. Melatonin at a dose of 5mg/kg body weight per day was given by intraperitoneally for same period. The dose of MLT was selected from earlier reports (Beni et al., 2003; Dakshayani et al., 2005). Apart from this, combination of HgCl₂ + MLT was also given for the same period. Melatonin was dissolved in ethanol and further diluted with normal saline. The final concentration of ethanol in the solution was <1%. Melatonin was given 25-30 minutes before mercury (HD) supplementation. The reason for the application of melatonin 25-30 minutes before mercury was due to the rapidity of melatonin metabolism.

The distribution of animals for each experimental group was as below:
Experimental Groups

Animals were assigned to five groups of ten rats each.

Group I

Group I served as control animals and were given distilled water

Group II

Groups II was received (2 mg/kg body wt, po) mercury chloride (MC). Mercury salt was dissolved in double distilled water and given using a feeding tube attached to a hypodermic syringe. This dose for mercury chloride is derived from its LD$_{50}$ value. Hence, low dose is 1/40th of LD$_{50}$.

Group III

Groups III were received (4 mg/kg body wt, po) mercury chloride (MC). Mercury salt was dissolved in double distilled water and given using a feeding tube attached to a hypodermic syringe. High dose of mercury chloride is derived from its LD$_{50}$ value. Hence, high dose is 1/20th of LD$_{50}$

Group IV

Group IV served as melatonin (5 mg/kg body wt, ip) and was dissolved in ethanol and further diluted with normal saline. The final concentrations of ethanol in solution was <1%.

Group V

The Group V was given melatonin (5mg/kg body wt, ip) 25 -30 minutes before mercury chloride (4 mg/kg body wt, po) administration. The reason for the application of melatonin 25-30 minutes before mercury feeding was rapidity of melatonin metabolism (Vakkuri et al., 1985). All the treatments
were repeated for 60 days and on day 61st animals were weighed and sacrificed by using anesthetic ether or chloroform for research work. The steroidogenic and non-steroidogenic organs were dissected out carefully, cleared adhering tissues and used for various biochemical parameters.

DATA COLLECTION

At the end of each treatment, the animals were first weighed on an animal weighing balance (Ohaus, USA) and sacrificed under light ether anaesthesia. The blood was collected by cardiac puncture and kept at room temperature for one hour and serum was separated by centrifugation for studying serum parameters. The testis, pancreas, adreanal and thyroid gland of all control and treated animals were dissected out carefully, blotted free of blood, weighed on digital balance (cotozen, Japan) to the nearest milligram and utilized to study the different parameters.

BODY AND ORGAN WEIGHTS

The body weights of all the experimental groups of animals were recorded to the nearest gm before the treatment and after the cessation of each respective treatment. Organ weights of the steroidogenic and non-steroidogenic organs were recorded after clearing to the nearest gm or milligram on a torsion balance.

GRAVIMETRIC ANALYSIS

Relative weights of the steroidogenic and non-steroidogenic organs were calculated as follows:
Absolute tissue weight
Relative tissue weight = \[
\frac{\text{Absolute tissue weight}}{\text{Body Weight}} \times 100
\]

**Steroidogenic organs (Testis and Adrenal)**

**BIOCHEMICAL PARAMETERS**

Biochemical assays were performed in steroidogenic organs of rats. The methods used are as following:

**3β HYDROXYSTEROID DEHYDROGENASE (E.C.1.1.1.145)**

**Principle**

The testicular and adrenal 3β hydroxysteroid dehydrogenase (3β HSD) activity was assayed by the method of Talalay (1962). The enzymed 3β hydroxyl steroid dehydrogenase acts on substrate 3β hydroxyl 5α-androstane-17-one (epiandrosterone) and reduces nicotinamide adenine dinucleotide (NAD) and the absorbance was measured at 340nm.

\[3\beta \text{ HSD}\]

Epiandrosterone + NAD \[\rightarrow\] Androstene 3, 17 diol + NADH

**Method**

A known amount of tissue was homogenized in 0.02M phosphate buffer (pH 7.5) along with Triton X-100 (2.5 mg/ml) (10:1 dilution). The homogenate was centrifuged at 8000 g for 30 minutes at 4°C. The supernatant was used for the assay. To 2ml of 0.1M sodium pyrophosphate buffer (pH 8.9), 0.2 ml of substrate [3mg epiandrosterone; Sigma Chemical Co., U.S.A] in 2 ml of N, N-
dimethyl formamide)] and 0.2 ml of homogenate were added. Then 0.2 ml of NAD and 0.4 ml of glass distilled water were added. The blank was prepared by adding 2 ml of buffer, 0.2 ml of substrate and 0.8 ml of distilled water. Final volume of the assay was 3 ml. the reduction in absorbance of NAD was read at every 15 seconds interval against blank at 340 nm on a systronics UV-Visible spectrophotometer (Model 118). The protein concentration in the tissue was estimated by the method of Lowry et al. (1951). The standard curve was prepared using 5α-androstane-3, 17 dione.

**Calculation**

\[
\text{Sample OD} \times \frac{\text{Std. Conc.}}{\text{Total volume of assay} \times 10} \times \frac{\text{Standard OD}}{\text{allquot volume}} \times \frac{3 \beta \text{ HSD}}{\text{Milligram protein}} = \text{X}
\]

The enzyme activity was expressed as nanomole of 5α-diol formed/ mg protein/ hour.

**17β HYDROXYSTEROID DEHYDROGENASE (E.C.1.1.1.62)**

**Principle**

The testicular and adrenal 17β hydroxysteroid dehydrogenase (17β HSD) activity was assayed by the method of Talalay (1962). The enzyme 17β hydroxysteroid dehydrogenase acts on substrate, testosterone and reduced nicotinamide adenine dinucleotide (NAD) to NADH and the absorbance was measured at 340nm.

\[
\text{17β HSD} \quad \text{Testosterone + NAD} \rightarrow \text{Androstat 4-ene 3, 17 diol + NADH}
\]
Method

A known amount of tissue homogenized in 0.02M phosphate buffer (pH 7.5) along with TritonX-100 (2.5 mg/ml) (10:1 dilution). The homogenate was centrifuged at 8000g for 30 minutes at 4°C. The supernatant was used for the assay. To 0.2ml of 0.2M sodium pyrophosphate buffer (pH 8.9), 0.2 ml of substrate (1.5 mg testosterone dissolved in 2 ml of methyl alcohol) and 0.2 ml of homogenate were added. Then 0.2 ml of NAD and 0.4 ml of glass distilled water were added. Blank was prepared by adding 2 ml of phosphate buffer, 0.2 ml of substrate and 0.8 ml of distilled water. Final volume of assay was 3 ml. the reduction in absorbance of NAD was readapt interval of 15 seconds on a systronics UV-Visible spectrophotometer (Model 118). Protein estimation in the tissue was carried out simultaneously by the method of Lowry et al. (1951). The enzyme activity was expressed in nanomoles of 5α-diol formed/ mg protein/ minute.

Calculation

\[
\text{Sample OD} \times \frac{\text{Std. Conc.}}{\text{Total volume of assay} \times 10} \times \frac{\text{X}}{\text{Standard OD} \times \text{Aliquot volume}} = \frac{X}{17 \beta \text{ HSD}} = \frac{\text{Milligram protein}}{\text{Milligram protein}}
\]

The enzyme activity was expressed as nanomole of 5α-diol formed/ mg protein/ hour.
SUCCINATE DEHYDROGENASE (SDH; E.C.1.3.99.1)

The estimation of SDH in steroidogenic organs were assayed by the method of Beatty et al. (1966)

Principle

The activity of succinate dehydrogenase (SDH) was estimated according to the method of Beatty et al. (1966), using INT as an electron acceptor. The electrons released by the enzymes SDH from the substrate are taken up by an electron acceptor i.e. INT which is reduced to red colored formazan. After extracting in ethyl acetate, the colour intensity was measured.

Method

A known amount of tissue was homogenized in desired volume of cold distilled water. To each sample tube, 1 ml of 0.2 M phosphate buffer, 1 ml 0.2 M sodium succinate and 1 ml of INT and 0.4 ml of tissue homogenate was added. Blank tube was run with 1 ml of distilled water instead of INT solution. After 15 minutes of incubation at 37°C; 0.1 ml of 30% TCA is added to all the tubes to terminate the reaction. The formazan was extracted into 7 ml of ethyl acetate by vigorous shaking for 30 seconds. All the tubes were centrifuged at 1500 rpm for 5 minutes. The supernatant was used to measure the colour intensity on a systronics spectrophotometer (Model 166) at 420 nm against the blank tube.

Calculation

The concentration of the formazan was calculated according to the standard regression formula
\[ X = 1.866 + 152.21 \times (y) \]

Where, \( X \) = concentration of formazan in \( \mu g. \)

\( Y \) = optical density (O.D) of unknown sample.

The final SDH activity was calculated as

\[
\text{SDH activity} = \frac{\text{Concentration (X) \times dilution \times 100}}{\text{Tissue weight \times aliquot volume}}
\]

The SDH activity was expressed as micrograms (\( \mu g. \)) formazan released/ 15 min/ mg protein by further dividing with protein content.

**ALKALINE PHOSPHATASE (ALKPase) (E.C.3.1.3.1)**

Alkaline phosphatase in testis and adrenal were estimated by Bessey et al. (1946)

**Principle**

The enzyme alkaline phosphatase hydrolyses the substrate \( p \)-nitrophenyl phosphate into organic phosphate and \( p \)-nitrophenol. The quantity of \( p \)-nitrophenol released under standardized condition was measured at 410nm on systronics digital spectrophotometer (Model 116).

**Method**

A known amount of tissue was weighed and homogenized in distilled water. 0.5 ml each of alkaline buffer and substrate was pipetted in test tube and incubated at 37°C for 5 minutes. 0.2 ml of the homogenate was added to the sample tubes whereas blank tube was run with same volume of distilled water. After incubation period of 30 minutes at 37°C, 10 ml of 0.02 N NaOH
was added in all the tubes. The absorbance was read at 410nm for calculating the enzyme activity.

**Calculation**

\[
\text{ALKPase activity} = \frac{\text{Sample OD} \times 0.741 \times \text{dilution} \times 100}{\text{Tissue weight} \times \text{aliquot volume}}
\]

Where, *conversion factor = 0.741*

The enzyme activity was expressed as μmoles p-nitrophenol released/30min/ mg protein by further dividing it with protein content.

**ACID PHOSPHATASE (ACPase; E.C.3.1.3.2)**

Acid phosphatase activity in testis and adrenal were estimated by the method of Bessey et al. (1946)

**Principle**

Acid phosphate, orthophosphoric monoester phosphohydrolase catalyses the hydrolysis of p-nitro phenyl phosphate at pH 4.8 liberating p-nitro phenol and inorganic phosphate. The liberated p-nitro phenol combines with 0.1 N NaOH to form an yellow colored complex, which is measured at 420nm.

**Method**

A known amount of tissue is homogenized in known amount of distilled water. 0.6 ml substrate buffer (16.5 mg of p-nitrophenyl phosphate dissolved in 10 ml citrate buffer) was taken in all the tubes and incubated at 37°C for 5 minutes. 0.2 ml of homogenate was added to all the tubes and blank tube was run with 0.2 ml distilled water. All the tubes were incubated at 37°C for 30
minutes. 4 ml of 0.1 N NaOH was added to all the tubes and optical density (O.D) of the samples were read at 420 nm wave length on a systronics digital spectrophotometer (Model 166).

Calculation

\[
\text{Enzyme activity} = \frac{\text{OD of Sample} \times \text{conversion Factor} \times \text{Dilution} \times 100}{\text{Tissue weight} \times \text{Aliquot volume}}
\]

Conversion factor = 0.741

The ACPase enzyme activity was expressed as \( \mu \) moles of p-nitrophenol released / 30 minutes/ mg protein by further dividing it with protein content.

ADENOSINE TRIPHOSPHATASE (ATPase; E.C. 3.6.11.3)

ATPase activity was assayed by the method of Quinn and White (1968) in the steroidogenic organs.

Principle

The enzyme adenosine triphosphatase hydrolyses the substrate ATP into adenosine diphosphate and inorganic phosphate (ip). The ip thus formed is measured according to the method of Fiske ans Subbarow (1925) on Systronics digital Spectorphometer (Model 166) at 660 nm

Method

The assay system consists of 0.3 ml substrate buffer (18 mg ATP disodium salt in 10 ml Tris HCl buffer of pH 7.4), 0.1 ml MgCl\(_2\) (3mM), 0.1 ml NaCl (150 mM), 0.1 ml KCl (30 mM), followed by the addition of 0.2 ml homogenate (made in distilled water) and 0.2 ml sucrose buffer (0.86% in tris
HCl of pH 7.4) in the sample tubes. In the blank tube, 0.2 ml of distilled water was added instead of homogenate. The assay system was mixed and incubated at 37°C for 30 minutes. For termination of the reaction 0.5 ml of 10% TCA was added to the medium and then the test tubes were mixed and kept at 4°C for 10 minutes for precipitation. The test tubes were centrifuged at 3000rpm for 15 minutes. The clear supernatant was then separated and was used for inorganic phosphate determination by adding 1 ml of 2.5% ammonium molybdate and 0.5 ml ANSA reagent (2,3 Amino Naphthol Sulphonic Acid). To the reaction mixture 7.5 ml of distilled water was added and kept for 20 minutes at room temperature. The colour developed was measured at 660nm on a systronics digital spectrophotometer (Model 166).

**Calculation**

ATPase activity was calculated as

\[
\text{ATPase} = \frac{X \times \text{conversion factor} \times \text{dilution}}{\text{Tissue weight} \times \text{aliquot volume}} \times 100
\]

Where, \(X\) = OD of sample

Conversion factor = 2.09
The enzyme activity was expressed as μ moles ip released/30 minutes/mg protein by further dividing it with protein content.

**PROTEIN**

The protein content in the steroidogenic organs were estimated according to the method of Lowry et al. (1951).

**Principle**

The protein containing sample when treated with phenol reagent of Folin-Ciocalteu, a deep blue colour develops. The colour development is due to two reactions occurring viz. the reaction of alkaline copper sulphate solution with peptide bonds and the reduction of phosphomolybdic acid and phosphotungstic acid by the aromatic amino acids present in the protein. The blue colour developed is quantitatively proportional to the total protein in the medium which is measured colorimetrically at 540nm.

**Method**

A known amount of tissue was homogenized in a definite volume of distilled water. To the sample test tubes containing 0.2 ml of tissue homogenate 0.6 ml of distilled water and 4 ml of alkaline copper sulphate solution [a mixture of 100 ml of alkaline Na-K tartrate (0.1 N NaOH, 2% sodium carbonate, 0.01% Na-K tartrate) and 2 ml of 0.5% copper sulphate] were added. In the blank test tube, instead of the homogenate same volume of distilled water was taken. The test tubes were kept for incubation at 37°C for 20 minutes. Then 0.4 ml of Folin-Phenol [one part of folin-phenol solution...
diluted with two parts of distilled water] was added to all the tubes and was mixed thoroughly. The test tubes were allowed to stand at room temperature for 20 minutes. The optical density was read at 540 nm on a systronics digital spectrophotometer (Model 106).

**Calculation**

\[
\text{Concentration of protein} = \frac{X \times \text{Dilution} \times 100}{T \times \text{aliquot volume} \times 100}
\]

\[
X = 807.35144(Y) - 74.8043
\]

Where, \(X\) = Concentration of Sample obtained from regression formula.

\(Y\) = Optical density (O.D) of unknown Sample.

The concentration in the testis was expressed as mg protein / 100mg fresh tissue weight.

**SERUM PROTEIN**

Protein concentration in the serum was calculated by using the regression formula described.

**Calculation**

\[
X = 807.35144(Y) - 74.8043
\]

Where, \(X\) = Concentration of Sample obtained from regression formula.

\(Y\) = Optical density (O.D) of unknown Sample.
Protein levels

\[ X \times \text{Dilution} \]

\[
\text{Protein levels} = \frac{X \times \text{Dilution}}{\text{Aliquot volume} \times 1000}
\]

Where, dilution=10ml

Aliquot volume=0.2ml

The concentration was expressed as mg protein/ml serum.

Cholesterol

The levels of cholesterol were estimated by the method of Zlatkis et al. (1953) in steroidogenic organs (testis and adrenal).

Principle

In the presence of concentrated sulphuric acid and glacial acetic acid, cholesterol forms a colored complex with ferric chloride that can be measured at 540 nm.

Method

The tissue was homogenized in glacial acetic acid. 0.2 ml of homogenate was added to the test tubes containing 5ml of working ferric chloride solution (1ml of stock 10% FeCl₃ solution diluted to 100ml with glacial acetic acid). 0.2ml of standard cholesterol (1mg/ml) and 0.2ml of glacial acetic acid were taken in the standard and blank tubes respectively. 3ml of concentrated sulphuric acid was added to all the test tubes. The test tubes were then allowed to stand at room temperature. The optical density was read at 540 nm on a Systronics digital Spectrophotometer (Model 166)
Calculation

Concentration of cholesterol

\[
\text{Concentration of cholesterol} = \frac{\text{OD of sample} \times \text{conc. of std} \times \text{dilution} \times 100}{\text{OD of Std.} \times \text{tissue weight} \times \text{aliquot volume} \times 1000}
\]

Where, dilution=1ml

\[
\text{Aliquot volume}=0.2\text{ml}
\]

\[
\text{Concentration of standard}=0.2\text{mg}
\]

The cholesterol levels were expressed as mg/100 mg fresh tissue weight.

SERUM CHOLESTEROL

The levels of cholesterol in the serum of control and all treated groups of rat was estimated by the method of Zlatkis et al (1953) as mentioned earlier by diluting the serum accordingly.

\[
\text{Serum cholesterol} = \frac{\text{OD of sample} \times \text{conc. of std} \times \text{dilution}}{\text{OD of Std.} \times \text{aliquot volume}}
\]

Where, dilution=10ml

\[
\text{Aliquot volume}=0.2\text{ml}
\]

\[
\text{Concentration of standard}=0.2\text{mg}
\]

The cholesterol levels were expressed as mg/ml serum.

TOTAL SULFHYDRYL (-SH) GROUPS

In testis Level of total sulfhydryl groups was estimated by using Ellman's reagent, modified by Sedlak and Lindsay (1968)
**Principle**

5-5' dithiobis-2-nitrobenzoic acid (DTNB) is reduced by -SH groups of samples to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of -SH. The reaction is carried out at pH 8.0. The nitromercaptobenzoic acid anion has an intense yellow colour and can be used to measure -SH groups.

**Method**

0.5 ml of aliquot of homogenate (made in 0.02M tris-EDTA) were mixed with 1.5 ml of 0.2M tris-EDTA buffer (pH 8.2), 0.1 M DTNB 999mg/ 25 ml methanol). The mixture was made upto 10 ml with 7.9 ml of absolute methanol. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The test tubes were allowed to stand with occasional shaking for 15 minutes. The the reaction mixtures were centrifuged at approximately 3000g at room temperature for 15 minutes. The absorbance of the supernatants was read in a spectrophotometer at 412 nm against blank tube.

**Calculation**

Levels of total -SH groups in tissue were calculated using the following formula:

\[
\frac{\text{OD of sample}}{\text{OD of standard}} \times \frac{\text{conc. of standard}}{\text{tissue weight}} \times \frac{\text{dilution}}{\text{aliquot volume}}
\]

The units were expressed as mg/ 100 mg tissue weight.
SUPEROXIDE DISMUTASE (EC 1.15.1.1)

The activity of SOD in testis and adrenal were assayed by the modified spectrophotometric method of Kakkar et al. (1984).

Principle

In this method, the formazan formed at the end of the reaction in presence of the enzyme was extracted and the activity was calculated. Here one unit of enzyme activity was calculated which is defined as the enzyme concentration required to inhibit 50% of the OD of chromagen formed in one minute at 560 mm under the assay condition and expressed as million units/million spermatozoa.

Method

The known amount of homogenate was centrifuged in cold condition. To the pellet 2ml of hypotonic solution (0.56% KCl) was added to yield the enzyme extract. After 15 minute, this was centrifuged at 3000 rpm and the supernatant was used as the sample. In the assay system, the control consisted of 2.4 ml pyrophosphate buffer (0.052m sodium pyrophosphate buffer at a PH 8.4 adjusted with 0.052 M NaH₂PO₄. 2H₂O), 0.1ml of freshly prepared phenazine methosulphate (186 mm freshly prepared nitro blue tetrazolium) and 0.2 ml of fresh NADH (780 mm NADH in double distilled water). To the sample system 0.2ml of enzyme solution was added prior to the addition of NADH in a total of 3ml of the assay system. There the reaction was stopped by the addition of 1ml of acetic acid, exactly 90 seconds after the addition of NADH. Then 4 ml of n-butanol was added to the tubes and shaken
vigorously to extract the formazan. Then the system was centrifuged for 10 minute at 2000 rpm and the supernatant was used for the measurement of OD at 560 mm against the butanol on a systronic 118v visible spectrophotometer. The activity was calculated and expressed as units/million spermatozoa.

Calculation

\[
X = \frac{\text{OD of control} \times \text{dilution}}{\text{accuracy factor} \times \text{Std enzyme units}} \frac{\text{OD of sample}}{\text{accuracy factor} \times \text{Std enzyme units}}
\]

Where, accuracy factor = 1.0

Std. enzyme unit = 3.0

Therefore, Enzyme activity = \[
\frac{X}{\text{mg protein}}
\]

The activity was expressed as unit SOD/mg Protein. The Protein levels were estimated by the method of Lowry et al., (1956).

GLUTATHIONE PEROXIDASE (EC 1.11.1.9)

The estimation of glutathione peroxidase in steroidogenic organs were done by the method of Mohandas et al. (1967).

Method

A known amount of tissue was homogenized in a known volume of 0.01% digitonin and centrifuged at 12,000 g for 30' at 0-15°C. The supernatant was used as an aliquot. Briefly, the assay mixture contained 1.59
ml phosphate buffer (100 mM, pH 7.6), 100 μl EDTA (10 mM), 100 μl sodium azide, 50 μl glutathione reductase, 100 μl H₂O₂ and 10 μl enzyme source. Disappearance of NADPH was measured immediately at 340 nm, against blanks containing all the components except the enzyme source, at 10 s intervals for 3 min on a Systronics spectrophotometer.

Calculation

\[
\text{GPx} = \frac{\Delta A/\text{min} \times \text{volume of assay system}}{6.22 \times 10^6 \times \text{Volume of enzyme source} \times \text{mg protein}}
\]

The activity of glutathione peroxidase in the testis was expressed as nmol NADPH oxidized/ min/ mg protein.

GLUTATHIONE REDUCTASE (EC 1.6.4.2)

The estimation of glutathione reductase in testis and adrenal were done by the method of Carlberg and Mannervik (1975).

Freshly weighed tissue were homogenized in a known volume of 0.2 M potassium phosphate buffer (pH 7.6) and centrifuged at 10,000 × g for 20 minutes at 4°C. The two ml assay mixture contains 1.75 ml phosphate buffer, 0.1 ml of NADPH, 0.1 ml of EDTA, 0.05 ml glutathione oxidized and 0.05 ml enzyme source was added to initiate the reaction. The decrease in absorbance at 340 nm was followed for 3 min. at exactly 1 minute interval. Blank tubes were run replacing sample supernatant (enzyme) with buffer. The difference in the absorbance was obtained by subtracting the blank readings from the sample readings.
Calculation

\[
\text{GR} = \frac{\Delta A / \text{ min} \times \text{volume of assay system}}{6.22 \times 10^6 \times \text{Volume of enzyme source} \times \text{mg protein}}
\]

The activity of glutathione reductase in testis and adrenal were expressed as nmol NADPH oxidized/ min/ mg protein.

LIPID PEROXIDATION

Lipid peroxidation in steroidogenic organs were estimated by the method of Ohkawa et al. (1979)

Principle

The method is based on the formation of red chromophore that absorbs 532nm. Following the reaction of thiobarbituric acid with malondialdehyde (MDA) and other break down products of peroxidised lipids collectively called as thiobarbituric acid reactive substances.

Method

A known amount of tissue was washed with 0.1 M Phosphate buffered saline (PBS) [pH 7.4] several times. The tissue was then blotted dry, weighed and analysed immediately or stored at 20°C for not more than 24 hours. The tissue was then homogenized in PBS to prepare 10 % homogenate. Then 0.2 ml of 10% tissue homogenate was added to the tubes containing 0.2ml of 8.1% sodium diodecyl sulphate (SDS), 1.5ml of 20% acetic acid (adjusted to pH 3.5 with 1 N NaOH ) and 1.5ml of 1% TBA solution. Blank was prepared for each sample by substituting the TBA solution with distilled water. The final
volume was made to 4ml with distilled water. The solution was mixed and heated in waterbath at 95°C for 60 minutes. The tubes were then immediately cooled and 2ml of aliquot was transferred to a centrifuge tube, to which an equal volume of 10% TCA was added. The solutions were mixed and centrifuged at 1000g for 15 minutes. The aliquot of the resulting supernatant fraction was read against blank on a systronics digital spectrophotometer (Model 166) at 532 nm.

**Calculation**

\[
\text{Concentration of MDA} = \frac{\text{OD of sample} \times \text{dilution factor} \times 10^9}{E \times \text{tissue weight (mg)}}
\]

Where, \( E = \text{Extinction Co-efficient for MDA} = 1.56 \times 10^5 \)

The results were expressed as nano moles of MDA / mg tissue weight / 60'

**TOTAL ASCORBIC ACID**

Levels of total ascorbic acid was estimated in the testis and adrenal of control and all treated groups of rat by the method of Roe and Kuether (1943).

**PRINCIPLE**

Total ascorbic acid (TAA) was oxidized to dehydroascorbic acid (DHA) by Norit Reagent in the presence of trichloro acetic acid (TCA). This couples with 2,4 dinitrophenyl hydrazine to yield a red coloured complex by the action of sulphuric acid which was measured colorimetrically.
METHOD

For total ascorbic acid (TAA), the homogenate was prepared in 10 ml Norit Reagent prepared by dissolving 2 g of activated charcoal in 100 ml 6% TCA and filtered through Whatman filter paper No.42. To 4 ml aliquot of homogenate, 1 ml of 2% 2,4-dinitrophenyl hydrazine reagent was added followed by a drop of 10% thiourea to activate the reaction. The blank tube was run with 4 ml of 6% TCA instead of homogenate and the standard tube contained 4 ml of ascorbic acid solution (10 µg/ml). The contents of the tubes were mixed well and kept in boiling water bath for 15 minutes and thereafter cooled in an ice bath. Then 5 ml of 85% sulphuric acid was added along the sides of the tube kept in an ice bath. The tubes were allowed to stand for 30 minutes and the optical density was measured at 540 nm against blank on a spectronic 103 colorimeter.

CALCULATION

The concentration of ascorbic acid was calculated by the formula:

\[
\text{Total Ascorbic Acid Activity} = \frac{\text{O.D.of sample}}{\text{concentration of standard}} \times \frac{\text{Dilution}}{\text{Aliquot volume}} \times \frac{\text{O.D.of standard}}{\text{tissue weight in mg}} \times 1000
\]

Where, Concentration of standard = 0.04 mg/ml

Dilution = 10 ml

Aliquot volume = 4 ml

The units are mg/g tissue weight.
GLUTATHIONE (GSH)

Glutathione levels in testis and adrenal were determined by the method of Glutnerd and Philips (1951).

PRINCIPLE

Glutathione (GSH) present in the tissue reacts with sodium nitroprusside to give a red colored complex in saturated alkaline medium.

METHOD

A known amount of tissue was homogenized in 3ml of 3% metaphosphoric acid and 1ml of distilled water and saturated with salt (1.5g NaCl crystals) and centrifuged. 2ml of aliquot was added to the sample tube containing 6ml of saturated NaCl solution and allowed to stand for 10 minutes at 20°C for equilibrium. The blank tube was run with 2ml of 2% metaphosphoric acid instead of the aliquot. Then 1ml each of (0.067 M) sodium nitroprusside and (1.5M) sodium cyanide mixture were added to blank and sample tubes respectively. The colored complex developed was measured at 520nm on a Bausch and Lomb Spectronic 88 Spectrophotometer within 1 minute.

CALCULATION

The level of glutathione was calculated using the regression formula:

\[ X = 272.01(Y) - 2.32 \]

Where, \( X \) = concentration of glutathione.

\( Y \) = optical density of the sample

The value of \( X \) was substituted in the formula.
Glutathione level = \frac{\text{Concentration}(X) \times \text{dilution} \times 100}{\text{Tissue weight (mg) \times \text{aliquot volume}}}

Where, \text{dilution} = 2\text{ml}

Glutathione levels were expressed as \mu\text{g}/100\text{mg} tissue weight

**CATALASE (CAT: EC; 1.11.1.6)**

The catalase activity in steroidogenic organs of control and all treated animals was assayed by the modified method of Luck (1963).

**METHOD**

A known volume of fresh tissue was homogenized in a known volume of 0.01% chilled digitonin and centrifuged at 10000rpm for 30 minutes at 4°C. The supernatant was used as an aliquot. The assay mixture consisted of 0.5 ml of 50 mM phosphate buffer (pH7.0) and 1 ml aliquot. The blank tube contained complete reaction mixture without the aliquot. The decrease in absorbance was noted every 5 seconds for 30 seconds at 240 nm on a systronics UV-Visible Spectrophotometer (Model No.118).

**CALCULATION**

\[
X = \frac{\text{O.D. of sample} \times \text{Total volume of assay (1.5ml)}}{\text{Aliquot volume (1ml) \times E}}
\]

Where = \text{Extinction coefficient} = 0.0041 \text{m mol}^{-1} \times \text{mm}^{-1}

\[
\text{Catalase activity} = \frac{X}{\text{mg protein concentration}}
\]
The enzyme activity was expressed as moles of H$_2$O$_2$/minute/mg protein. The protein levels were estimated by the method of Lowry et al (1951).

**MERCURY LEVELS**

Mercury levels were done in the steroidogenic organs by the acid digestion method of Allen (1989) as mentioned by Subramaniam et al (1995).

**Method**

A known amount (200 or 500 mg) of air dried or oven dried ground sample (tissue) is weighed and dissolved into a 50 ml kjeldahl flask. 1 ml of 60% perchloric acid, 5 ml of concentrated nitric acid and 0.5 ml of sulphuric acid were added into this tissue sample. Mixed gently and digested slowly at moderate heat (within 45°C). Then the digested sample was cooled and transferred to volumetric flask and diluted to a required volume. Blank digestions were carried out in the same way.

The digested sample was then analyzed by making use of the cold vapor atomic absorption spectrometry technique. The instrument model used is MA 5840, Mercury Analyzer of Electronics Corporation of India Ltd., Hyderabad, India.

Standard curve was plotted using mercury concentration from 10-100 ppb.

Quality control studies using reference materials viz. human hair (GBW-09, 101), tuna fish (IAEA 350) and Copepod homogenate (MA-A-a) for the
Mercury estimation were also done with recovery percentage of 94-95%. Mercury levels were calculated using the following formula:

\[
\text{Mercury content (ng/g)} = \frac{\text{Mercury content in aliquot (ng)} \times 3}{\text{weight of the sample taken (g)}}
\]

\[
\text{Mercury content (ng/ml)} = \frac{\text{Mercury in the aliquot taken (ng)}}{\text{Volume of the aliquot taken (ml)}}
\]

Non-steroidogenic organs (Pancreas and Thyroid gland)
Organ weights of the non-steroidogenic organs were recorded after clearing to the nearest gm or milligram on a torsion balance.

Biochemical analysis in non-steroidogenic organs
In the thyroid and pancreas the activities of antioxidative enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPx; EC 1.11.1.9) and glutathione reductase (GR; EC 1.6.4.2) were assayed by the spectrophotometric method of Kakker et al. (1984), modified method of Luck (1963), Mohandas et al. (1967) and Carlberg and Mannervik (1975) respectively.

The assay of glutathione (GSH) and Lipid peroxidation (LPO) were determined by Grunert and Philips (1951) and Ohkawa et al. (1979) correspondingly in the non-steroidogenic organs.

In the non-steroidogenic organs the estimation of alkaline phosphatase (ALPase; E.C.3.1.3.1) and acid phosphatase (ACPase; E.C.3.1.3.2) were done by the methods of Bessey et al. (1946) while protein, adenosine
triphosphatase (ATPase; E.C.3.6.11.3) and succinate dehydrogenase (SDH; E.C.1.3.99.1) were determined by the methods of Lowry et al. (1951), Quinn and White (1968) and Beatty et al (1966) respectively.

Mercury levels were measured in the thyroid and pancreas by the acid digestion method of Allen (1989) as mentioned by Subramaniam et al (1995). These techniques were mentioned earlier.

SERUM AMYLASE (EC; 3.2.1.1)

PRINCIPAL

Amylase in the specimen acts on the substrate, starch. The products formed are dextrins and maltose. After the incubation, the end products are treated with the colour reagent (iodine reagent). A decrease in the blue colour is directly proportional to the amylase concentration in the specimen and gives the measure of amylase present in the specimen.

METHOD

2.5ml of buffered substrate was incubated at 37°C for 5 minutes. 0.1ml of serum was added in test sample tubes. The tubes were mixed and incubated for 7½ min at 37°C. Then 2.5 ml of working color reagent was added to all the tubes, solutions were then mixed thoroughly, and read intensities of test sample

CALCULATION

\[
\text{Serum Amylase (C.U.)} = \frac{\text{O.D. of Blank} - \text{O.D. of sample}}{\text{O.D. of Blank}} \times 400
\]
Caraways units: Caraways defined the enzyme unit as the amount of enzyme that hydrolyses 10mg of starch in 30 minutes to a colorless stage.

ANALYSIS OF BLOOD GLUCOSE

The estimation of glucose in blood of control and all treated groups of rat was carried out by method of Nelson and Somogyi (1945).

PRINCIPLE

The protein free blood filtrate was heated with alkaline copper tartarate reagent in a Florin-Wu tube to prevent oxidation and then treated with arsenomolybdate color reagent giving a blue colored substances (Molybdenum blue). The intensity of the blue color was measured using a systronics 106 colorimeter at 680 nm and compared with the standard.

METHOD

0.1ml of freshly collected heparinised blood was taken in 3.5ml of distilled water, into which 0.2 ml of 0.3 N Barium hydroxide (Ba(OH)$_2$) and 0.2 ml of 5% Zinc sulphate (ZnSO$_4$) were added. The contents were mixed in the tubes and then centrifuge the clear protein free supernatant was used for estimation.

Protein free supernatant was pipette into a tube followed by 1ml of alkaline copper tartarate reagent. The standard tube contained 1ml of sugar solution containing 0.025mg/ml, while 1 ml of distilled water was added to the blank tube. The tubes were heated in boiling water bath for fifteen minutes after which they were cooled under running tap water and 1ml of
arsenomolybdate reagent was added. The contents were diluted up to 10ml with distilled water and mixed well. The optical Density was read in a Systronics 106 colorimeter at 680 nm against blank. The concentration of glucose, present in the blood serum was calculated according to the formula.

**CALCULATION**

\[
\text{Conc. Of Glucose} = \frac{\text{O.D. of sample}}{\text{Conc. Of Standard}} \times \frac{\text{O.D. of Standard}}{0.025} \times 100
\]

The concentration of blood sugar was expressed as mg/100ml of blood.

**HISTOLOGICAL ANALYSIS**

The steroidogenic and non-steroidogenic organs were immediately fixed in Bouins fluid for 24 hrs. Tissue was then cut in 2-4 mm thick pieces and thoroughly washed overnight under running tap water. The tissue was dehydrated in ethanol series, cleared in xylene and embedded in paraffin wax (58°-60°C). 5μm paraffin sections of above tissues were stained with Haematoxylin and Eosin stain.

**Composition of Bouins fluid:**

- Saturated aqueous picric acid solution 75 ml
- 40% formaldehyde 25 ml
- Glacial acetic acid 05 ml
HAEMATOXYLIN AND EOSIN (HE) STAINING

Hematoxylin (Harris, 1900)

Hematoxylin 2.5 ml
Absolute alcohol 25 ml
Aluminum ammonium sulphate 50g
Distilled water 500 ml
Mercuric oxide 1.25 g

The hematoxylin was dissolved in absolute alcohol and then added to aluminum ammonium sulphate, which was previously dissolved in warm distilled water. The mixture was rapidly brought to boil and then mercuric oxide was added. The stain was rapidly cooled by plunging the flask into cold water. The stain was kept in light for one week to allow further natural ripening prior to use.

EOSIN

Eosin Y 500 mg
70% ethyl alcohol 100 ml

After dissolving eosin, 2 drops of glacial acetic acid were added to obtain sharp staining and proper differentiation.

ALCOHOL SERIES:

A graded series of alcohol, viz. 30%, 50%, 70%, 90% and 95% alcohol were prepared from absolute ethyl alcohol.
Procedure:

The sections were deparaffinized and hydrated through xylene, alcohol series and distilled water and then immersed in hematoxylin. After 4 minutes, sections were thoroughly washed under running tap water. Differentiation and potentiation of nuclear staining was done in acidic water (2 drops of conc. HCL in a coupling jar full of distilled water) and ammonia water (2-4 drops of ammonium hydroxide solution in a coupling jar full of distilled water). The sections were rinsed in 70% ethanol, counterstained with eosin, differentiated and dehydrated in alcohol series, cleared in xylene and mounted in D.P.X. All the stained slides were seen under microscope and photographs were taken at different magnifications in Leica binocular microscope with a camera attached.

HYSTOCYTROMETRY STUDIES

Transverse sections of the steroidogenic and non-steroidogenic organs were studied under the microscope. Treated and control groups were critically analysed for following specific histological changes.

Steroidogenic organs

Testis
1. Seminiferous tubular diameter.
2. Seminiferous epithelial cell height
3. Lumen diameter
4. Leydig cell diameter
5. Leydig cell nucleus diameter

Adrenal

1. Diameter of the cortex region
2. Diameter of the medullar region

Non-steroidogenic organs

Pancreas

1. Diameter of the islets of langerhance
2. Diameter of the islets of langerhance cell nuclei

Thyroid

1. Diameter of the follicular cell
2. Diameter of the follicular cell nuclei

Calculations were done by using Abercrombies (1947) formula:

\[ A = \frac{C \times T}{T + D} \]

Where, \( A \) = Absolute count \( T \) = Thickness of the section (m) \( C \) = Crude count \( D \) = Diameter of the cell (nucleus) (um)

Histocytometry in different organs were observed by using an ocular lens with a micrometer scale. The nuclear diameters of cells were measured at 1000x magnification.
Statistics

For each parameter a minimum of 8 replicates were done and the results were expressed as Mean ± SE (Standard Error). All the treated groups have compared with control groups. The data were then statistically analyzed by Student 't' test and Analysis of variance (ANOVA).