CHAPTER 3

MATERIAL AND METHODS

3.1 MATERIALS

3.1.1 Sources of Drugs for study

Doxorubicin and cisplatin injections were gift sample from Serum Institute of India Ltd., Pune. Standardized powdered, ethyl acetate extract of green tea leaves (*Camellia sinensis*) was gift sample from Cherain Chemicals, Baroda, India with total polyphenolic content 35%. Lovastatin was gift sample from Mercury Labs Pvt Ltd, Vadodara, Gujarat, India. Melatonin, and resveratrol were purchased from Sigma Aldrich; USA.

3.1.2 Sources of Fine Chemicals

(a) Dimethylbenzanthracycline (DMBA) 1, 1, 3, 3-tetraethoxypropane, crystalline beef liver catalase, superoxide dismutase, and 1, 1-diphenyl-2-picryl hydrazyl (DPPH) (Sigma Chemicals, St. Louis, M.O., U.S.A).

(b) Epinephrine bicarbonate, Thiobarbituric acid, tris buffer, sucrose, ATP, reduced glutathione, 5,5'-dithiobis (2-nitro benzoic acid), bovine serum albumin, riboflavin, naphthylenediamine di hydrochloride, nitro blue tetrazolium (NBT) and pyrogallol (Hi-Media Laboratories Pvt. Ltd., Mumbai, India).

(c) Trichloroacetic acid, ammonium molybdate, methylene blue, citric acid monohydrate, sodium nitrate, sulphanilic acid, hydrogen peroxide, copper sulphate, sodium potassium tartarate, methanol, ethanol, ethylenediaminetetraacetic acid disodium salt and Folin's phenol reagent (S.D. Fine Chemicals, Mumbai, India).

(d) Sodium hydroxide, sodium carbonate, sodium bicarbonate, magnesium chloride, sodium chloride, potassium chloride, calcium chloride, disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate, carbon tetrachloride, chloroform, ether, hydrochloric acid and conc. sulphuric acid (Qualigens Chemicals Ltd., Mumbai, India).

All the reagents and chemicals used in the entire study were of analytical grade.

3.1.3 Sources of standard kits

Aspartate aminotransferase (GOT), creatinine, urea and uric acid (Span Diagnostics Ltd., Surat, India), Lactate dehydrogenase (LDH) and Creatine kinase (CK) (Reckon Diagnostics Pvt. Ltd., Baroda). Testosterone level was estimated by direct chemiluminescent assay (ADVIA CENTAUR).

3.1.4 Chemical analysis of green tea extract

TLC fingerprint profile of the extract was established using HPTLC. For development of TLC fingerprint, 500 mg of powdered green tea extract was extracted with (3x25 ml) of methanol. Extracts were pooled, filtered and concentrated to 25 ml. Suitably diluted stock solution of methanolic extract with gallic acid standard solution and catechin were spotted on a pre-coated Silica gel G60 F254 TLC plate (E.Merck) using CAMAG Linomat IV Automatic Sample Spotter and the plate was developed in the solvent system of Toluene: Ethyl acetate: Formic acid (6: 6: 1). The plate was dried at room temperature and scanned using CAMAG TLC Scanner 3 at UV 254 nm and Rf values, and peak area of the resolved bands were recorded. Relative percentage area of each band was calculated from peak areas. The TLC plate was derivatised by spraying with 5% methanolic ferric chloride solution for the detection of phenolic compounds.

3.1.5 Animals

Albino rats of Wistar strain weighing between 200-250gm and Sprague Dawley strain weighing between 45-55gm were used for the experiments. All the animals were from in house animal breeding. The animals were fed *ad libitum* with standard pellet diet and had free access to water.

All the protocols of animal experiments were approved by the Institutional Ethics Committee in accordance to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, New Delhi.

3.2 METHODS (EXPERIMENTAL DESIGN)

The study was designed to evaluate the antioxidant activity of green tea extract (GTE), melatonin, resveratrol and lovastatin on the oxidative stress induced by anticancer drugs such as doxorubicin and cisplatin. The study was categorized into two sections: *in vitro* and *in vivo* studies.

3.2.1 *IN VITRO* STUDIES

To ascertain free radical scavenging property of green tea extract (GTE), melatonin, lovastatin and resveratrol, *in vitro* methods were performed in four systems, namely radical scavenging activity by DPPH reduction (DPPH assay), superoxide radical scavenging activity in riboflavin/light/NBT system, nitric oxide scavenging activity and lipid peroxidation inhibition.

Drug solutions for *in vitro* studies:

Green tea extract was dissolved in distilled water to get 1000 µg/ml concentration. Lovastatin, melatonin and resveratrol were dissolved in methanol to get 1000 µg/ml concentration. The concentrations from 20 µg/ml to 100 µg/ml were selected for study.

3.2.1.1 DPPH assay

Principle

DPPH assay was used as a rapid method to provide an evaluation of antioxidant activity due to free radical scavenging. Antiradical activity was measured by a decrease in absorbance at 516 nm of a methanolic solution of coloured DPPH brought about by the sample. 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) a purple coloured stable free radical is reduced into the yellow coloured diphenylpicryl hydrazine (Vani et al., 1997).

Reagents

1. DPPH stock solution

1.3 mg of 1, 1-diphenyl-2-picryl hydrazyl (DPPH) was dissolved in 1ml of methanol.

Procedure

100 µl of suitably diluted stock solution of either aqueous or methanolic solutions of the drugs were mixed with 3ml of methanol. 75µl of DPPH solution was added and decrease in the absorbance was noted after 15

minutes at 516nm against methanol as blank. Curcumin was used as positive control.

The obtained data was used to determine the ability of the sample to scavenge the DPPH free radicals. The results were expressed as the mean ± SEM of three replicates.

3.2.1.2 Assay for superoxide radical scavenging activity

Principle

The assay was based on the capacity of the methanolic or aqueous solutions of drugs to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system (Beauchamp and Fridovich, 1971).

Reagents

1. *Phosphate Buffer (50mM, pH 7.6)*

(a) 2.72 gm of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water.

(b) 800mg of sodium hydroxide (NaOH) was dissolved in 60 ml of distilled water. The volume was then made up to 100 ml with distilled water.

50 ml of (a) was mixed with 42.4ml of (b). The pH of the mixture was adjusted to 7.6 with 1N hydrochloric acid (HCl). The volume was then made up to 200 ml with distilled water.

2. *Riboflavin*

5mg of riboflavin was dissolved in 25ml of phosphate buffer (50mM, pH 7.6).

3. *EDTA (12mM)*

402 mg of EDTA was dissolved in 5ml of buffer. The volume was then made up to 10 ml with phosphate buffer.

4. *Nitro blue tetrazolium (NBT) (0.1%)*

5mg of NBT was dissolved in 2ml of buffer. The volume was then made up to 5ml with phosphate buffer.

Procedure

The reaction mixture contained 2.5ml buffer, 100µl riboflavin solution, 200µl of EDTA solution, 100µl of diluted methanolic or aqueous solutions of drugs and 100µl of NBT solution, added in the mentioned sequence. Reaction was started by illuminating the reaction mixture in UV light for 5 minutes.

Immediately after illumination the absorbance was measured at 290nm. Ascorbic acid was used as positive control. The percent inhibition by sample exposure was determined by comparison with a methanol-treated control group.

3.2.1.3 Assay for nitric oxide scavenging activity

The assay is based on the principle that sodium nitroprusside in aqueous solution at physiologic pH liberates nitric oxide at steady rate which is converted to nitrite that can be measured by reaction with Griess reagent. The agents having nitric oxide scavenging activity will reduce the nitrite formation. (Sreejayan and Rao, 1997)

Reagents

1. Phosphate buffered saline (PBS) pH *7.4*

1.38 gm of disodium ethylenediaminetetraacetic acid, 0.19 gm of potassium dihydrogen phosphate and 8 gm of sodium chloride was dissolved in 900 ml of distilled water and adjusted pH using dilute hydrochloric acid. The volume was adjusted to 1000 ml using distilled water.

2. Griess reagent

1gm of sulphanilamide, 2gm phosphoric acid and 0.1 gm naphthylenediamin dihydrochloride was dissolved in 100 ml of distilled water.

3. Sodium nitroprusside solution (5 mM)

15 mg of Sodium nitroprusside was dissolved in 10 ml of phosphate buffered saline.

Procedure

All the reagents were freshly prepared. All the drug solutions were added (20 to 100 µl) to 1.5 ml sodium nitroprusside solution and incubated at room temperature for 150 minutes. Blank without test but equivalent amount of either methanol or distilled water was conducted in identical manner. After incubations the solutions were removed and equal amount of Griess reagent was added. The absorbance was chromophore was measured at 546 nm. Curcumin was used as standard.

3.2.1.4 Measurement of effect on lipid peroxidation in rat liver homogenate-

The study was based on principle that ferric chloride in presence of ADP and ascorbic acid induces oxidative stress to liver homogenate thereby producing MDA as lipid peroxidation product. Antioxidant will minimise the formation of MDA (Sreejayan and Rao, 1993 and 1994).

Reagents

1. Tris buffer (pH 7.4)

1.21gm tris was dissolved in 900 ml of distilled water and the pH was adjusted to 7.4 with 1M hydrochloric acid. The resulting solution was diluted to 1000 ml with distilled water.

2. Trichloroacetic acid (10% w/v)

10gms of trichloroacetic acid was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

3. Adenosine diphosphate (ADP) (1.7 μM)

1.5 mg of Adenosine diphosphate (ADP) was dissolved in 10 ml tris buffer (pH 7.4).

4. Ascorbate solution (500 μM)

17.6 mg of Ascorbic acid was dissolved in 10 ml tris buffer (pH 7.4).

5. Ferric chloride solution (100 μM)

16.22 mg of Ferric chloride was dissolved in 100 ml tris buffer (pH 7.4).

6. Thiobarbituric acid solution

0.67gm of thiobarbituric acid was dissolved in 50 ml of hot distilled water and the final volume was made up to 100 ml with hot distilled water.

Procedure

Rat liver was excised and processed in the manner similar to heart as described in section to get homogenate. 1.5 ml of homogenate was mixed with 10 μl ADP solution, 100 μl ascorbic acid, 200 μl of ferric chloride solution and 20 to 100 μl of drug solutions in sequence. The volume was made to 2 ml with tris buffer. The reaction was started by incubating at 37 ^{0} C for 20 minutes. The reaction was stopped by adding 2 ml of TCA solution and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation and 2.0 ml of clear supernatant solution was mixed with 2.0 ml of freshly prepared

thiobarbituric acid (TBA). The resulting solution was heated in a boiling water bath for 10 minutes. It was then immediately cooled in an ice bath for 5 minutes. The colour developed was measured at 532nm against reagent blank.

3.2.2 *IN VIVO* STUDIES

The drugs green tea extract, melatonin and lovastatin were tested for their antioxidant activity by using *in vivo* experimental models like doxorubicin induced cardiotoxicity, cisplatin induced nephrotoxicity in addition to testicular toxicity where as resveratrol was tested against DMBA induced mammary gland cancer. Doxorubicin and cisplatin injection was reconstituted in sterile water for injection. Powdered green tea extract was reconstituted in distilled water. Melatonin, lovastatin and resveratrol were suspended in 0.5% Sodium Carboxy Methyl Cellulose (CMC) solution just before use. The experimental models were divided into various sets, which are follows:

- **Set 1: Doxorubicin induced cardiotoxicity**
- **Set 2: Cisplatin induced nephrotoxicity**
- **Set 3: Doxorubicin induced testicular toxicity**
- **Set 4: Cisplatin induced testicular toxicity**
- **Set 5: DMBA induced mammary gland cancer**

SET 1: DOXORUBICIN INDUCED CARDIOTOXICITY

A: Acute study

The experiment was performed using rats from Wistar strain of either sex weighing 200 to 250 grams. The animals were divided into following groups. Each group consisted of six rats.

Group 1: Control animals, received vehicle (3 ml/kg/day p.o. for 30 days) followed by sterile water for injection (1 ml/kg, i.v.) on 30th day.

Group 2: Animals received vehicle (3 ml/kg/day p.o.for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 3a: Animals received green tea extract (25 mg/kg/day p.o.for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 3b: Animals received green tea extract (50 mg/kg/day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 3c: Animals received green tea extract (100 mg/kg/day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 4a: Animals received melatonin (3 mg/kg /day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 4b: Animals received melatonin (6 mg/kg /day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 5a: Animals received lovastatin (3 mg/kg /day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 5b: Animals received lovastatin (6 mg/kg /day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

The change in body weight was recorded weekly. After 48 hours of the injection of doxorubicin or vehicle, electrocardiographic changes were recorded. Blood was collected and serum was separated for estimations of creatine kinase (CK), lactate dehydrogenase (LDH), and SGOT. The animals were sacrificed and the heart was dissected out and weighed. It was then processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione; membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on heart were also carried out.

B: Chronic study

The experiment was performed using rats from Wistar strain of either sex weighing 200 to 250 grams. The animals were divided into following groups. Each group consisted of six rats.

Group 1: Control animal received vehicle (3 ml/kg /day p.o. for 30 days) and sterile water for injection (1ml/kg, i.p.) on day 1, 7, 14, 21, 28)

Group 2: Animals received vehicle (3 ml/kg /day p.o. for 30 days) and doxorubicin injection (3 mg/kg, i.p.) every week (days 1, 7, 14, 21 and 28).

Group3a: Animals received green tea extract (25 mg/kg/day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group3b: Animals received green tea extract (50 mg/kg/day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group3c: Animals received green tea extract (100 mg/kg/day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 4a: Animals received melatonin (3 mg/kg/day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 4b: Animals received melatonin (6 mg/kg /day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and28).

Group 5a: Animals received lovastatin (3mg/kg /day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) on 30^{th} day.

Group 5b: Animals received lovastatin (6mg/kg /day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

The change in body weight was recorded weekly. The study was done in two sets for each group. One for measurement of markers of oxidative stress and other for haemodynamic measurement. After 48 hours of the last dose of doxorubicin or vehicle, blood was collected and serum was separated for estimations of creatine kinase (CK), lactate dehydrogenase (LDH), and SGOT. The animals were sacrificed and the heart was dissected out and weighed. It was then processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione; membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on

heart were also carried out. Second set of animals used for hemodynamic measurements.

Electrocardiographic measurements: After 48 hours of the last injection of either doxorubicin or vehicle, ECG was recorded under light ether anesthesia through needle electrodes (Lead II) using Biopac MP30 data acquisition system (Biopac Systems, Santa Barbara, CA). The changes in QT interval, ST interval and Heart rate were determined from ECG.

Hemodynamic measurements:

Measurement of Blood pressure by Non invasive method (indirect method): For arterial blood pressure measurements using tail cuff method, rats were trained for at least one week until the blood pressure was recorded with minimal stress and restraint. Systolic BP, Diastolic BP and Mean BP were measured once in a week (Tail cuff) using LE 5002 storage pressure meter.

Measurement of Blood pressure by Invasive method (direct method): After completion of treatment schedule, animals were anesthetized with urethane (i.p. 1.2 g/kg) and then a catheter was introduced into the left carotid artery for measurement of arterial BP and HR. The arterial BP was monitored continuously by Biopac MP30 data acquisition system (Biopac Systems, Santa Barbara, CA). Femoral vein was cannulated with a fine polyethylene catheter for administration of drugs. After 30 min. of stabilization of BP, vascular reactivity to various drugs like noradrenaline, adrenaline, isoprenaline, phenylephrine and angiotensin were recorded.

SET 2: CISPLATIN INDUCED NEPHROTOXICITY

A: Acute study

The experiment was performed using rats from Wistar strain of either sex weighing 200 to 250 grams. The animals were divided into following groups. Each group consisted of six rats.

Group 1: Control animals, received vehicle (3 ml/kg/day p.o. for 30days) followed by sterile water for injection (1ml/kg, i.p.) on 30th day.

Group 2: Animals received vehicle (3ml/kg/day p.o.for 30days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

Group 3a: Animals received green tea extract (25 mg/kg/day p.o.for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

Group 3b: Animals received green tea extract (50 mg/kg/day p.o. for 30 days) and cisplatin injection (5 mg/kg i.v.) on 30th day.

Group 3c: Animals received green tea extract (100 mg/kg/day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

Group 4a: Animals received melatonin (3 mg/kg /day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

Group 4b: Animals received melatonin (6 mg/kg /day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

Group 5a: Animals received lovastatin (3 mg/kg /day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

Group 5b: Animals received lovastatin (6 mg/kg /day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

The change in body weight was recorded weekly. Blood was collected 5 days after cisplatin injection and serum was separated for estimations of creatinine, urea, uric acid and blood urea nitrogen (BUN). The animals were then sacrificed and the kidney was dissected out, weighed and processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione, membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on kidney were also carried out.

B: Chronic study

The experiment was performed using rats from Wistar strain of either sex weighing 200 to 250 grams. The animals were divided into following groups. Each group consisted of six rats.

Group 1: Control animal received vehicle (3ml/kg /day p.o. for 30 days) and sterile water for injection (1ml/kg, i.p.) every week on day 1, 7, 14, 21, 28.

Group 2: Animals received vehicle (3ml/kg /day p.o. for 30 days) and cisplatin injection (3 mg/kg, i.p.) every week (days 1, 7, 14, 21 and 28).

Group3a: Animals received green tea extract (25 mg/kg/day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group3b: Animals received green tea extract (50 mg/kg/day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group3c: Animals received green tea extract (100 mg/kg/day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 4a: Animals received melatonin (3mg/kg/day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 4b: Animals received melatonin (6mg/kg /day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 5a: Animals received lovastatin (3mg/kg /day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 5b: Animals received lovastatin (6mg/kg /day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and28).

The change in body weight was recorded weekly. After 48 hours of the last dose of cisplatin or vehicle, blood was collected and serum was separated for estimations of creatinine, urea, uric acid and blood urea nitrogen (BUN). The animals were then sacrificed and the kidney was dissected out, weighed and processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione, membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on kidney were also carried out.

SET 3: DOXORUBICIN INDUCED TESTICULAR TOXICITY

A: Acute study

The experiment was performed using male rats from Wistar strain weighing 200 to 250 grams. The animals were divided into following groups. Each group consisted of six rats.

Group 1: Control animals, received vehicle (3ml/kg/day p.o. for 30 days) followed by sterile water for injection (1ml/kg, i.v.) on 30th day.

Group 2: Animals received vehicle (3ml/kg/day p.o.for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 3a: Animals received green tea extract (25 mg/kg/day p.o.for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 3b: Animals received green tea extract (50 mg/kg/day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 3c: Animals received green tea extract (100 mg/kg/day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 4a: Animals received melatonin (3 mg/kg /day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 4b: Animals received melatonin (6 mg/kg /day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 5a: Animals received lovastatin (3 mg/kg /day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

Group 5b: Animals received lovastatin (6 mg/kg /day p.o. for 30 days) and doxorubicin injection (10 mg/kg i.v.) on 30th day.

The change in body weight was recorded weekly. After 48 hours of the last dose of doxorubicin or vehicle, blood was collected and serum was separated for estimation of testosterone level. Epididymis was removed; the epididymal sperm count was done immediately. The animals were sacrificed and the testes was dissected out and weighed. It was then processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione; membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on testes were also carried out.

B: Chronic study

The experiment was performed using male rats from Wistar strain weighing 200 to 250 grams. The animals were divided into following groups. Each group consisted of six rats.

Group 1: Control animal received vehicle (3 ml/kg /day p.o. for 30 days) and sterile water for injection (1ml/kg, i.p.) on day 1, 7, 14, 21, 28.

Group 2: Animals received vehicle (3 ml/kg /day p.o. for 30 days) and doxorubicin injection (3 mg/kg, i.p.) every week (days 1, 7, 14, 21 and 28).

Group3a: Animals received green tea extract (25 mg/kg/day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group3b: Animals received green tea extract (50 mg/kg/day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group3c: Animals received green tea extract (100 mg/kg/day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 4a: Animals received melatonin (3 mg/kg/day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).
Group 4b: Animals received melatonin (6 mg/kg /day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).
Group 5a: Animals received lovastatin (3 mg/kg /day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).
Group 5b: Animals received lovastatin (6 mg/kg /day p.o. for 30 days) and doxorubicin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).
The change in body weight was recorded weekly. After 48 hours of the last dose of doxorubicin or vehicle, blood was collected and serum was separated for estimation of testosterone level. The epididymal sperm count was done immediately. The animals were sacrificed and the testes was dissected out and weighed. It was then processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione; membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on testes were also carried out.

SET 4: CISPLATIN INDUCED TESTICULAR TOXICITY

A: Acute study

The experiment was performed using male rats from Wistar strain weighing 200 to 250 grams. The animals were divided into following groups. Each group consisted of six rats.
Group 1: Control animals, received vehicle (3 ml/kg/day p.o. for 30days) followed by sterile water for injection (1 ml/kg, i.p.) on 30th day.
Group 2: Animals received vehicle (3ml/kg/day p.o.for 30days) and cisplatin injection (5 mg/kg i.p.) on 30th day.
Group 3a: Animals received green tea extract (25 mg/kg/day p.o.for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.
Group 3b: Animals received green tea extract (50 mg/kg/day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.
Group 3c: Animals received green tea extract (100 mg/kg/day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.
Group 4a: Animals received melatonin (3 mg/kg /day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

Group 4b: Animals received melatonin (6 mg/kg /day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

Group 5a: Animals received lovastatin (3 mg/kg /day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

Group 5b: Animals received lovastatin (6 mg/kg /day p.o. for 30 days) and cisplatin injection (5 mg/kg i.p.) on 30th day.

The change in body weight was recorded weekly. Blood was collected 5 days after cisplatin injection and serum was separated for estimations of testosterone level. Epididymis was removed; the epididymal sperm count was done immediately. The animals were sacrificed and the testes was dissected out and weighed. It was then processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione; membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on testes were also carried out.

B: Chronic study

The experiment was performed using male rats from Wistar strain weighing 200 to 250 grams. The animals were divided into following groups. Each group consisted of six rats.

Group 1: Control animal received vehicle (3ml/kg /day p.o. for 30 days) and sterile water for injection (1ml/kg, i.p.) every week on day 1, 7, 14, 21, 28.

Group 2: Animals received vehicle (3ml/kg /day p.o. for 30 days) and cisplatin injection (3 mg/kg, i.p.) every week (days 1, 7, 14, 21 and 28).

Group 3a: Animals received green tea extract (25 mg/kg/day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 3b: Animals received green tea extract (50 mg/kg/day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 3c: Animals received green tea extract (100 mg/kg/day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 4a: Animals received melatonin (3mg/kg/day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 4b: Animals received melatonin (6mg/kg /day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

Group 5a: Animals received lovastatin (3mg/kg /day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) on 30th day.

Group 5b: Animals received lovastatin (6 mg/kg /day p.o. for 30 days) and cisplatin injection (3 mg/kg i.p.) every week (days 1, 7, 14, 21 and 28).

The change in body weight was recorded weekly. After 48 hours of the last dose of cisplatin or vehicle, blood was collected and serum was separated for estimation of testosterone level. The epididymal sperm count was done immediately. The animals were sacrificed and the testes was dissected out and weighed. It was then processed for assays of lipid peroxidation, superoxide dismutase, catalase, reduced glutathione; membrane bound enzymes (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) and proteins. Histopathological studies on testes were also carried out.

SET 5: DMBA INDUCED MAMMARY GLAND CANCER

Female Sprague-Dawley rats of weighing between 45-55 gm were used for the experiments. The animals were divided into following groups. Each group consisted of eight rats.

Group 1: Control animals, received a single intragastric (i.g.) dose of vehicle (sesame oil, 1ml/rat).

Group 2: Animals received a single intragastric (i.g.) dose of DMBA (15 mg/rat) in sesame oil.

Group 2a: Animals received a single intragastric (i.g.) dose of DMBA (15 mg/rat) and after 10 weeks, received doxorubicin injection (3 mg/kg, i.p.) every week (days 1, 7, 14, 21 and 28).

Group 2b: Animals received a single intragastric (i.g.) dose of DMBA (15 mg/rat) and after 10 weeks, received resveratrol (6 mg/kg/day p.o. for 30 day).

Group 2c: Animals received a single intragastric (i.g.) dose of DMBA (15 mg/rat). After 10 week, Resveratrol (6mg/kg) was given orally daily for 30 day along with doxorubicin injection (3 mg/kg, i.p.) every week (days 1, 7, 14, 21 and 28).

During the experimental period, animals were observed daily to assess their general health. Animals were treated with resveratrol and doxorubicin or

combination of resveratrol and doxorubicin 10 weeks after DMBA administration.

After 48 hours of the last dose of doxorubicin, blood was collected and serum was separated for estimations of creatine kinase (CK), lactate dehydrogenase (LDH), and SGOT. The animals were sacrificed and the heart and tumors were dissected out and weighed. Heart was then processed for assays of lipid peroxidation, superoxide dismutase, catalase and reduced glutathione and proteins. Histopathological studies on heart and tumors were also carried out.

3.2.3 BIOCHEMICAL ESTIMATIONS

3.2.3.1 Tissue Parameters

1. Lipid peroxidation or malondialdehyde (MDA) formation
2. Endogenous antioxidants
   a) Superoxide dismutase (SOD)
   b) Catalase (CAT)
   c) Reduced glutathione (GSH)
3. Membrane bound enzymes, namely
   a) Na^+K^+ATPase
   b) Ca^{2+}ATPase
   c) Mg^{2+}ATPase
4. Inorganic phosphorus
5. Total proteins
6. Epididymal sperm count

3.2.3.2 Serum Parameters

1. Creatine Kinase
2. Lactate Dehydrogenase
3. SGOT
4. Creatinine
5. Urea
6. Uric acid
7. Blood Urea Nitrogen
8. Testosterone

Apart from these, histopathological studies were also carried out.

3.2.4 Removal and Processing of Serum and Tissues for Various Estimations

Serum:

At the end of the treatment period, rats were anaesthetised with anesthetic ether. Blood was collected from the retro-orbital plexus without any anti-coagulant and allowed to clot for 10 minutes at room temperature. It was then centrifuged at 2500 rpm for 20 minutes. The serum obtained was stored at 4°C till further use.

Tissues:

Reagents

1. Phosphate Buffered Saline pH 7.4-

1.38 gm of disodium ethylenediaminetetraacetic acid, 0.19 gm of potassium dihydrogen phosphate and 8 gm of sodium chloride was dissolved in 900 ml of distilled water and adjusted pH using dilute hydrochloric acid. The volume was adjusted to 1000 ml using distilled water.

2. Sucrose solution (0.25M)

85.58gms of sucrose was dissolved in 200 ml of water and diluted to 1000 ml with distilled water.

3. Tris hydrochloride buffer (10mM, pH 7.4)

1.21gm tris was dissolved in 900 ml of distilled water and the pH was adjusted to 7.4 with 1M hydrochloric acid. The resulting solution was diluted to 1000 ml with distilled water.

Procedure

The animals were euthanasiously sacrificed, after blood collection; heart was quickly transferred to ice-cold phosphate buffered saline (pH 7.4). It was blotted free of blood and tissue fluids, weighed on a Single Pan Electronic Balance (Precisa 205 ASCS). The hearts were cross-chopped with surgical scalpel into fine slices, suspended in chilled 0.25M sucrose solution and quickly blotted on a filter paper. The tissues were then minced and homogenised in chilled tris hydrochloride buffer (10mM, pH 7.4) to a concentration of 10% w/v. Prolonged homogenisation under hypotonic condition was designed to disrupt, as far as possible, the structure of the

cells so as to release soluble proteins. The homogenate was centrifuged at 7,000 rpm at 0°C for 25 minutes using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the determination of superoxide dismutase and catalase. The sediment was re-suspended in ice-cold tris hydrochloride buffer (10mM, pH 7.4) to get a final concentration of 10% w/v and was used for the estimation proteins.

3.3 TISSUE ESTIMATIONS

The tissue levels of lipid peroxidation (MDA content), Superoxide Dismutase (SOD) Catalase (CAT) and Reduced Glutathione (GSH) were estimated as biomarkers of oxidative stress.

3.3.1 Determination of Lipid Peroxidation (MDA content)

It was estimated using the method described by Slater and Sawyer (1971).

Reagents

1. Thiobarbituric acid (0.67% w/v)

0.67gm of thiobarbituric acid was dissolved in 50 ml of hot distilled water and the final volume was made up to 100 ml with hot distilled water.

2. Trichloroacetic acid (10% w/v)

10gms of trichloroacetic acid was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

3. Standard Malondialdehyde stock solution (50mM)

A standard malondialdehyde stock solution was prepared by mixing 25µl of 1, 1, 3, 3-tetraethoxypropane up to 100 ml with distilled water. 1.0 ml of this stock solution was diluted up to 10 ml to get solution containing 23µg of malondialdehyde/ml. One ml of this stock solution was diluted up to 100 ml to get a working standard solution containing 23ng of malondialdehyde/ml.

Procedure

2.0 ml of the tissue homogenate (supernatant) was added to 2.0 ml of freshly prepared 10% w/v trichloroacetic acid (TCA) and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitate was separated by centrifugation and 2.0 ml of clear supernatant solution was mixed with 2.0 ml of freshly prepared thiobarbituric acid (TBA). The resulting solution was heated in a boiling water bath for 10 minutes. It was

then immediately cooled in an ice bath for 5 minutes. The colour developed was measured at 532nm against reagent blank. Different concentrations (0-23nM) of standard malondialdehyde were taken and processed as above for standard graph.

The values were expressed as nM of MDA/mg protein.

3.3.2 Assay of Endogenous Antioxidants

3. 3. 2.1 Superoxide Dismutase (SOD) (Superoxide: Superoxide oxido reductase, EC-1.15.1.1)

Superoxide dismutase was estimated using the method developed by Misra and Fridovich (1972).

Reagents

1. Carbonate Buffer (0.05M, pH 10.2)

16.8gms of sodium bicarbonate and 22gms of sodium carbonate was dissolved in 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.

2. Ethylenediaminetetra acetic acid (EDTA) solution (0.49M)

1.82gm of EDTA was dissolved in 200 ml of distilled water and the volume was made up to 1000 ml with distilled water.

3. Hydrochloric acid (0.1N)

8.5ml of conc. hydrochloric acid was mixed with 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.

4. Epinephrine solution (3mM)

0.99gm epinephrine bitartarate was dissolved in 100 ml of 0.1N hydrochloric acid and the volume was adjusted to 1000 ml with 0.1N hydrochloric acid.

5. Superoxide Dismutase (SOD) standard (100 U/L)

1mg (1000 U/mg) of SOD from bovine liver was dissolved in 100 ml of carbonate buffer.

Procedure

0.5ml of tissue homogenate was diluted with 0.5ml of distilled water, to which 0.25ml of ice-cold ethanol and 0.15ml of ice-cold chloroform were

added. The mixture was mixed well using cyclo mixer for 5 minutes and centrifuged at 2500 rpm. To 0.5ml of supernatant, 1.5ml of carbonate buffer and 0.5ml of EDTA solution were added. The reaction was initiated by the addition of 0.4ml of epinephrine and the change in optical density/minute was measured at 480nm against reagent blank.

SOD activity was expressed as units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

3.3.2.2 Catalase (CAT) (Hydrogen peroxide oxido reductase, EC-1.11.1.6)

It was estimated by the method of Hugo Aebi as given by Colowick et al. (1984).

Reagents

1. Phosphate Buffer (50mmol/l, pH 7.0)

(a) 6.81gms of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in distilled water and made up to 1000 ml.
(b) 8.90gms of disodium hydrogen orthophosphate (Na_2HPO_4) was dissolved in distilled water and made up to 1000 ml.
The solutions (a) and (b) were mixed in the proportion of 1: 1.5 (v/v).

2.Hydrogen Peroxide (30 mmol/l)

0.34ml of 30% hydrogen peroxide was diluted with phosphate buffer to 100 ml. This solution was prepared fresh each day.

3. Catalase standard (65,000 U/mg protein; 1mg protein/ml)

Crystalline beef-liver catalase suspension was centrifuged to isolate the crystals of the enzyme that was dissolved in 0.01M phosphate buffer (pH 7.0) to give a final concentration of 1.0mg protein/ml. Before assay, it was diluted with distilled water to obtain 1000 U/ml.

Procedure

To 2ml of diluted sample 1ml of hydrogen peroxide (30 mmol/l) was added to initiate the reaction. The blank was prepared by mixing 2ml of diluted sample (similar dilution) with 1ml of phosphate buffer (50mmol/l; pH 7.0).

The dilution should be such that the initial absorbance should be approximately 0.500. The decrease in absorbance was measured at 240nm. Catalase activity was expressed as μmoles of H_2O_2 consumed/min/mg protein.

3.3.2.3 Reduced Glutathione (GSH)

Reduced glutathione was determined by the method described by Moron et al. (1979).

Reagents

1. Trichloroacetic acid (20% w/v)

20gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made up to 100 ml with distilled water.

2. Phosphate Buffer (0.2M, pH 8.0)

0.2M sodium phosphate was prepared by dissolving 30.2gms sodium phosphate in 600 ml of distilled water, the pH was adjusted to 8.0 with 0.2M sodium hydroxide solution and the final volume was adjusted up to 1000 ml with distilled water.

3. DTNB reagent (0.6mM)

60mg of 5, 5'-dithiobis (2-nitro benzoic acid) was dissolved in 50 ml of buffer and the final volume was adjusted to 100 ml with buffer.

4. Standard Glutathione

10mg of reduced glutathione was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

Procedure

Equal volumes of tissue homogenate (supernatant) and 20% TCA were mixed. The precipitated fraction was centrifuged and to 0.25ml of supernatant, 2ml of DTNB reagent was added. The final volume was made up to 3ml with phosphate buffer. The colour developed was read at 412nm against reagent blank. Different concentrations (10-50μg) of standard glutathione were taken and processed as above for standard graph.

The amount of reduced glutathione was expressed as μg of GSH/mg protein.

3.3.3 Assay of Membrane Bound Enzymes and Inorganic Phosphorus

3.3.3.1 Sodium-Potassium dependent adenosine triphosphatase (Na^+K^+ ATPase) (ATP phosphohydrolase, EC-3.6.1.3)

Na^+K^+ATPase was assayed according to the method described by Bonting (1970).

Reagents

1. Tris hydrochloride buffer (92mM, pH 7.5)

11.13gms of tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.5 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

2. Magnesium sulphate solution (5mM)

1.232gm of magnesium sulphate dissolved and made upto 1000ml with distilled water.

3. Potassium chloride solution (5mM)

0.372gm of potassium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

4. Sodium chloride solution (60mM)

3.231gms of sodium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

5. EDTA solution (0.1mM)

0.372gm of EDTA was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

6. Adenosine triphosphate (ATP) solution (40mM)

0.220gm of ATP was dissolved in 4ml of distilled water and the final volume was made upto 10ml with distilled water.

7. Trichloroacetic acid (10% w/v)

10gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made upto 100ml with distilled water.

Procedure

1.0ml of tris-hydrochloride buffer and 0.2ml each of magnesium sulphate, sodium chloride, potassium chloride, EDTA, ATP were added to test tube

containing 0.2ml of homogenate. The mixture was incubated at 36°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated as described in section 3.3.3.4.

The enzyme activity was expressed as μM of inorganic phosphorus liberated/mg protein/min.

3.3.3.2 Calcium dependent adenosine triphosphatase (Ca^{2+}ATPase) (ATP phosphohydrolase, EC-3.6.1.3)

Ca^{2+}ATPase was assayed according to the method described by Hjerken and Pan (1983).

Reagents

1. Tris hydrochloride buffer (125mM, pH 7.5)

15.12gms of tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.5 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

2. Calcium chloride solution (50mM)

5.55gms of calcium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

3. Adenosine triphosphate (ATP) solution (10mM)

0.551gm of ATP was dissolved in 4ml of distilled water and the final volume was made upto 100ml with distilled water.

4. Trichloroacetic acid (10% w/v)

10gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made up to 100ml with distilled water.

Procedure

The incubation mixture contained 0.1ml each of tris-hydrochloride buffer, calcium chloride, ATP and homogenate in a test tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated as described in section 3.3.3.4.

The enzyme activity was expressed as μM of inorganic phosphorus liberated/mg protein/min.

3.3.3.3 Magnesium dependent adenosine triphosphatase (Mg^{2+}ATPase) (ATP phosphohydrolase, EC-3.6.1.3)

Mg^{2+}ATPase was assayed according to the method described by Ohinishi et al. (1982).

Reagents

1. Tris hydrochloride buffer (374mM, pH 7.6)

45.24gms of tris was dissolved in 900ml of distilled water and the pH was adjusted to 7.6 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

2. Magnesium chloride solution (25mM)

6.16gms of magnesium chloride was dissolved in 400ml of distilled water and the final volume was made upto 1000ml with distilled water.

3. Adenosine triphosphate (ATP) solution (10mM)

0.551gm of ATP was dissolved in 4ml of distilled water and the final volume was made upto 100ml with distilled water.

4. Trichloroacetic acid (10% w/v)

10gms of trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was made upto 100ml with distilled water.

Procedure

The incubation mixture contained 0.1ml each of tris-hydrochloride buffer, magnesium chloride, ATP and homogenate in a test tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by addition of 1.0ml of 10% TCA, mixed well and centrifuged. The phosphorus content of the supernatant was estimated as described in section 3.3.3.4.

The enzyme activity was expressed as μM of inorganic phosphorus liberated/mg protein/min.

3.3.3.4 Determination of Inorganic Phosphorus (P_i)

Inorganic Phosphorus (P_i) was estimated by the method described by Fiske and Subbarow (1925).

Reagents

1. Ammonium molybdate reagent (2.5% w/v)

2.5gms of ammonium molybdate was dissolved upto 100ml with 3M sulphuric acid.

2. 1-Amino 2-naphthol 4-sulphonic acid (ANSA) reagent

(a) 0.25% w/v of ANSA reagent in 15% w/v of sodium metabisulphite and 20% w/v of sodium sulphite.

(b) 15gms of sodium metabisulphite was dissolved in 100ml of distilled water.

(c) Accurately weighed 20gms of sodium sulphite was dissolved in 100ml of distilled water.

250mg of ANSA was dissolved in 97.5ml of 15% w/v of sodium metabisulphite and 2.5ml of 20% w/v of sodium sulphite, mixed well and stored at room temperature.

3. Standard Phosphorus

35.1mg of potassium dihydrogen orthophosphate was dissolved in 100ml of distilled water. This contained 80µg of phosphorus/ml.

Procedure

1ml of the supernatant was taken and the volume was made upto 5.0ml with distilled water. To this, 1ml of 2.5% ammonium molybdate reagent and 0.5ml of ANSA reagent was added. The colour developed in 20 minutes was read using blank containing water instead of sample at 620nm. A standard graph was prepared taking different concentrations of standard phosphorus (16-80µg).

The values were expressed as µM of inorganic phosphorus liberated/mg protein/min.

3.3.4 Estimation of Total Protein

The method of Lowry et al. (1951) was used for the estimation of total protein.

Reagents

1. Sodium hydroxide (0.1M)

4gms of sodium hydroxide was dissolved in 400 ml of distilled water and the final volume was made up to 1000 ml with distilled water.

2. Lowry C reagent

(a) Copper sulphate in 1% sodium potassium tartarate (1% w/v) 0.5gm of copper sulphate was dissolved in 1% sodium potassium tartarate (Prepared by dissolving 1gm of sodium potassium tartarate in 100 ml of distilled water).

(b) Sodium carbonate in 0.1M sodium hydroxide (2% w/v)

2gms of sodium carbonate was dissolved in 100 ml of 0.1M sodium hydroxide.

2ml of solution (a) was mixed with 100 ml of solution (b) just before use.

3. Standard Protein (Bovine serum albumin)

20mg of bovine serum albumin was dissolved in 80 ml of distilled water and few drops of sodium hydroxide were added to aid complete dissolution of bovine serum albumin and to avoid frothing. Final volume was made up to 100 ml with distilled water and stored overnight in a refrigerator.

4. Folin's phenol reagent

Folin's phenol reagent was diluted with distilled water in the ratio of 1:2. (i.e. 1ml of Folin's phenol reagent was mixed with 2ml of distilled water).

Procedure

Diluted membrane fraction aliquots (0.1ml) were taken in test tubes. To this, 0.8ml of 0.1M sodium hydroxide and 5ml of Lowry C reagent was added and the solution was allowed to stand for 15 minutes. Then 0.5ml of 1N Folin's phenol reagent was added and the contents were mixed well on a vortex mixer. Colour developed was measured at 640nm against reagent blank containing distilled water instead of sample.

Different concentrations (40-200μg) of standard protein (Bovine serum albumin) were taken and processed as above for standard graph. The values were expressed as mg of protein/ gm of wet tissue (mg/ gm).

3.3.5 Epididymal sperm count

Epididymal sperm count was determine by the method of Narayana et al. (2002)

Reagent

1. Formalin-bicarbonate solution

5 gms of sodium bicarbonate dissolved in 95ml of distilled water. 1ml formalin was added to sodium bicarbonate solution and the final volume was made up to 100 ml with distilled water.

Procedure

Epididymis was removed, cleared off the adhering tissues and weighed. Epididymal sperm was collected by slicing the epididymis in 5 mL Formalin-bicarbonate solution. An aliquot of the suitably diluted epididymal sperm suspension was used for spermatozoa count using Neubauer haemocytometer. Spermatozoa were counted in 4 squares. The values were expressed as sperm count /mg epididymis.

3.4 SERUM ESTIMATIONS

3.4.1 Creatine kinase (CK)

Quantitative estimation of creatine kinase was performed as per instructions provided for ENZOPAK-CK-NAC kit using RA 50 auto analyser. Units were expressed as U/L.

3.4.2 Lactate dehydrogenase (LDH)

Quantitative estimation of lactate dehydrogenase (LDH) was performed as per instructions provided for ENZOPAK LDH L→P using RA 50 auto analyser. Units were expressed as U/L.

3.4.3 Aspartate aminotransferase (GOT) (L-aspartate: L-aspartate; 2-oxoglutarate aminotransferase, EC-2.6.1.1)

Quantitative estimation of Serum glutamate oxaloacetate transaminase (GOT) or Aspartate aminotransferase (AST) was done as per method of Reitman and Frankel and performed according to instructions provided for diagnostic reagent kit using SHIMADZU 1601 UV spectrophotometer.

3.4.4 Creatinine

Quantitative estimation of creatinine was performed as per instructions provided for diagnostic kit Span Diagnostics Ltd., Surat, India using RA 50 auto analyser. Units were expressed as mg/dl.

3.4.5 Uric acid

Quantitative estimation of uric acid was performed as per instructions provided for diagnostic kit Span Diagnostics Ltd., Surat, India using RA 50 auto analyser. Units were expressed as mg/dl.

3.4.6 Urea and Blood urea nitrogen (BUN)

Quantitative estimation of urea and blood urea nitrogen (BUN) performed as per instructions provided for diagnostic kit Span Diagnostics Ltd., Surat, India using RA 50 auto analyser. Units were expressed as mg/dl.

3.4.7 Testosterone

The ADVIA Centaur testosterone assay is a competitive immunoassay using direct chemiluminescent technology. Testosterone in the sample competes with acridinium ester-labeled testosterone in the Lite reagent for limited amount of polyclonal rabbit antitestosterone antibodies bound to monoclonal mouse anti-rabbit antibody which is coupled to paramagnetic principles in the Solid Phase. The assay uses Testosterone Releasing Agent to release bound testosterone from the endogenous binding protein in the sample. Units were expressed as ng/ml.

3.5 HISTOPATHOLOGICAL STUDIES

After the treatment period, the animals were sacrificed and the organs were excised, blotted free of blood and tissue fluids and preserved in 10% v/v formal saline solution. The specimens were given for further processing to Baroda Pathological Laboratory, Vadodara where routine procedure for sectioning, staining and mounting was observed by the laboratory personnel. Briefly, after a week the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax. Sections of 5μ thickness were cut on a microtome and taken

on glass slides coated with albumin. The sections were deparaffinated in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The haematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada balsam. The stained sections were examined under Olympus BX40 Photomicroscope and photographed. Either samples were coded to perform blind study or expert guidance from the veteran pathologist was sought to determine histopathological changes.

3.6 STATISTICAL ANALYSIS OF DATA

Results of all the above estimations have been indicated in terms of mean ± SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) with Tukey-Kramer post comparison test using GraphPad InStat version 3.00, GraphPad Software, California USA. The level of significance was set at $P<0.05$.