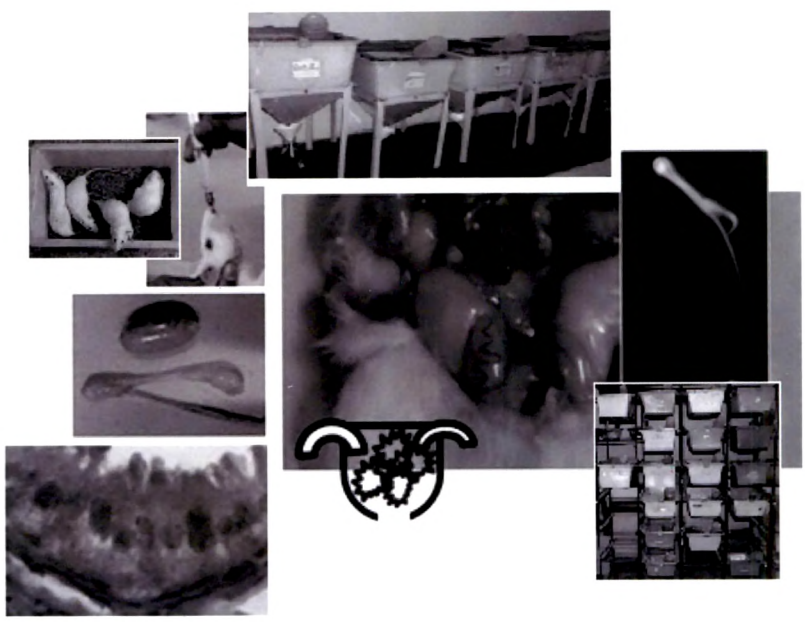
MATERIALS AND METHODS



1. EXPERIMENTAL ANIMALS AND THEIR MAINTENANCE
2. CHEMICALS
3. EXPERIMENTATION
    3.1. Rationale for the Selection of Study Chemicals
4. PREPARATION OF DOSAGE
5. DURATION OF THE STUDY
    5.1. The Spermatogenic and Seminiferous Epithelial Cycle Studies
    5.2. Treatment and Withdrawal Phase Studies
    5.3. Protocol for Toxicity Study
    5.4. Protocol for Withdrawal Study
6. PARAMETERS EVALUATED
    6.1. Clinical Signs, Body Weight and Food and Water Consumption
    6.2. Histology of Tissues
        6.2.1. Light microscopy
        6.2.2. Qualitative spermatogenesis analysis
    6.3. Blood Collection and Hematological Analysis
    6.4. Serum Biochemistry
        6.4.1. Liver function tests
        6.4.2. Lipid profile
        6.4.3. Kidney function tests
        6.4.4. Urine analysis:
    6.5. Biochemical Studies in Liver, Kidney and Testis
        6.5.1. General Parameters
            Protein
            Succinate dehydrogenase
            Acid phosphatase
            Alkaline phosphatase
            Cholesterol
        6.5.2. Parameters of Oxidative Stress
            Superoxide dismutase
            Glutathione Peroxidase

1 EXPERIMENTAL ANIMALS AND THEIR MAINTENANCE

Adult male Wistar rats (*Rattus rattus norvegicus*) weighing 300-330 g were obtained from Sun Pharma Advance Research Company Ltd., Vadodara. The animals were allowed to acclimatize in departmental animal house for a period of one week. These animals were housed in plastic cages with bedding of husk under standard conditions (22±3^{0}C, L: D 12:12), fed with commercial rat chow (Pranav Agro Ltd., Vadodara) and water *ad libitum*. The experimentation on animals was approved by Institutional Animal Ethics Committee; the animal handling and all procedures on animals were carried out in accordance with the approved guidelines.

Cage cards indicating details of group number, animal number, dose level etc. were used to identify the different experimental groups and the animals were marked using diluted picric acid solution as head, back, tail etc for proper identification of individuals in each experimental group.

2 EXPERIMENTATION

Present investigations were designed to study the effect of administration of mixture of chemicals below individual "no-observed-adverse-effect-level" (NOAEL). A sixty day oral toxicity study was performed in which the toxicity (hematology, clinical chemistry, biochemical studies and pathology) of combination of four compounds was examined in different organs. The study comprised nine groups (ten animals/ group) and the animals were exposed (through oral gavage) daily for a period of 60 days to heterogeneous chemical mixture (HCM). Following 60 days of HCM exposure the treatment was withdrawn for further 60 days for recovery phase studies.

3 CHEMICALS

The test compounds, cadmium chloride, chromium trioxide, Phthalic acid dibutyl ester and 1, 2-dichlorobenzene were purchased from SISCO

Research Laboratories, Gujarat and used for the preparation of HCM. All chemicals used in the study were of analytical grade or of the highest grade commercially available.

3.1 Rationale for the Selection of Study Chemicals

The troubles in risk assessment of chemical mixtures are because of their interactions that alters the outcome of mixture toxicity. It is very much possible that with increase in the number of chemicals in a mixture formulation there may be greater chance of chemical interactions at various end points in toxicodynamics as well as in toxicokinetic phases. Because of this multitude of interactions, each possible combination cannot be tested for its toxicity. One way to overcome this problem is to select few chemicals for mixture formulation and treat the mixture as a single compound at different doses (Mumtaz *et al.,* 1998; Gorten *et al.,* 1997). Moreover, for mixture toxicity studies, the three to five component mixtures is generally considered suitable, for minimizing the number of groups involved in mixture toxicity testing (Jonker *et al.,* 1996; Mumtaz *et al.,* 1998). Therefore in present sixty days oral toxicity study the chemical mixture of four compounds was examined.

In mixture toxicological studies, the focus of health risk assessment should be on chemicals of actual public health concern, that is those chemicals should be used for formulation of mixture that are found as the major pollutants in the vicinity. Studies reported the presence of selected components of toxicant mixture in different concentrations in the effluent arising from various industries situated in central and south Gujarat, India (Labunska *et al.,* 1999). Unfortunately, the effluent is used by local farmers for irrigation and in this way pollutants pave their way to various components of ecosystem. Studies carried out earlier in our department have demonstrated high levels of heavy metals particularly chromium and cadmium in fruits, vegeTables, and fishes and in human hairs (Sharma, 1995). Similarly few organicals like dichlorobenzene, phthalate ester *etc.* and their metabolites

were also detected in human samples, when occupationally exposed to these compounds (Kumagai and Matsunaga, 1995; Pant *et al.,* 2008). Therefore the chemicals selected for formulation of chemical mixture are highly relevant to general population in terms of usage pattern, level and frequency of exposure. Moreover two heavy metal and two organic compounds were selected to prepare heterogeneous chemical mixture (with different chemical structure and actions), as mixtures in the environment are usually composed of multiple components.

4 PREPARATION OF DOSAGE

For the preparation of chemical mixture, Phthalic acid dibutyl ester and 1, 2–dichlorobenzene were mixed in corn oil and cadmium chloride and chromium trioxide were dissolved in distilled water at concentrations equal to 1/1000 (0.1%) and 1/100 (1%) of their LD_{50} values (Table 1). The 0.1% dose is very much equivalent to NOAEL of individual compound (Table 1). At the time of dosing all the toxicants were mixed to a total volume of 0.8 ml and administered through oral gavage.

Table 1: Toxicological details of selected compounds

Selected compound	LD 50	NOAEL	$\frac{LD_{50}}{100}$	$\frac{LD_{50}}{1000}$
Cadmium chloride	88 mg/kg BW (Onwuka *et al.,* 2010)	9-4 mg/kg BW (Guilhermino *et al.,* 1998)	0.88	0.088
Chromium hexavalent	80 mg/kg BW (Szelag *et al.,* 2003)	2.4 mg/kg BW (EPA, 1991 a & b)	0.80	0.080
Phthalic acid dibutyl ester	8000 mg/kg BW (ATSDR, 2001)	50-138 mg/kg BW (Mylchreest *et al.,* 2000; NTP, 1995)	80	8
1,2-dichloro benzene	500 mg/kg BW (NTP, 1985)	25 mg/kg BW (Robinson *et al.,* 1991)	5	0.5

5 DURATION OF THE STUDY

5.1 The Spermatogenic Cycle and Seminiferous Epithelial Cycle Studies

The study was performed to investigate the sub chronic effect of chemical mixture on different systems of male rats. To study occurrence, cause, and manifestation of adverse effect of chemical mixture on reproductive system, animals should be dosed for at least one complete cycle of spermatogenesis in order to elicit any adverse effect on spermatogenesis, as many of significant changes are considered being associated with particular stages of seminiferous epithelial cycle.

Seminiferous epithelium of adult male rat is composed of various generations of germ cells associated with Sertoli cells (Leblond and Clermont, 1952). In rat testis one complete cycle of spermatogenesis requires around 56-58 days. Therefore, the experimental schedule was planned in such a way that the treatment was continued for one complete cycle of spermatogenesis, and similarly the withdrawal phase also constitute of one complete cycle of spermatogenesis.

5.2 Treatment and Withdrawal Phase Studies (Table 2)

Protocol for toxicity study: Each experimental group comprised of 20 rats except for group I designated as zero (initial) day control group and that comprised of 10 rats. Rats of groups II and III were designated respectively as control group receiving basal diet and vehicle treated control group receiving corn oil along with basal diet. Group IV and V rats were exposed to chemical mixture (Phthalic acid dibutyl ester, 1, 2–dichlorobenzene, Cadmium chloride and Chromium trioxide) at concentrations equal to 1/1000 (0.1%) and 1/100 (1%) of their LD_{50} values, respectively. The schedule was continued for 60 days with daily dose of chemical mixture and vehicle. On 61st day 10 rats from each group were sacrificed after urine and blood collection using appropriate methodology (detailed in later section).

Protocol for withdrawal study: Remaining 10 animals from above groups except group I were kept for recovery study. All the treatments were stopped in recovery group to check possibility of withdrawal period recovery towards normalization. This schedule was kept till 120 days from the initiation of the experiments. On 121st day rats were sacrificed after urine and blood collection using appropriate methodology. During recovery phase the group nomenclature was: control animals were designated as Group VI and VII and toxicant exposed groups were designated as group VIII and IX, respectively.

Table 2: Experimental protocol

Groups	Treatment	Autopsy day
I	Zero (initial) day control	01
II	Control (No treatment)	61
III	Control (Vehicle treatment)	61
IV	Heterogeneous chemical mixture treated group (1% of LD_{50} dose/ animal/ day - NOAEL dose level)	61
V	Heterogeneous chemical mixture treated group (0.1% of LD_{50} dose/ animal/ day)	61
VI	Control (No treatment)	121
VII	Control (Vehicle treatment)	121
VIII	Heterogeneous chemical mixture treated group (1% of LD_{50} dose/ animal/ day) for 60 days followed by withdrawal phase of 60 days - NOAEL dose level.	121
IX	Heterogeneous chemical mixture treated group (0.1% of LD_{50} dose/ animal/ day) for 60 days followed by withdrawal phase of 60 days.	121

6 PARAMETERS EVALUATED

6.1 Clinical Signs, Body Weight and Food and Water Consumption

Morphological changes were noticed in the animals of all the groups by careful visual examination to see any physical changes in the body such as hair fall, overgrowth and infection. The body weight of animals was recorded every alternate day to assess the general growth pattern. After urine and blood collection, the animals were sacrificed for tissue examination. The organs under study were excised from the animal and weighed. The weights of the liver, kidney, testes and male reproductive accessory organs were noted and studied for any abnormal gain or loss of weight from normal. This gives a preliminary confirmation regarding the adverse effects (if any) of the heterogeneous chemical mixture. Relative weight of above tissue was calculated as follows:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight} \times 100}{\text{Body weight}}$$

Water intake and food consumption was also recorded over the exposure period in all groups.

6. Histology of Tissues

6.2.1. Light microscopy:

The tissues (Liver, kidney, Epididymis) from all groups of animals were dissected out and fixed in 10% formalin and testis was fixed in Bouins fluid for 24 h. After few hours of fixation, the tissues were cut into 5-6 mm thick pieces and dehydrated in ethanol series, cleared in xylene and embedded in paraffin wax (58^0- 60^0C). 5-7 µm paraffin sections of these tissues were stained with Hematoxylin and Eosin and assessed under light microscope at different magnifications. All the alterations observed in treatment groups were compared with the normal structures. In testis histology qualitative and quantitative spermatogenic analysis were carried out at different seminiferous epithelial stages to understand cell kinetics.

Composition of Bouins fluid:

Saturated aqueous picric acid solution	75 ml
40% Formaldehyde	25 ml
Glacial acetic acid	05 ml

Hematoxylin (Harris, 1900)

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

The hematoxylin was dissolved in absolute alcohol and then added to alum, 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.

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.

Procedure

The sections were deparafinized in xylene, hydrated through alcohol (50%, 70%, 80%, 90%, and 100%) series and distilled water and stained with hematoxylin for 3-4 min followed by thorough wash under tap water. Differentiation and potentiation of nuclear staining was done in acidic alcohol (2 drops of concentrated HCl in a coupling jar full of 70% alcohol) and ammonical alcohol (2 drops of ammonium hydroxide solution in a coupling jar full of 70% alcohol). The sections were rinsed in 70% ethanol, counterstained with eosin, differentiated and dehydrated in alcohol (50%, 70%, 80%, 90% and 100%) series, cleared in clove oil and xylene and mounted in D.P.X.

6.2.2. Qualitative spermatogenesis analysis

The stages of seminifercus epithelium (SE) were identified as per the description of Leblond and Clermont (1952) and Hess (1990). The SE stage identification was done mainly to divide the observation in different categories of tubules at early (Stages I-IV), mid (stages V-VI and stages VII-VIII) and late (stages IX- XIV) stages of spermatogenic cycle.

For the simplicity of study, the area where the spermatogonia, preleptotene primary spermatocytes and basal part of Sertoli cells were present was considered as basal portion of the tubule. The remaining part was considered as crypt and adluminal portion of the tubule. This distinction was arbitrary and not based upon the location of Sertoli- Sertoli junctional complex. Transverse section of at least 20 tubules in each stage was critically assessed for following specific histological changes in each group.

1 Seminiferous tubular diameter
2 General status of basal and adluminal portions of the tubules
3 Height of seminiferous epithelium and lumen diameter
4 Status of spermatogenic cell nuclei
5 Presence and extent of cytoplasmic vacuolation
6 Cell sloughing and cytoplasmic blebbing
7 Deformation of round spermatids
8 Deformation of elongating spermatids
9 Premature release of spermatids
10 Retention of residual bodies within the adluminal portion of seminiferous epithelial
11 Delayed spermiation
12 Clogging of the flagella of spermatozoa
13 Status of peritubular membrane
14 Status of interstitium and interstitial cells
15 Type of cell loss

6.3 Blood Collection and Hematological Analysis

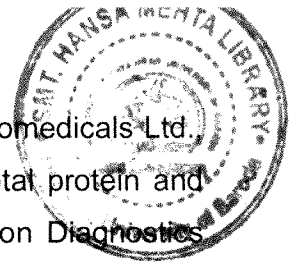
After the treatment period, as per the experimental protocol shown in Table, all the rats were fasted overnight and anesthetized with diethyl ether for blood collection from retro orbital plexus. The blood samples were collected (from retro-orbital plexus under mild anesthesia) in non heparinized vials for serum biochemistry or in heparinized vials for hematological parameter studies. The hematological measurements and calculations were performed by using hematology analyzer (Celltac MEK-6318, Nihon Kohden, USA). Hematological evaluation included red blood cell count, hemoglobin concentration, hematocrite, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, Platelet count, white blood cell count and differential white blood cell count.

6.4 Serum Biochemistry

The non heparinized blood was left to clot for about 15 minute and centrifuged at 3000 rpm for 15 minute. The supernatant serum samples were drown in clean tubes and kept deep freezed at -20 ^{0}C until the biochemical analysis. Clinical chemistry determination was performed by using semi automatic biochemistry analyser (ERBA CHEM-5 Plus). Parameters included tests of liver function, lipid profile and kidney function. All the parameters were analyzed using commercially available kits.

6.4.1 Liver function tests

Activity of enzyme and concentration of certain biochemical parameters were determined in serum as follows: serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase activities (SGPT) and lactate dehydrogenase (LDH) were determined according to the method described by International Federation of Clinical Chemistry (IFCC) and kits used were purchased from Transasia Biomedicals Ltd., India whereas Gamma glutamyl transpeptidase (GGT) and Alkaline phosphatase (ALP)



activity were measured using kit purchased from Transasia Biomedicals Ltd., India and Reckon Diagnostic Pvt Ltd., India respectively. Total protein and albumin were determined by using standards kits from Beacon Diagnostics Pvt Ltd., India. Globulin concentration was calculated by subtracting albumin level from total protein. Serum bilirubin concentrations were estimated by using the standard kit from Bayer Diagnostic India Ltd.

6.4.2 Lipid profile

Cholesterol (TC) and triglyceride (TG) were estimated by enzymatic method described in the kit purchased from Eve's Inn Diagnostics, Baroda and Bayer Diagnostic India Ltd, respectively. High density lipoprotein levels were estimated as described in kit of Transasia Biomedicals Ltd., India. Low density lipoprotein cholesterol (LDL-c) and very low density lipoprotein cholesterol (VLDL-c) were calculated according to Friedwald's equations (Friedwald *et al.,* 1972)

$$VLDL\text{-}c = TG/5$$

$$LDL\text{-}c = TC\text{-}[HDL\text{-}c + (TG/5)]$$

6.4.3 Kidney function tests

Urea and creatinine concentration were determined according to Urease method based on Berthelot's reaction (Bayer Diagnostic India Ltd.) and kit method of Jaffe reaction provided by Transasia Biomedicals Ltd., India respectively. Fasting (12 hours) blood glucose was measured in separated serum using kit purchased from Reckon diagnostic Pvt Ltd., India. Calcium and chlorine were estimated by using the standard kit from Avecon Healthcare Pvt Ltd. Potassium and sodium concentrations were determined by flame photometry (Systronics Flame Photometer-129).

6.4.4 Urine analysis

Urine samples were collected over a 24 h period using metabolic cages. The collected samples were stored on dry ice packs, centrifuged and stored at – 20 ^{0}C till assayed. The urine volume and urinary concentration of creatinine were estimated to calculate Glomerular Filtration Rate (GFR). GFR was calculated using the following formula (Shetty *et al.,* 2005)

$$\text{GFR (ml/min)} = \frac{\text{urinary creatinine (mg/dl) x urine volume (ml) x 1000(g)}}{\text{Serum creatinine (mg/dl) x body weight (g) x 1440 (min)}}$$

6.5 Biochemical Studies

The tissues (Liver, Kidney, Testis and Epididymis) were weighed and homogenized as per the requirement of different procedures.

6.5.1. General Parameters

Protein (Lowry *et al.,* 1951)

Principle: When protein containing sample is treated with phenol reagent of Folin-Ciocalteu, a deep colour develops. This colour is due to two reactions occurring simultaneously:

i. Biuret reaction of alkaline copper sulphate solution with peptide bonds and

ii. Reduction of phosphomolybdic acid and phosphotungstic acid by the aromatic amino acids present in the proteins. The blue colour developed is quantitatively proportional to the total protein present in the reaction medium.

Reagents

1 Reagent A: 2% Sodium carbonate in 0.1 N NaOH solution

2 Reagent B: 0.5% Copper sulphate in 1% sodium potassium tartarate solution

3 Reagent C: 50 ml of reagent A and 1 ml of reagent B (mixed just before use)

4 Folin–phenol reagent: 1 ml of the reagent, add 1 ml distilled water before use.

5 Standard: Bovine serum albumin (10 mg) was dissolved in 10 ml of distilled water. For use 0.02 to 0.1 ml (20 – 100 µg) of stock solution was taken and diluted to 1 ml and then below mentioned procedure was followed.

Procedure: To 0.1 ml of tissue homogenate, 0.9 ml of distilled water and 5 ml of reagent C were added. Then 0.5 ml of folin- phenol was added and the optical density was read at 660 nm just after 30 minutes of incubation. The blank tube was run with 1 ml distilled water instead of homogenate. A standard curve of optical density at 660 nm versus concentration of BSA was plotted to determine the amount of protein in the sample.

Succinate dehydrogenase (SDH) (Beatty *et al.,* 1966)

Principle: The electrons released by the enzyme SDH from the substrate are taken up by an electron acceptor (INT) which is reduced to red coloured formazan. After extracting it in ethyl acetate the colour intensity can be measured in a specetrophotometer at 420 nm.

Reagents

1 Phosphate buffer (0.2 M, pH 7.6)

2 Sodium succinate (0.1 M)

3 0.1% 2-(-4-iodo phenyl)-3-(4-nitrophenyl) -5phenyl tetrazolium chloride (INT)

4 30% Trichloroacetic acid

5 Standard: Different concentrations of INT (25, 50, 100, 150, 200 µg) were used to prepare the standard curve. Stock INT solution was prepared and known volume of INT solution was added into five different tubes so that test tube will contain 25, 50, 100, 150, 200 µg of INT. The total volume was made up to 6 ml by adding required volume of water and pinch of sodium hydrosulphite was added so that the INT gets reduced to red formazan which was read on a colorimeter at 420 nm.

Procedure: Tissues were homogenized in desired volume of phosphate buffer. To each sample tube, 1ml of 0.2 M phosphate buffer, 1 ml of 0.1 M sodium succinate, 1 ml of INT and 0.4 ml of tissue homogenate was added. Blank test tube was run with 1 ml of distilled water instead of the INT solution. After addition of above reagents the tubes were incubated at 37 ^{0}C for 15 minute, and then 0.1 ml of 30% TCA was added to terminate the reaction. The formazan was extracted into 7 ml of ethyl acetate by vigorous shaking for 30 seconds. The tubes were centrifuged for 3-5 minutes at 1500 rpm. The supernatant was used to measure the colour intensity on the spectrophotometer at 420 nm against the blank. A standard curve of optical density at 420 nm versus concentration of reduced INT to formazan was plotted to determine the activity of succinate dehydrogenase.

Acid phosphatase (Bessey *et al.,* 1946)

Principle: Acid phosphatase orthophosphate monoester phoshohydrolase catalyses the hydrolysis of p-nitro phenyl phosphate at pH 4.8 and liberates p-nitro phenol and inorganic phosphate. The liberated p-nitro phenol combines with NaOH solution to form a yellow coloured complex which is directly proportional to the acid phosphatase activity.

Reagents

1 Citrate Buffer (0.05 M, pH 4.8): 0.41 g of citric acid and 1.125 g of sodium citrate were dissolved in 100 ml distilled water and the pH was maintained with 0.1 N HCl.

2 Substrate buffer: 165 mg of p-nitrophenyl phosphate of disodium salt in 100 ml of 0.05 M citrate buffer.

3 0.1 N NaOH solution

Procedure: In the sample tube 0.02 ml of tissue homogenate (prepared in cold distilled water) was added with 0.4 ml of substrate buffer. In blank test tube 0.02 ml of distilled water was added instead of 0.2 ml of homogenate. The tubes were incubated for 30 minutes at 37 ^{0}C and thereafter 4 ml of 0.1 N NaOH was added. The colour intensity was read on spectrophotometer at 410 nm. For calculation of acid phosphatase activity, conversion factor obtained

from standard Graph was used to express as μ moles of p-nitrophenol liberated / mg protein/ 30 minutes.

Alkaline phosphatase (Bessey *et al.*, 1946)

Principle: The enzyme alkaline phosphatase hydrolyses the substrate p-nitro phenyl phosphate at pH 10.4 and liberates p-nitro phenol and inorganic phosphate. The quantity p-nitro phenol released under standardize condition can be measured at 410 nm.

Reagents

1. Glycine buffer: 7.5 g of glycine, 0.095 g magnesium chloride, and 85 ml of 1 N NaOH and total volume was made up to 1 liter.
2. p-nitro phenyl phosphate solution: 0.4% was freshly prepared
3. Substrate buffer: equal volume of glycine buffer and stock pNPP mixed and pH was adjusted to 10.4
4. 0.05 N NaOH solution
5. Standard: Different concentrations of p-nitro phenol (pNP) solution (0.02, to 0.2 μ mole) were used to prepare the standard curve and to determine the unknown from the Graph.

Procedure: Known amount of tissue was weighed and homogenized in desired amount of cold phosphate buffer saline. 0.4 ml of working buffer substrate was added in the sample and blank test tube and placed in the water bath for 5 minutes at 37 ^{0}C. Then 0.02 ml of homogenized tissue and 0.02 ml of distilled water was added in sample and blank tubes respectively. After incubation period of 30 minutes, 4 ml of 0.02 N NaOH was added. The developed colour intensity was read at 410 nm. Standard curve of optical density versus concentration of pNP was plotted to determine the unknown from the Graph.

Cholesterol (Pearson *et al.*, 1953)

Principle: Cholesterol reacts at room temperature with acetic anhydride and concentrated H_2SO_4 to form a compound with a brownish green colouration, depending on the protein precipitation by p-Toluene sulphonic acid.

Reagents

1 p-Toluene sulphonic acid: 4 gm of p-Toluene sulphonic acid in 100 ml of (3:2) acetic anhydride : Glacial acetic acid.

2 Standard cholesterol solution: Different concentrations of cholesterol solutions (100- 10 µg) in glacial acetic acid were used to prepare the standard curve.

Procedure: Known amount of tissue was homogenized in desired volume of glacial acetic acid. In sample, blank and standard tubes, 0.2 ml of homogenate, glacial acetic acid and standard cholesterol were added, respectively. After this 5 ml of p-Toluene sulphonic acid was added to all the tubes. 1 ml of concentrated H_2SO_4 was added and allowed to develop colour and read at 620 nm. Standard curve of optical density versus concentration of cholesterol was plotted to determine the cholesterol level from the Graph.

6.5.2. Parameters of Oxidative Stress

Superoxide dismutase (Marklund and Marklund, 1974)

Principle: The assay of superoxide dismutase is based on the ability of the enzyme to inhibit the auto-oxidation of pyrogallol, as pyrogallol is known to auto-oxidized rapidly especially in alkaline medium (pH 8.2).

Reagents

1 Tris-Hcl buffer pH 8.2

2 Pyrogallol: 2 mM pyrogallol dissolved in 0.5 N HCl

Procedure: Initially, the rate of auto-oxidation of pyrogallol was noted. The reaction mixture consisted of 2 ml of Tris–HCl buffer (pH 8.2), 0.2 ml of 2 mM pyrogallol and 0.8 ml of distilled water for auto-oxidation for pyrogallol. This was considered as 100% auto- oxidation. The assay mixture for enzyme contained 2 ml of Tris–HCl buffer (pH 8.2), 0.75 ml of distilled water, 50 µl of homogenate (enzyme source) and 0.2 ml of 2 mM pyrogallol. The samples were immediately read at 420 nm against blank containing all the content except homogenate and pyrogallol at every 1 min interval, for 3 minute on spectrophotometer. The enzyme activity was calculated as IU/ mg protein. One unit of superoxide dismutase activity was defined as the amount of enzyme that inhibited pyrogallol self-oxidation by 50% under assay conditions.

Catalase (Sinha *et al.,* 1972)

Principle: Catalase is a tetramer of four polypeptide chain containing four porphyrin heme groups, which catalyzes the decomposition of hydrogen peroxide to water and oxygen. The method is based on the fact that dichromate in acetic acid is reduced to green coloured chromic acetate when heated in the presence of hydrogen peroxide with the formation of perchromic acid as an unsTable intermediate. As the enzyme reaction mixture hit the acetic acid, its activity is destroyed; and hydrogen peroxide which hasn't been split by the catalase will react with the dichromate to give blue precipitate of perchromic acid. This unsTable precipitate is then decomposed by heating to give green colour solution. The developed colour intensity was measured on spectrophotometer at 570 nm to determine the quantity of hydrogen peroxide left.

Reagents

1 phosphate buffer, 0.01 M, pH 7.0

2 0.2 M Hydrogen peroxide (H_2O_2): H_2O_2 was prepared freshly and kept in dark bottle.

3 Dichromate acetic acid reagent: this reagent was prepared by mixing 5% solution of potassium dichromate in distilled water with glacial acetic acid in the ratio 1:3.

4 Standard: Different concentrations of H_2O_2 in micromoles were used to prepare the standard curve.

Procedure: The assay mixture containing 0.5 ml of 0.2 M H_2O_2, 1 ml of phosphate buffer and 0.4 ml distilled water was mixed with 0.1 ml of enzyme extract to initiate reaction. Then 2 ml of dichromate- acetic acid reagent was added after 30 and 60 seconds to arrest the reaction. To the control tube the enzyme was added after addition of dichromate- acetic acid reagent. The tubes were then heated for 10 minute, allowed to cool and the developed green colour was read at 570 nm against the blank containing all components except the enzyme. For standard curve different concentrations of H_2O_2 ranging from 10 to 150 µ moles were taken in test tubes and 2 ml of the dichromate acetic acid reagent was added. The tubes were heated for 10

minute after formation of blue precipitate, allowed to cool and the absorbance was read at 570 nm. Sample values were calculated based on the standard curve plotted from the values of absorbance versus micromoles of H_2O_2 in cuvette.

Glutathione Peroxidase (Rotruck *et al.,* 1973)

Principle: Glutathione peroxidase catalyse the reduction of hydrogen peroxide by reduce glutathione resulting in H_2O and oxidize glutathione which is then instantly and continuously converted in to reduced glutathione by glutathione reductase. The oxidation of reduce glutathione by 5,5' dithio bis 2, nitro benzoic acid DTNB was used to measure the total glutathione content.

Reagents

1 10 mM Sodium azide

2 4 mM Reduce glutathione

3 10% Trichloroacetic acid

4 0.3M di-Sodium hydrogen phosphate

5 0.2 mM Hydrogen peroxide

6 Ellman's reagent: 40 mg of 5, 5 – dithiobis-2-nitro benzoic acid (DTNB) in 100 ml of 1% Sodiun Citrate

7 Standard: Different concentrations of reduce glutathione can be used to prepare the standard curve.

Procedure: The assay mixture contained 0.4 ml of sodium phosphate buffer, 0.1 ml of sodium azide, 0.2 ml of reduced glutathione, 0.1 ml of homogenate, 0.1 ml of H_2O_2 and 1.1 ml of distilled water. The reaction was terminated by addition of 0.5 ml of trichloro-acetic acid after 10 minutes of incubation at 37^0 C and centrifuged at 4000 rpm for 10 minutes. To determine the residual glutathione content 1 ml of supernatant was added to 3 ml of disodium hydrogen phosphate and 1 ml of Ellman's reagent solution. The developed colour was read at 412 nm against reagent blank containing phosphate solution and Ellman's reagent. Suitable aliquots of the standard (reduced glutathione) were taken and treated in the same manner to calculate the activity of glutathione peroxidase.

Glutathione- S-transferase (Habig *et al.,* 1974)

Principle: Glutathione transferases catalyze the conjugation of 2,4 dinitro-chlorobenzene (CDNB) or 3,4 dichloronitrobenzene (DCNB) with reduced Glutathione (GSH) to produce a yellow product that has an absorbance maxima at 340 – 360nm and the rate of product formation, that indicates the enzyme activity, can be calculated by following the increase in absorbance at 340nm.

Reagents

1 Phosphate buffer saline (PBS), pH 6.5, 0.1M

2 50mM of Reduced Glutathione in PBS

3 2, 4 dinitro-chlorobenzene (CDNB) in ethanol

Procedure: The homogenate was prepared in 0.1 M, pH 6.5 of phosphate buffer saline containing EDTA (0.01 M) and centrifuged at 10,000 rpm for 15 minutes. The assay mixture contained 2.77 ml of phosphate buffer, 0.03 ml of homogenate, and 0.15 ml of reduced glutathione and incubated at 37 ^{0}C for 10 min. After this 0.05 ml of 1-chloro-2, 4-dinitrobenzene was added and the optical density was measured against blank (distilled water instead of homogenate) at 340 nm immediately for 5 minute at every 60 second of interval. The enzyme activity was calculated using extraction coefficient between CDNB-GSH conjugate and CDNB.

Measurement of oxidative degradation of lipid (Buege and Aust, 1978)

Principle: Lipid peroxidation leads to the formation of an endoperoxide i.e malondialdehyde (MDA), which reacts with thiobartburic acid and gives a characteristic pink colour that can be measured spectrophotometrically at 532 nm.

Reagents

1 Thiobarbituric acid (TBA) reagent: 100 mg TBA, 46 mg EDTA, 10 ml 20% TCA, 5 ml of 2.5 N HCl and make volume up to 20 ml with distilled water.

2 Standard: standard curve was prepared using different aliquotes of tetraethoxy propane and thiobarbitburic acid in 1:1 ratio.

Procedure: 1ml of Tissue homogenate; prepared in 0.1 M phosphate buffer saline (pH 7.4) was thoroughly mixed with 1ml of thiobarbituric acid reagent. The mixture was boiled at 90^0C in water bath for 20 minute, cooled and centrifuged at 300 rpm for 15 minute. The supernatant was taken and malondialdehyde content was determined from the absorbance at 532nm.

Reduce Glutathione (Beutler *et al.,* 1963)

Principle: Glutathione is a major non protein thiol present in the tissue. The sufahydryl group in glutathione reduces the 5, 5 – dithiobis-2-nitro benzoic acid (DTNB) to form one mole of 5-thio-2-nitrobenzoate (TNB) per mole of SH by the following reaction:

2GSH + DTNB $\longrightarrow$ GSSG + TNB

TNB anion has an intense yellow colour with absorbance maxima at 412 nm and is used to measure the –SH group.

Reagents

1. Phosphate buffer (pH 7.4, 0.1M)
2. 1% Sodiun Citrate
3. Ellman's reagent: 5, 5 – dithiobis-2-nitro benzoic acid (DTNB): 40 mg in 100 ml of 1% Sodiun Citrate
4. Precipitating Reagent: 1.67 g Glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl g dissolved in 100 ml of distilled water
5. 0.3 M Di-sodium hydrogen phosphate solution
6. Standard: standard curve was prepared using different concentrations of reduced glutathione.

Procedure: The assay mixture contained 1 ml of homogenate and 1.5 ml precipitating reagent, was centrifuged at 3000 rpm for 15 minutes to obtain supernatant. 0.5 ml of supernant, 2 ml of Di-sodium hydrogen phosphate solution forms a coloured complex with 0.25 ml of Ellman's reagent, which was measured at 412nm. The calculation was done according to the slope calculated from the standard Graph.

Ascorbic acid (Roe and Kuether, 1943)

Principle: The ascorbic acid is oxidized to dehydro form by norit reagent in the presence of trichloroacetic acid (TCA). This is coupled with 2, 4 dinitrophenyl hydrazine which give red colour complex, by the action of H_2SO_4. This is measured spectrophotometrically.

Reagents

1. 6% and 4% Trichloroacetic acid (TCA)
2. 2, 4 dinitrophenyl hydrazine (DNPH): 2% of DNPH in 9 N H_2SO_4.
3. 10% of Thiourea in 50% alcohol
4. Norit reagent: 2 g of activated charcoal in 100 ml 6% TCA. Shake well and keep for 10 minutes. Filter through Whatman paper No. 42.
5. Standard: 50 mg of ascorbic acid was dissolved in 50 ml of 6% TCA. One ml of this solution was diluted to 100 ml with 4% TCA to prepare working ascorbic acid solution. Different concentrations of ascorbic acid solution (10 to 100 µg) were used for standard curve preparation to determine the unknown from the Graph.

Procedure: The tissue was homogenized in norit reagent. To 4 ml of homogenate, 1 ml of 2, 4- dinitrophenyl hydrazine was added followed by addition of a drop of thiourea, to activate the reaction. Blank tube was run with 4 ml of 6% TCA instead of homogenate. The content of test tubes was boiled for 15 minutes. Then 85% H_2SO_4 was added and incubated for 30 minutes. The colour developed was read at 540 nm. The ascorbic acid content in the tissue was calculated using standard curve equation.

6.5.3. Steroidogenic Parameters in Testicular Tissues

3β-Hydroxysteroid dehydrogenase (Shivanandappa and Venkatesh, 1997)

Principle: The method is based on the formazan formation due to reduction of the tetrazolium salt. The reaction mixture contain the substrate (dehydroepiandrosterone for 3β-Hydroxy dehydrogenase and estradiol for 17β-Hydroxy dehydrogenase), NAD, iodonitrotetrazolium in 0.1 M Tris-HCl

buffer and the enzyme, incubated for 1 hour at 37 ^{0}C. Absorbance at 490 nm is read in a spectrophotometer.

Reagents

1 Phthalate buffer (50 mM, pH 3.0): 2.55 g of potassium hydrogen phthalate dissolved in a mixture of 51 ml 0.1N HCl and 2.5 ml Tween 20; pH was adjusted to 3.0 and the volume made up to 250 ml with distilled water.

2 Tris-HCl buffer (0.1 M, pH 7.8)

3 NAD (500 µM)

4 Colouring Reagent: 40 mg of INT and 0.5 ml of Tween 20 were dissolved in 50 ml of distilled water.

5 substrate: dehydroepiandrosterone (100 µM) 3β and estradiol (100 µM) for 17β was first dissolved in 0.3 to 0.5 ml of dimethyl formamide and the further desired volume of solution was prepared with Tris HCL buffer.

6 Standard curve: 1 mM solution of NADH was freshly prepared in distilled water. Aliquots of graded concentrations of NADH (0 to 150 n mol) were reacted with colouring reagent (40 mg INT, 10 mg Phenazene methosulfate (PMS) and 0.5 ml Tween 20 were dissolved in 50 ml distilled water for the standard curve), after this phthalate buffer was added and the absorbance read at 490 nm. A standard curve was prepared by plotting NADH concentration versus absorbance.

Procedure: 10 % homogenate was prepared in 0.1 M Tris-HCl (pH 7.8) and centrifuged at 3000 rpm at 4 ^{o}C. The supernatant was used as the enzyme extract for assay. The enzyme was assayed in 0.1 M Tris HCl buffer (pH 7.8) containing NAD and of substrate, in a total volume of 3.0 ml. The reaction was started by adding the enzyme (50 µl) and incubated at 37 ^{0}C for 60 minutes. The reaction was stopped by addition of 2.0 ml of phthalate buffer (pH 3.0). The turbidity was removed by centrifugation at 3000 rpm for 20 minutes and the supernatant was read at 490 nm in a spectrometer. The enzyme activity was calculated from the standard curve of NADH and expressed as mmol NADH formed per 60 minutes per mg protein.

17β-Hydroxysteroid dehydrogenase (Talalay, 1992)

Principle: 17β-Hydroxysteroid dehydrogenase acts on substrate testosterone and reduce nicotinamide adenine dinucleotide (NDA) to NADH. This reduction in absorbance was measured.

Reagents

1 0.02 M Phosphate buffer

2 Tritone X-100: 2.5 mg/ ml

3 Substrate: 1.5 mg of testosterone dissolved in 2 ml of methyl alcohol.

4 0.1 M Sodium pyrophosphate buffer

Procedure: A known amount of tissue was homogenized in 0.02 M phosphate buffer along with triton X 100. The homogenate was centrifuged at 8000 g for 30 minutes at 4 ^{0}C. The supernatant was used for the assay. To 2.0 ml of 0.1 M sodium pyrophosphate buffer, 0.2 ml of testosterone and 0.2 ml of homogenate were added. Then 2.0 ml of NAD and 0.4 ml of distilled water were added. Blank was prepared by adding 2.0 ml of buffer, 0.2 ml of substrate, 0.8 ml of distilled water. Final volume of the assay was made up to 3.0 ml. The reduction in terms of the absorbance of NAD was read at every 15 second for 1 min at 340 nm. The enzyme activity was expressed as nanomoles of 5x-diol formed/ mgprotein/ hour.

6.6 BIOCHEMICAL INDICATORS OF ACCESSORY REPRODUCTIVE ORGAN FUNCTIONS

6.6.1. Glycerylphosphorylcholine in Caput and Cauda Epididymis (White, 1959)

Principle: The glucose and other interfering substances of protein free filterate were removed after the copper sulphate and calcium hydroxide treatment. The glycerol is extracted with alcohol and is oxidized by periodate and the excess periodate destroyed by arsenite. An aliquote of the resulting solution is heated with concentrated sulphuric acid. The formaldehyde is then estimated colorimetrically by the formation of a complex with chromotropic acid. The method is useful for determining the rate of breakdown of glycerol

when added to washed spermatozoa suspension and is extended to the estimation of glycerylphosphorylcholine. Glycerylphosphorylcholine is apparently absorbed on copper-lime precipitate to the same extent as the glycerol and again gave about half the colour that would be expected from its glycerol content when both it and free glycerol with which it was compared, were subject to copper lime treatment. It is thus possible to estimate the glycerylphosphorylcholine content in sampleby comparing the colour developed on periodate oxidation with standard glycerol solutions which have been treated with zinc and copper-lime in exactly the same way as the sample. In order to express the results as glycerylphosphorylcholine the glycerol values are multiplied by 5.7.

Reagents

1 0.5 N Sodium Hydroxide

2 10% Zinc Sulphate

3 20% Copper Sulphate

4 0.1 M Sodium periodate solution

5 Sodium Arsenite solution: dissolve 13.33 gm of sodium arsenite in 100 ml distilled water.

6 Chromotrophic acid (Fresh solution): 1.08 g of chromotrophic acid sodium salt was dissolved in 100 ml distilled water and 450 ml of 65% sulphuric acid was added slowly.

7 Standard curve: stock solution of glycerol (4 mg/ ml) was prepared. After diluting the stock solution 1 in 10, Suitable aliquots were taken in test tubes and the volumes were made up to 2 ml with distilled water and below mention procedure was followed.

Procedure: 0.2 ml of tissue homogenate was used for assay. For the estimation, 0.2 ml of homogenate, 1.8 ml of Distilled water, 1 ml of 0.5 N NaOH, 1 ml of 10 % $ZnSO_4$ was added in test tube. Mixed well and centrifuged for 30 minutes at 2000 rpm. Clear supernatant was taken in separate tube and 0.1 ml of concentrated sulphuric acid and 0.3 ml of 20% copper sulphate was added. After mixing the content of the tube 0.23 g of $Ca(OH)_2$ powder was mixed well with glass rod and kept for 20 minutes at room temperature. The test tube content was heated with 2 ml of ethyl alcohol

at 75 ^{0}C for 10 minutes and centrifuged for 15 minutes at 2000 rpm. After this 0.5 ml of 2 N H_2SO_4, 0.5 ml of sodium periodate and 2 ml of supernatant was mixed for 5 minutes. After 5 minutes 0.5 ml of sodium arsenite was added and again mixed. After 10 minutes test tube total volume was mad up to 10 ml with distilled water. 1 ml of of above solution was taken in another test tube and 10 ml of chromotrophic acid was added and heated in a boiling water bath for 30 minutes. The developed colour was read at 580 nm. The optical density has been plotted against standard concentration to measure the amount of glycerol content in samples and the measured glycerol was multiplied with 5.7 to express the results as glycerylphosphorylcholine.

6.6.2. Sperm-Specific Parameters

Sperm count

The sperm count was determined from the cauda epididymis using Haemocytometer Neubauer chamber.

Reagents

1 Physiological saline (0.87%)

2 5% $NaHCO_3$

Procedure: A known amount of cauda epididymal tissue was taken in physiological saline and teased gently to release the spermatozoa. The cauda suspension is sucked up to the 0.5 mark in the WBC pipette. The suspension was diluted upto the 11 mark with 5% $NaHCO_3$. Sodium bicarbonate acts as a spermicide and kills the spermatozoa to facilitate the counting. A drop of this fluid was then placed gently on Neubauer chamber and covered with coverslip. The total numbers of sperms were then counted in 64 small squares (WBC square). The total sperm count was calculated in millions per ml using formula:

$$\text{Total sperm count} = \frac{N \times \text{Dilution} \times 1000}{0.4}$$

Where N is total number of spermatozoa counted in 64 squares

Dilution from 0.5 mark to 11 mark of WBC pipette = 20 times

Volume of 64 squares = 0.4 cumm

Sperm Motility

A drop of cauda suspension was placed on a glass-slide, and covered with cover-slip and observed under microscope. The motile spermatozoa are counted along with the total number of spermatozoa in each focus. A total of minimum 10-12 separate fields were scored and the percentage motility was calculated using formula:

$$\text{Percentage motility} = \frac{\text{No. of motile sperm x100}}{\text{Total no. of spermatozoa}}$$

Sperm Dead and Live ratio

Principle: Live spermatozoa retain the selective semi-permeability of their cell membrane and hence do not take up stain (Trypan blue a supravital stain). And therefore remain unstained. Dead spermatozoa, on the other hand lose their membrane selective permeability and permeable to stain and hence appear stained under the microscope. The ratio of unstained to stained spermatozoa gives the live: dead sperm ratio.

Procedure: 0.2 ml of sperm suspension from cauda epididymis was taken in a test tube. 0.2 ml of 0.1 % Trypan blue was added to the sample and incubated for 15 minutes at 37 ^{0}C. Now a drop of this suspension was placed on the slide and observed under 400X magnification. The numbers of stained and unstained spermatozoa were scored in 20 separate fields.

Hypo-Osmotic Swelling test

Principle: The hypo osmotic test is a simple test to evaluate the functional integrity of the membrane of the spermatozoa. Spermatozoa swell under hypo-osmotic condition due to the influx of water molecules, resulting in the expansion of the membrane.

Reagents

1 Hypo- Osmotic solution: 13.51 g fructose + 7.35 g sodium citrate dissolved in 1000 ml with distilled water.

Procedure: The hypo-osmotic swelling technique consists of mixing 0.1 ml of cauda suspension with 0.9 ml of hypo-osmotic solution. The mixture was then

incubated at 37 ^{0}C for at least 30 minutes, in a tightly capped tube. After incubation, a drop was placed on the Neubauer chamber and observed under microscope. At least 100-200 spermatozoa were scored and the percentage of swollen spermatozoa was determined as follows:

$$\text{Percentage of swollen spermatozoa} = \frac{\text{No. of swollen spermatozoa} \times 100}{\text{Total no. of counted spermatozoa}}$$

Acridine orange and ethidium bromide staining

Principle: The principle is based on the fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes such as Ethidium bromide (EB) and Acridine orange (AO) staining. Acridine orange permeates all cells and makes the nuclear appear green while Ethidium bromide is only taken up by cells when cytoplasmic membrane integrity is lost and stain the nucleus reddish orange. Thus live cells have a green nucleus; apoptotic cells display condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus.

Reagents

1. Acridine orange: dissolve 10 mg of Acridine orange in 1 ml of normal saline
2. Ethidium bromide: dissolve 10 mg of Ethidium bromide in 1 ml of normal saline

Procedure: 10 µl of Acridine orange and 10 µl of Ethidium bromide and 100 µl of sperm suspension from cauda epididymis was mixed thoroughly. A drop of this mixture was then placed on a slide and allowed to settle down for one minute, after placing a cover slip. The slide was then observed under 400X magnification in fluorescence light microscope.

6.6.3. Fructose in Seminal vesicle (modified method of Foreman *et al.*, 1973)

Principle: The modified method is specific for fructose as it gives no reaction with glucose or glucose phosphate. When heated with concentrated HCl

fructose forms oxymethyl furfural which gives a red colour complex with resorcinol. This red colour complex can be measured calorimetrically.

Reagents

1 Resorcinol: 0.1% of resorcinol in 95% alcohol

2 5% Perchloric acid: 5 ml of concentrated perchloric acid is diluted to 70 ml with distilled water.

3 30% HCl: 30 ml of concentrated HCl is made upto 36 ml with distilled water

4 Standard fructose Solution: Different concentrations of fructose solutions 10-100 µg were prepared and read at 410 nm after following the procedure.

Procedure: A known amount of tissue was homogenized in a definite volume of 5% perchloric acid. 0.2 ml of homogenate was taken in sample tube and 1.8 ml of 5% perchloric acid was added. The blank tube was run with 2 ml of 5% perchloric acid. 1 ml of 0.1 % resorcinol and 3 ml of 30% HCl was added to all tubes and heated in water bath at 80 ^{0}C for 1 hour and cooled at room temperature. The developed red colour was read on spectrophotometer at 410 nm against blank. The concentration of fructose was calculated using the regression formula obtained from the standard Graph.

7 HORMONAL ASSAYS

7.1. Total testosterone level

IMMULITE 2000 Total Testosterone In-Vitro Diagnostic Test Kit was utilized to measure the total testosterone level on IMMULITE 2000 system analyzer.

Principle and Procedure: Serum level of testosterone was estimated by a competitive chemiluminescence enzyme immunoassay using IMMULITE 2000 Total Testosterone which utilized specific antibody- coated polystyrene beads as a solid phase. After the sample was incubated with alkaline phosphatase-labeled reagent, the bound label was then quantified using a specific chemoluminescent substrate and light emission was detected by photomultiplier tube. The test procedure was applied according to the provided kit guide book.

7.2. Cortisol Level

Immunoassay kit of Elecsys and Cobase, for the In-Vitro quantitative determination of cortisol in serum and plasma was utilized for measurement of cortisol.

Principle and Procedure: The principle is based on the Solid-phase, competitive chemiluminescence enzyme immunoassay. 20 µl of sample is incubated with a Cortisol- specific biotinylated antibody and a ruthenium complex labeled cortisol derivative. Depending on the concentration of the analyte in the sample and the formation of the respective immune complex, the labeled antibody binding site is occupied in part with sample analyte and in part with ruthenylated hapten. After addition of streptavidin-coated microparticles, the complex becomes bound to solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of voltage to the electrode then induces chemiluminescence emission which is measured by a photomultiplier. The test procedure was applied according to the provided kit guide book.

8 METAL DETECTION

An acid digestion procedure was used for sample preparation as it eliminates elemental loss by volatilization because the digestion takes place at a low temperature. Cadmium, chromium and selenium concentrations in different samples were determined by Inductively Coupled Plasma (ICP-Atommic emission spectroscopy, ARCOS- MS. Spectro, Germany) Spectroscopy and Zinc level was determined by Atomic absorption Spectroscopy.

8.1. Tissues Samples (Liver, Kidney, Testis, Epididymis)

Procedure: About 5 g of sample was digested into tube with 5 ml of HNO_3 and 5 ml of H_2SO_4. The reaction was allowed to proceed. As and when it

slows down the tubes were placed in the digestion apparatus (oven) and heated at (60 °C) for 30 minutes. The tubes were removed from digestion apparatus and cooled. Again the tubes were returned to digestion rack and heated slowly to 120 °C after addition of 10 ml of HNO_3. The temperature was increased to 150 °C. The tubes were removed when the samples turn black in colour and cooled. 1 ml of H_2O_2 was added to the tubes and placed back to digestion apparatus to obtain a clear sample solution. A vigorous reaction may occur after addition of H_2O_2. The tubes were removed and diluted up to 50 ml with deionized water and used for analysis.

8.2. Blood and urine samples:

Procedure: For determination of cadmium, chromium, selenium and zinc the serum and urine samples were directly diluted 1: 25 with deionized water with the addition of few drops of concentrated acid and used for analysis.

9 METHOD FOR DETECTION OF DICHLOROBENZENE AND PHTHALATE ESTER AND THEIR METABOLITES BY GCMS

9.1 Blood and urine samples (Kumagai and Matsunaga, 1995; Hattori *et al.*, 1981)

1 ml of sample was placed in a screw cap glass tube and 0.3 ml of concentrated hydrochloric acid was added. To hydrolyze the conjugated metabolites the mixtures was heated for few minutes. When mixture had cooled, 1 ml of diethyl ether and 1 ml of methyl alcohol was added. The mixture was centrifuge for 5 minutes at 1500 rpm and the organic layer was transferred to another glass tube. In order to secure complete dehydration 0.5g of Na_2SO_4 was added to the organic layer and shaken with vortex type mixer. A 3.0 µl aliquot of the organic layer was injected into the port of gas chromatograph of GC/MS.

9.2 Tissue Samples (Brunstrom et al., 2001)

Tissues samples were extracted using a slightly modified version of a general method for analysis. All the tissues samples were homogenized with two fold of water and the homogenates were saturated with sodium chloride and extracted with hexane: acetone (2:5, V/V) (3 ml per g tissue sample). The extract was submitted to gas chromatoGraphy.

The GC separation was made on 30m x 250µ x 0.250µ gas capillary column packed with PE-5 MS, on Perkin Elmer Autosystem XL with turbo mass GC/MS.

The GC condition was: temperature programmed for 80^0C – 290 ^{0}C at 10 ^{0}C/ min, helium was used as the carrier gas at a flow rate of 1 ml/min.

10 STATISTICAL ANALYSIS

All data were expressed as mean ± SE and the statistical significance was evaluated using one way ANOVA followed by Bonferroni's compare all pairs of column using Graph Pad Prism Version 3.0 for Windows, Graph Pad Software, San Diego CA, USA. All statistical tests were run at a 95 % confidence interval and P 0.05 was taken as the level of statistical significance. Some of the Graphs were prepared in Microsoft Office Excel 2007 to simplify the result outcome of the experiments. For statistical analysis, groups IV and V were compared with group III in toxicity study and for recovery study groups VIII and IX were compared with group VII.