Material and Methods:

Animals:

Around 180 days old male *Wistar* rat (*Ratus norvegicus*) weighing 325-375gms were used for all the experiments. All animals were housed in clean polypropylene cages(Tarsons, India) and maintained under controlled temperature 25±2 °C with photoperiod schedule of L:D 13:11. Animals were fed with standard rat pellet diet (Pranav Agro Ltd., Sangli, India) and provided with drinking water *ad libitum*. The animals were maintained and handled as per the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA Reg. No.: 827/ac/04/CPCSEA).

Treatment Schedule: (I) → Animal treated for 15 days.

Set 1: Animals given normal drinking water and given and *i.p.* injection of 0.9% saline.

Set 2: Animals given normal drinking water and given *i.p.* injection of melatonin 10mg/ kg BW/ day at 18.00hrs.

Set 3: Animals given exposure to metal alone or in combination and *i.p.* injection of 0.9% saline.

Set 3 a: → Cr(VI) – 20mg/ kg BW/ day **(Chapter: 1 and 5)**

Set 3 b: → Cd – 9mg/ kg BW/ day **(Chapter: 2 and 6)**

Set 3 c: → Ni – 40mg/ kg BW/ day **(Chapter: 3 and** 7)

Set 3 d: → Mixture of all the three metals **(Chapter: 4 and 8)**

(Cr(VI), Cd and Ni) at the same concentration

Set 4: Animals given exposure to metal alone or in combination same as in of

Set: 3 and also co-exposed to *i.p.* injection of melatonin (10mg/ kg BW/ day).

Treatment Schedule: (II) → Animals treated for 30 days.

Exactly same experimental setup as described in **Treatment Schedule: (I)** except that animals were treated for **30 days**

Treatment Schedule: (III) → Animals treated for 60 days.

Exactly same experimental setup as described in **Treatment Schedule: (I)** except that animals were treated for **60 days**

Rats were sacrificed at the end of each treatment schedule under mild anesthesia (Diethyl Ether) and blood was collected by brachial vein puncture in epenndorf tubes and then allowed to stand at room temperature for 8-10 min. Tubes were then centrifuged (Plastocraft) at 3000 rpm for 15 min at 4 °C. Serum was then separated carefully and utilized for the analysis of serum parameters including hormones. The viscera was then cut open and reproductive organs were excised and washed in physiological saline, blotted properly and weighed accurately using digital weighing balance (Systronics). The sera and tissues were stored at -80 °C (Cryosoft) until the assay. For biochemical assay, tissues were homogenized in cold PBS (Phosphate buffer saline)

Lipid Peroxidation

Tissue lipid peroxidation was measure by the method of Benge and Aust (1978).

Principle:

Lipid peroxidation leads to the formation of an endoperoxide i.e. malondialdehyde (MDA), which reacts with thiobarbituric acid (TBA) and forms a pink chromogen [TBA]- malondialdehyde adduct (thiobarbituric reactive substance [TBARS]) and, is measured by its absorbance at 532nm.

Reagents:

i. TBA Reagent: TBA – 100mg, EDTA – 46mg, 20% TCA – 10ml, 2.5N HCL – 5ml, total volume made up to 20ml with double distilled water.

ii. Standard malondialdehyde: A stock solution of malondialdyhyde was prepared in double distilled water, using 1,1,3,3 tetraethoxypropane. This was stored at 4 °C and diluted just before use, to have a working standard containing 50 nmoles/ml.

Procedure:

Reagent	Blank	Tissue
Sample	-	1.0ml
Double Distilled Water	1.0ml	-
TBA Reagent	1.0ml	1.0ml

Keep in boiling water bath for 20 minutes, cool the test tubes and centrifuge at 3000 rpm for 15 minutes and read the absorbance at 532 nm.

Calculation: According to the slope calculated from the standard graph.

Unit: nmoles of MDA formed/ 100mg of tissue.

Reduced Glutathione

Principle: Cell contains GSH as a non protein sulfhydryl compound. 5,5'-dithiobis (2-nitrobenzoic acid) [DTNB] is a disulfide compound, which is readily reduced by

sulfhydryl compounds forming a highly coloured yellow anion, which can be read at 412nm.

Reagents:

i. Precipitating Reagent: Glacial metaphosphoric acid – 1.67gm, EDTA – 0.20gm, NaCl – 30gm and total volume was made up to 100ml with double distilled water.

ii. Phosphate Solution: 0.3M Na2HPO4 prepared in double distilled water.

iii. DTNB: 5,5'-dithiobis (2-nitrobenzoic acid) – 40mg dissolved in 100ml 1% Sodium Citrate.

iv. Sodium Citrate: 1% Sodium citrate prepared in double distilled water.

v. Stadard GSH Solution: 2mM GSH stalk solution was prepared in double distilled water.

Procedure:

Reagents	Blank	Tissue
Sample	-	1.0ml
Double distilled water	1.0ml	-
Precipitating Reagent	1.5ml	1.5ml
Keep the test tubes for 5 minutes and than centrifuge at 3000rpm for 15 minutes and collect the supernatant		
Supernatant	0.5ml	0.5ml
Phosphate solution	2.0ml	2.0ml
DTNB	0.25ml	0.25ml

Read the absorbance at 412nm within a minute after adding DTNB.

Calculation: Calculation was done according to the slope value from the standard graph.

Unit: µg GSH/ 100mg of Tissue.

Ascorbic acid

Ascorbic acid content was measured by the method of Omaye *et al.* (1971).

Reagents:

i. Trichloroacetic acid: 10% TCA, w/v

ii. Trichloroacetic acid: 5% TCA, w/v

iii. Sulphuric acid: 60% H_2SO_4, v/v

iv. 2,4,-dinitrophenyl hydrazine (DNPH) – thiourea – copper sulphate (DTC) reagent: DNPH – 3.0gm, Thiourea – 0.4gm, 0.05gm $CuSO_4$ dissolved in 100ml of 9N H_2SO_4.

v. Standard Ascorbic acid: Ascorbic acid – 50mg in 100ml of 5% TCA

Procedure:

Aliquots of the tissue homogenate were prepared in ice-cold 10% TCA and centrifuged at 3500 rpm for 20 minutes. And supernatant was collected for the assay.

Reagents	Blank	Sample
Supernatant	-	1.0ml
Double Distilled water	1.0ml	-
DTC reagent	0.2ml	0.2ml
Test tubes were incubated for 3 hours at 37°C		
60% H_2SO_4	1.5ml	1.5ml
Mix it well and allow it to stand for 30 more minutes at room temperature and than read the absorbance at 520nm.		

Standard ascorbic acid was also treated similarly.

Calculation: Calculation was done according to the slope value from the standard graph.

Unit: µg/ 100 mg of tissue

Catalase

The activity of catalase was assayed by the method of Sinha (1972).

Principal: Dichromate in glacial acetic acid is reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2) with the formation of perchloric acid as an unstable intermediate. The chromic acetate formed was measured at 570nm.

Reagents:

i. Hydrogen peroxide: 0.2M H_2O_2, v/v.

ii. Sodium Phosphate buffer: 0.01M, w/v, pH 7.0

iii. Dichromate-acetic acid reagent: 5% solution of potassium dichromate with glacial acetic acid in the ration of 1:3 (v/v). This solution was further diluted to 1:5 with double distilled water, before use.

Procedure:

Reagents	Blank	Sample
Double distilled water	0.4ml	0.4ml
Sodium phosphate buffer	1.0ml	1.0ml
H_2O_2	5.0ml	5.0ml
Tissue homogenate	0.1ml *	0.1ml #
Dichromate-acetic acid reagent	2.0ml @	2.0ml @
Heat the test tubes for 10 minutes and allow it to cool		

The green colour developed was read at 570 nm.

Note:

#	0.1ml tissue homogenate was added to initiate the reaction.
@	2.0ml dichromate-acetic acid was added after 15, 30, 45 and 60 seconds to stop the reaction
*	To the blank test tube tissue homogenate was added after the addition of the dichromate-acetic acid reagent.

Calculation:

$$\frac{\Delta OD/min \times 3.4 \times 1000 \times 1 \times \text{Dilution factor} \times 1}{\text{O time OD} \times 34 \times \text{volume of tissue extract} \times \text{mg protein}}$$

Unit: the activity of catalase was expressed as Units/ mg protein (1unit is the amount of enzyme that utilizes 1µmoles of H_2O_2/ minute).

Superoxide dismutase (SOD)

The enzyme activity was determined according to the method of Marklund and Marklund (1974).

Principle: The degree of inhibition of auto-oxidation of Pyrogallol, at an alkaline pH, by superoxide dismutase was used as a measure of the enzyme activity.

Reagents:

i. Tris-HCl – EDTA buffer: ph-8.2,Tris-HCl – 50mM, w/v, containing EDTA – 1mM, w/v

ii. Tris-HCl buffer: Tris-HCl – 50mM, pH-7.5

iii. Pyrogallol stock solution: 25.2mg pyrogallol was dissolved in 1.0ml of 50mM Tris-HCl buffer, pH-7.5 in dark container having cover lid.

iv. Pyrogallol working solution: 0.5ml of the above stock solution was diluted to 5.0ml with 50mM Tris-HCl buffer to give a 2mM working solution. The working solution was prepared for freshly after every 5 reactions.

Procedure:

Reaction mixture for 100% auto-oxidation of pyrogallol contained 2.4ml Tris-HCl-EDTA buffer pH-8.2, 0.3ml of 2mM pyrogallol and 0.1ml of double distilled water. The rate of auto-oxidation of pyrogallol was noted at an interval of 1 minute to 3 minutes to get the 100% auto-oxidation of pyrogallol.

The enzyme assay mixture contained 2.4ml Tris-HCl-EDTA buffer pH-8.2, 0.3ml of 2mM pyrogallol and 0.1ml of enzyme source. The rate of inhibition of pyrogallol auto-oxidation after the addition of enzyme was recorded. The percentage inhibition in auto-oxidation of pyrogallol in the presence of enzyme source was converted to units of inhibition. The amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation is considered as 1 unit of enzyme activity. The exact unit of enzyme activity was then calculated using the formula.

Calculation:

$$\frac{\text{Units of inhibition} \times \text{assay volume} \times \text{dilution factor} \times 1}{\text{Volume of tissue extract} \times \text{mg protein}}$$

Unit: unit/ mg protein

Glutathione peroxidase (GPx)

The activity of glutathione peroxidase (GPx) was determined by the method of Rotruck *et al.* (1973).

Principle: Glutathione peroxidase converts reduced glutathione (GSH) into oxidized form using hydrogen peroxide during its reaction. The amount of GSH utilized is estimated by measuring it in the assay mixture before and after enzyme activity. GSH reacts with the DTNB to give a yellow colour, which is read at 412nm.

Reagents:

i. Sodium phosphate buffer: 0.4M, w/v, pH 7.0

ii. Sodium azide: NaN_3 – 10mM, w/v

iii. Reduced Glutathione: GSH – 4mM, w/v

iv. Hydrogen peroxide: H_2O_2 – 2.5 mM, v/v

v. Trichloro acetic acid: 10% TCA, w/v

vi. Disodium hydrogen phosphate solution: 0.3M, w/v

vii. DTNB: 5,5'-dithiobis (2-nitrobenzoic acid) – 40mg dissolved in 100ml 1% Sodium Citrate

viii. Standard reduce glutathione: GSH – 20mg in 100ml double distilled water

Procedure:

Reagents	Blank	Sample
Sodium phosphate buffer	-	0.5ml
NaN_3	-	0.1ml
Reduced Glutathione	-	0.2ml
H_2O_2	-	0.1ml
Tissue homogenate	-	0.5ml
Double distilled water	-	0.6ml
Tubes were Incubated at 37°C for 3 minutes		
10% TCA	-	0.5ml
To determine the residual glutathione content the test tubes were centrifuged at 3000 rpm for 10 minutes and than the supernatant was collected		
Disodium hydrogen phosphate solution	4.0ml	4.0ml
DTNB	1.0ml	1.0ml

* Suitable aliquots of standard were also run similarly.

Calculation:

$$\frac{\text{OD of unknown} \times \text{Standard Conc.} \times 1 \times \text{Dilution factor} \times 1}{\text{OD of the standard} \times \text{volume of tissue extract} \times \text{mg protein}}$$

Unit: The enzyme activity was expressed as Units/mg protein (one unit is the amount of enzyme that converts 1 µmole of GSH to GSSG in the presence of H_2O_2/ minute).

Glutathione Reductase (GR)

The activity of glutathione reductase (GR) was determined by the method of Racker (1955).

Reagents:

i. 1M Potassium phosphate buffer (pH 7.6)

ii. 10mM EDTA

iii. 1mM NADPH

iv. 10mM

Reagents	Blank	Sample
Tissue homogenate	0.0ml	0.1ml
Double Distilled water	0.5ml	0.5ml
EDTA	5.0ml	5.0ml
Phosphate buffer	0.05ml	0.05ml
NADPH	0.1ml	0.1ml
Reaction was started by addition of GSSG as given below		
GSSG	0.1ml	0.1ml
Activity was measured by monitoring the decrease in absorbance at 340nm at per one minute interval		

Unit: nmoles of NADPH oxidized/ min/ mg protein

Protein

The tissue protein content was estimated by the method of Lowry *et al.* (1951).

Principle: Protein reacts with Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to reaction of the alkaline copper with protein and the reduction of phophomolybdate by tyrosine and tryptophan (aromatic amino acids) in

protein. The intensity of colour depends on the amount of aromatic amino acids present and will thus vary for different protein.

Reagents:

i. Lowry's reagent:
   Solution A: 2% Sodium carbonate I 0.1N Sodium hydroxide
   Solution B: 0.5% Copper sulphate in 2% Sodium potassium tartarate
   50ml Lowry's reagent was freshly prepared by adding 1.0ml of solution B to 49.0ml of solution A.

ii. Folin-Ciocalteau reagent (1N): The commercially available 2N solution was diluted 1:1 with double distilled water, just before the use.

iii. Standard: Standard bovine serum albumin (BSA) containing 20mg/ 100ml were used to generate a standard graph. The concentration of the standard solution was 200µg of protein/ ml.

Procedure:

Reagents	Blank	Sample
Tissue homogenate	-	0.1ml
Double Distilled water	1.0ml	0.9ml
Lowry's reagent	5.0ml	5.0ml
Test tubes were allowed to stand at room temperature for 10 minutes		
Folin-Ciocalteau reagent	0.5ml	0.5ml
Mix it well and allow it to stand for 30 more minutes in the dark condition at room temperature.		

Standard bovine serum albumin (BSA) at different concentrations (20-100µg) was treated similarly.

Calculation: Protein content was calculated using the slope value of standard graph.

Unit: mg protein/ 100mg of tissue.

3β-Hydroxy Steroid Dehydrogenase (3β-HSD)

3β-HSD enzyme activity was assayed by the colorimetric method of Shivanandappa and Venkatesh (1997).

Principle: This enzyme catalyses the reaction of converting Dehydroepiandrosterone (DHEA) to Androsternedione with concomitant reduction of NAD to NADH which, combines with iodonitrotetrazolium chloride (INT) and forms formazone, a pink chromophore which absorbs light at 490nm.

Regents:

i. 50mM Pthalate buffer (pH-3.0): 51.0ml of 0.1N HCl, 2.5ml of Tween 20. Mix it well and add 2.55gm potassium hydrogen thalate to the mixture. Adjust pH-3.0 and make up the volume to 250ml with double distilled water.

ii. Tris-HCl buffer: 100mM, pH-7.8

iii. Sucrose buffer: 250mM, pH-7.4

iv. NAD: 500μM

v. Colour reagent: INT-40mg, 0.5ml Tween 20. Dissolve in 50ml double distilled water. For standard curve 10mg phenazene methosulfate (PMS) was added.

vi. Substrate [DHEA (5-adrostean-3β-ol-17 one) OR Prenenolone (5-pregne-3β-ol-2 one)]: DHEA (100μM) or Pregnenolone (100μM) was first dissolved in 0.05ml of dimethyl formamide (DMF) and than prepared in 10.0ml of Tris-HCl buffer(100mM, pH-7.8).

vii. Standard curve: 1mM solution of NADH (nicotinamide adenine dinucleotide reduced) was freshly prepared in double distilled water. Aliquots of graded concentrations of NADH (0 to 150nmlo) were reacted with the colour reagent and after the colour formation 2.0ml of phthalate buffer was added to each tube and read at 490nm.

Procedure:

Reagents	Blank	Sample
Tris-HCl buffer	1.0ml	0.5ml
NAD	-	0.5ml
Substrate (DHEA or Pregnenolone)	0.5ml	0.5ml
Enzyme source	0.05ml	0.05ml
Colour reagent	0.25ml	0.25ml
Test tubes were incubated at 37 °C temperature for 60 minutes		
Phthalate buffer	1.0ml	1.0ml
Read at 490nm.		

Calculation: The enzyme activity was calculated from the standard graph of NADH.

Unit: nmol of NADH formed/ min/ mg protein.

17β-Hydroxy Steroid Dehydrogenase (17β-HSD)

17β-HSD enzyme activity was assayed by the colorimetric method of Shivanandappa and Venkatesh (1997). The original method is for 3β-HSD however, the method is also applicable to NADP-dependent steroid dyhydrogenases such as 17β-HSD by providing suitable substrate.

Principle: This enzyme catalyses the reversible reaction of converting estradiol to estrone with the concomitant reduction of NADP to NADPH which, combines with iodonitrotetrazolium chloride (INT) and forms formazone, a pink chromophore which absorbs light at 490nm.

Regents:

i. 50mM Phthalate buffer (pH-3.0): 51.0ml of 0.1N HCl, 2.5ml of Tween 20. Mix it well and add 2.55gm potassium hydrogen thalate to the mixture. Adjust pH-3.0 and make up the volume to 250ml with double distilled water.

ii. Tris-HCl buffer: 100mM, pH-7.8

iii. Sucrose buffer: 250mM, pH-7.4

iv. NADP: 500μM

v. Colour reagent: INT-40mg, 0.5ml Tween 20. Dissolve in 50ml double distilled water. For standard curve 10mg phenazene methosulfate (PMS) was added.

vi. Substrate(Estradiol): Estradiol(100μM) was first dissolved in 0.05ml of dimethyl formamide (DMF) and than prepared in 10.0ml of Tris-HCl buffer (100mM, pH-7.8).

vii. Standard curve: 1mM solution of NADPH (nicotinamide adenine dinucleotide phosphate reduced) was freshly prepared in double distilled water. Aliquots of graded concentrations of NADPH (0 to 150nmlo) were reacted with the colour reagent and after the colour formation 2.0ml of phthalate buffer was added to each tube and read at 490nm.

Procedure:

Reagents	Blank	Sample
Tris-HCl buffer	1.0ml	0.5ml
NADP	-	0.5ml
Substrate (Estradiol)	0.5ml	0.5ml
Enzyme source	0.05ml	0.05ml
Colour reagent	0.25ml	0.25ml
Test tubes were incubated at 37 °C temperature for 60 minutes		
Phthalate buffer	1.0ml	1.0ml
Read at 490nm.		

Calculation: The enzyme activity was calculated from the standard graph of NADPH.

Unit: nmol of NADPH formed/ min/ mg protein.

Serum Testosterone

Serum Testosterone was measured by using the RIA Testosterone, direct (REF - IM119) kit obtained from IMMUNOTECH SAS (A BECKMAN COULTER COMPANY), France.

Principle: The radioimmunoassay of testosterone is a competition assay. Samples or calibrators are incubated with ^{125}I-lableled testosterone in antibody-coated tubes. After incubation, the liquid content of tubes is aspirated and the bond radioactivity is determined in a gamma counter, a standard curve is prepared. Unknown values are obtained from the curve by interpolation.

Performance Characteristics of the Assay: The analytical sensitivity of the testosterone assay was 0.025ng/ml and measuring range 0.025 – 20ng/ml. The

antibody used in the immunoassay is highly specific for testosterone and extremely low cross reactivity was obtained against several related molecules such as 5αDihydrotestosterone, Δ4-Androstenedione, etc. The intra- and inter-assay coefficient of variations was 7.2 – 14.8% and 6.9 – 15.0%, respectively.

Reagents: Anti-testosterone antibody-coated tubes (ready to use), ^{125}I-lableled testosterone tracer (ready to use), Calibrators (ready to use) and control serum (ready to use) were provided by the manufacturer.

Procedure: As per the manual of direction for use supplied by the manufacturer.

Calculation: As per the instruction given in the manual of the kit manufacturer.

Unit: ng of Testosterone/ ml of Serum

Serum Estradiol

Serum Estradiol was measured by using ELISA kit procured from dbc-Diagnostics Biochem Canada Inc. (Cat. No. # CAN-E-430)

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario. Competition occurs between an unlabeled estradiol (present in standards, control and patient samples) and an enzyme-labelled estradiol (conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the colour formed is inversely proportional to the concentration of unlabeled estradiol in the sample. A set of standards is used to plot a standard curve from which the amount of estradiol in patient samples and controls can be directly read.

Performance Characteristics of the Assay: The analytical sensitivity of the estradiol assay was 10 pg/ml. The antibody used in the ELISA is highly specific for estradiol and extremely low cross reactivity was obtained against several related molecules such as estriol and estrone. The intra- and inter-assay coefficient of variations was 9.3 – 4.6% and 10.1 – 9.6%, respectively.

Reagents: Necessary reagents were provided by the manufacturer

Procedure: As per the manual of direction for use supplied by the manufacturer.

Calculation: As per the instruction given in the manual of the kit manufacturer.

Unit: pg of Estradiol/ ml of Serum

Serum Melatonin

Serum Melatonin levels were determined by Melatonin Direct RIA kit (REF – BA 3300) procured from Labor Diagnostika Nord GmbH & KG (LDN), Germany.

Principle: The assay procedure follows the basic principle of radioimmunoassay, involving competition between a radioactive and non-radioactive antigen for a fixed number of antibody binding sites. The amount of ^{125}I-lableled antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the antibody bound radioactivity is precipitated with a second antibody in the presence of polyethelylene glycol. The precipitate is counted in a gamma counter. Quantification of unknown samples is achieved by comparing their activity with a reference curve prepared with unknown calibrators.

The sensitivity of the Melatonin assay was 2.0pg/ml, the intra – and inter – assay coefficient of variation was 9.8 – 12.3% and 9.6 – 16.2% respectively. The cross reactivity was of Melatonin with N-Acetylserotonin and Serotonin were 0.80 and <0.01% respectively.

Reagents: Calibrator, Control, Equalizing reagent, enzyme, Enzyme buffer, Melatonin Antiserum, ^{125}I-Melatonin and Precipitating Regent were provided by the manufacturer.

Procedure: As per the manual for direction for use supplied by the manufacturer.

Calculation: As per the instruction given in the manual of the kit manufacturer.

Unit: pg of Melatonin/ ml of Serum

Estimation of Tissue metal load (Chromium, Cadmium and Nickel):

The tissue Cr, Cd and Ni estimation was carried out by the method described in NIOSH Manual of Analytical Methods (NMAM), Fourth Edition, 1994. Prepared samples were then analyzed at Earth Science Department, Indian Institute of Technology – Bombay (IIT-B), Powai, Mumbai, using Inductive Coupled Plasma Atomic Emission Spectrophotometer **ICP – AES** of HORIBA Jobin Yvon, France, model No. ULTIMA-2. The values were expressed as µg/gm of tissue. And finally the percentage of increased or decreased metal content compared to control was calculated for representation.

Histology (TREATMENT SCHEDULE – III 60 DAYS)

Testis and epididymis from control and experimental animals were fixed in 10% formalin. 5 µ thick sections were cut and stained with haematoxylin and eosin, and were observed under the light microscope (Image analyzer Lieca).

TUNEL Assay

Plain (without HE staining) parafinated slides were deparafinated and following the instruction manual of manufacturer TUNEL assay was carried out using R & D Systems Kit (MN, USA).

Caspase-3

Caspase-3 activity was determined by following the instruction manual of the manufacturer provided with the Kit (R & D Systems, MN, USA).

Serum NO

NO kit was procured from R & D Systems (MN, USA) to determine serum titre of NO and was determined by the instruction manual of the manufacturer provided with the kit.

Semen Parameters (Sperm Count, Sperm Motility and Sperm Abnormality): (Gopalkrishnan ***et al.***, 1980)

→ Cauda epididymis was dissected out and washed with saline and immediately given cut so as to allow the semen to ooze out. Semen was collected and diluted with PBS. Sperm counts were made using a Neubauer's chamber and expressed in value x 10^6/ ml.

→ Sperm motility was determined by using a hanging drop preparation in cavity slide and expressed in terms of percentage.

→ Sperm abnormalities (curved tail, headless, tailless, abnormal head) were observed at 400X in the microscope (Image analyzer - Lieca) and final calculation was expressed in terms of percentage.

Separation of Leydig cells:

Testes were decapsulated under aseptic conditions with fine forceps without breaking the seminiferous tubules and were incubated with 5ml. medium- L in 25ml. conical flask for 15 minutes at 34°C. After this incubation the flask were gently shaken and fresh DMEM (10 ml.) was added. Allowed to stand for 5 minutes at 37°C and the supernatant was transferred to sterilized centrifuge tubes using Pasteur pipette. This

procedure was repeated once again. To remove the remaining Leydig cells in the testis. The supernatant were combined and centrifuged at 1000xg for 15 minutes at 4°C. this process was repeated twice (for washing the cells) and the pellet was resuspended in 5 ml. DMEM. This suspension is called crude Leydig cell suspension.

Purification of Leydig cells:

Leydig cells were purified by discontinuous Percoll density gradient method (Rigaudiere *et al.*, 1988). 2 ml. 75% Percoll gradient was added slowly to a graduated centrifuge tube. Above this layer 2ml. each of 60%, 45%, 30%, 15% and 5% percoll were gently laid one over the other, taking care to avoid mixing, in an aseptic condition. 1 ml. crude Leydig cell suspension was added above the percoll gradients without disturbing the gradient and centrifuged at 1000x g for 20 minutes at 4°C. After centrifugation, purified Leydig cells observed 30% and 45% gradients were removed carefully using a Pasteur pipette. 5 ml. DMEM was added, mixed and centrifuged at 800xg for 15 minutes at 4°C. The supernatant was discarded. This procedure was repeated thrice so as to remove the residual percoll and the pellet was suspended in 2 ml. DMEM. The purified Leydig cell preparation was used for in vitro studies. The purified Leydig cells were identified by histochemical localization of 3β-HSD by using the procedure of Aldred and Cooke (1983). 100 µl Leydig cell suspension was incubated with 100 µl each of β- NAD. NBT and pregnenolone at 30°C for 60 minutes, after the incubation the cells were washed thrice with phosphate buffer and resuspended in the same buffer. The percentage of stained cells was counted using a haemocytometer under the microscope. The purity of Leydig cells was 82%. The principle contaminants were spermatogonia, spermatocytes, sperm and peritubular myoid cells.

Viability of isolated Leydig cells:

The viability of the isolated testicular cells was assessed by trypan blue exclusion test (Aldred and Cooke, 1983). 100 µl of 4% trypan blue solution and 100 µl cell suspensions were incubated for 5 minute at room temperature. The cells were then washed twice with saline and observed under the microscope. The number of unstained and stained cells, which represents viable and damaged cells, respectively was counted. The percentage of viable cells was then calculated from the total number of cells counted. The viability of the cells was assessed both before and after the experiment. The viability of Leydig cells was found to be between 83% to 87%.

Leydig cell culture and treatment:

10^5 Leydig cells (in 100µl medium) per well cultured in 96-well plates and placed in a 5% CO_2 and 95% air in a CO_2 incubator (Thermo) at 37 °C. Leydig cells were incubated with or without single (Cr(VI), Cd or Ni) or multiple metals (all the three metals) at the concentration of 1mM for different time period (3, 6 and 12 hrs.) with serum free medium to check viability and basal testosterone production. The similar set was repeated to assess the hCG (1 IU/ ml medium) stimulated testosterone production. At the end of the respective incubation period media were collected and centrifuged at 1500 rpm for 10 minutes in cold centrifuge 4 °C and supernatants were used for testosterone assay.

Statistical Analysis:

The data were statistically analyzed by one-way analysis of variance (ANOVA) and Bonferroni multiple comparison test to get the significance between different group means at $p< 0.05$ using computer based software **Prism 3.0** and routine mean and slope values were calculated using **MS-Excel**.