CHAPTER - 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Sources of Fine Chemicals

1, 1, 3, 3-tetraethoxy propane, crystalline beef liver catalase, superoxide dismutase, urethane and egg lecithin, were obtained from Sigma Chemicals, St.Louis, M.O., U.S.A.

Alpha lipoic acid, sodium selenite, Co-enzyme Q10, thiobarbituric acid, tris-buffer, sucrose, ATP, ferric chloride hexahydrate, triolein, reduced glutathione,1-amino-2-naphthol-4-sulphonicacid, sodium metabisulphite, sodium sulphite, sodium sulphate and 5,5'-dithiobis (2-nitro benzoic acid) were obtained from Hi-Media Laboratories Pvt. Ltd.,

Cadmium chloride, trichloroacetic acid, ammonium molybdate, acetylacetone, hydrogenperoxide, ammonium thiocyanate, sodium phosphate and sodium metaperiodate were obtained from S.D.Fine Chemicals, Mumbai, India.

Sodium hydroxide, sodium carbonate, sodium bicarbonate, magnesium chloride, sodium chloride, potassium chloride, calcium chloride, potassium hydroxide, sodium dihydrogen orthophosphate, potassium dihydrogen orthophosphate, chloroform, ether, hydrochloric acid, sulphuric acid, glacial acetic acid and isopropanol were purchased from Qualigens Chemicals Ltd., Mumbai, India.

Ethylenediaminetetraacetic acid disodium salt, epinephrine bitartarate, cholesterol, were obtained from BDH Chemicals, Mumbai, India.

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

3.1.2 Animals

Adult albino rats of Wistar strain of either sex weighing 200-250gm were used in the entire study. Each pair of animal was housed in a spacious cage containing husk as nesting material, which was maintained at 25±3°C in a well-ventilated animal house under natural photoperiod condition.All the animals were from in house animal breeding. All the animals were fed with standard pellet diet *ad libitum* and had free

access to water. The investigation conformed to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).The Institutional Animal Ethical Committee approved the protocol of the study.

3.2 METHODS (EXPERIMENTAL DESIGN):

Pilot study was carried out wherein animals were divided into four groups as shown below.

Group1(Control)	Animals received double deionized water (DDW) and a normal diet.
Group2(10CD)	Animals received 10 ppm of cadmium chloride dissolved in DDW along with normal diet.
Group 3(30 CD)	Animals received 30 ppm of cadmium chloride dissolved in DDW along with normal diet.
Group4(100CD)	Animals received 100 ppm of cadmium chloride dissolved in DDW along with normal diet.

The following parameters were investigated after 30 days of treatment:

Tissue Parameters

1. Lipid peroxidation or malondialdehyde formation
2. Endogenous Antioxidants
   a) Superoxide dismutase (SOD)
   b) Catalase (CAT)
   c) Reduced glutathione (GSH)

Serum Parameters

Blood was collected from retro orbital plexus under mild ether anaesthesia. Serum was separated and they were estimated using commercially available kits (Bio Systems Ltd., Spain).

1. Serum glutamate pyruvate transaminase (GPT)
2. Serum glutamate oxaloacetate transaminase (GOT)
3. Alkaline phosphatase (Alkp)
4. Lactate dehydrogenase (LDH)

Pilot study carried out on 10ppm, 30ppm and 100ppm cadmium showed that most of the changes in the various enzymes and cellular components have occurred in animals exposed to 100ppm of cadmium chloride. Hence further studies were carried out with 100 ppm of cadmium chloride alone and in combination with alpha lipoic acid, selenium and coenzyme Q10.

The study was designed by dividing the animals (rats) into groups as was required for pharmacological and toxicological studies. After 30 days of several treatments in rats as mentioned below, various investigations were carried out.

Group 1 (Control)	Animals received double de-ionized water (DDW) and a normal diet.
Group 2 (100 CD)	Animals received cadmium chloride (100 ppm) dissolved in DDW along with normal diet.
Group 3 (ALA)	Animals received Alpha-lipoic acid i.p along with normal diet and DDW.
Group 4 (ALA+100CD)	Animals received Alpha-lipoic acid i.p along with normal diet and 100 ppm cadmium chloride in DDW.
Group 5 (Se)	Animals received Selenium i.p along with normal diet and DDW.
Group 6 (Se+100CD)	Animals received Selenium i.p along with normal diet and 100ppm cadmium chloride in DDW.
Group 7 (CoQ10)	Animals received Coenzyme Q10 i.p along with normal diet and DDW.
Group 8 (CoQ10+100CD)	Animals received Coenzyme Q10 i.p along with normal diet and 100ppm cadmium chloride in DDW.

Solutions of cadmium chloride was prepared by dissolving it in double de-ionized water and given as ad libitum (Schroeder and Vinton, 1962).Alpha-Lipoic acid given in the dose of 25 mg/kg i.p. in arachis oil.Co-enzyme Q10 given in the dose of 10mg/kg i.p. in arachis oil. Selenium in the form of Sodium Selenite given in the dose of 1mg/kg i.p. in saline.

3.2.1 Pharmacological Investigations

The following parameters were investigated after thirty days of treatment with cadmium alone and its combination with alpha lipoic acid, co-enzyme Q10 and selenium.

Electrocardiogram and Hemodynamic measurements:

Blood pressure, heart rate and Electrocardiogram (E.C.G.) were recorded with the help of Biopac MP30 data acquisition system (Biopac Systems, Santa Barbara, CA).

BIOCHEMICAL ESTIMATIONS

Tissue Parameters

1. Lipid peroxidation or malondialdehyde 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^{++}-ATPase
    c) Mg^{++}-ATPase
4. Inorganic Phosphorous (Pi)
5. Estimation of Lipids
    a) Cholesterol (CHOL)
    b) Triglycerides (TGLY)
    c) Phospholipids (PLIPI)

Serum Parameters

Blood was collected from retro orbital plexus under mild ether anaesthesia. Serum was separated and they were estimated using commercially available kits (Bio Systems Ltd., Spain).

1. Serum glutamate pyruvate transaminase (GPT)

2. Serum glutamate oxaloacetate transaminase (GOT)
3. Alkaline phosphatase (Alkp)
4. Lactate dehydrogenase (LDH)
5. Total bilirubin (TBIL)
6. Total protein
7. Cholesterol
8. Triglyceride

Toxicological study:

The deposition of metal in liver, kidney, lung, heart and brain was investigated in animals exposed to cadmium chloride alone and in combination with alpha lipoic acid, CoenzymeQ10 and Selenium.

Apart from this body weight, organ weight, histopathological studies were also carried out.

3.3 ELECTROCARDIOGRAM AND HAEMODYNAMIC MEASUREMENTS

After completion of treatment schedule Blood pressure, heart rate and Electrocardiogram (E.C.G.) was recorded with the help of Biopac MP30 data acquisition system (Biopac Systems, Santa Barbara, CA).

Electrocardiographic measurements:

After completion of treatment schedule rats from each group were anaesthetized with light ether anesthesia and ECG was recorded through needle electrodes (Lead II) using Biopac MP30 data acquisition system (Biopac Systems, Santa Barbara, CA).The changes in the ECG were noted.

Hemodynamic measurements

Measurement of Blood pressure by Direct and Indirect Method :

Indirect Method (Non-Invasive Method):

Blood pressure was measured non invasively by tail cuff method using LE 5002 storage pressure meter (LETICA scientific instruments, SPAIN) in all the above mentioned groups.For the blood pressure measurements animals were trained for at least 1 week until blood pressure was steadily recorded with minimal stress and restraint. The mean of 7-8 measurements of trained animals was recorded. Blood pressure can be measured indirectly in the tail by occluding the artery with a small inflatable tubular cuff and by sensing the pulse. Cardiovascular parameters-Systolic BP, Diastolic BP and Mean BP were measured weekly for 4 weeks by indirect non invasive tail cuff method. Heart rate was also determined at the end of 30 days.

Direct Method (Invasive Method):

After completion of treatment schedule rats from each group were anaesthetized with urethane (120mg/100g, i.p.).Femoral vein was cannulated with fine polyethylene catheter for administration of drugs.Tracheostomy was performed and blood pressure was recorded from left common carotid artery using pressure transducer by direct method on BIOPAC data Acquisition System (BIOPAC MP30 SYSTEM, USA).Heparinised saline (500U/ml) was filled in the transducer and in the fine polyethylene catheter cannulated to the carotid artery to prevent clotting. After 30 minute of stabilization, blood pressure and vascular reactivity to various drugs like noradrenaline (1and 2µg/kg), adrenaline (1and 2µg/kg) and isoprenaline (1and 2µg/kg) were recorded in the rats.

3.4 Removal and Processing of Serum and Tissues for Various Investigations

At the end of 30 days, animals were anaesthetized with ether and blood was collected from the retro-orbital plexus without any anticoagulant and allowed to clot for 10 minutes at room temperature. It was then centrifuged at 2500 rpm for 20 minutes. The serum obtained was kept 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 (0.25 M)*

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

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

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

Procedure

The animals were euthanasiously sacrificed, after blood collection; liver, lung, heart, kidney and brain were isolated carefully and quickly transferred to ice-cold phosphate buffered saline (pH 7.4). The organs were blotted free of blood and tissue fluids, weighed on a Single Pan Electronic Balance (Precisa 205 ASCS) and the weights were recorded. The organs were cross-choppedwith 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 homogenized in chilled 10mM Tris-hydrochloride buffer (pH 7.4) to a concentration of 10% w/v with 25 strokes of Teflon pestle inglass homogenizer at a speed of 2500 rpm. Prolonged homogenization under hypotonic condition was designed to disrupt, as far as possible, the structure of the cells so as to release soluble proteins. Suitable aliquots of homogenates were taken out separately for the estimation of metals by atomic absorption spectrophotometry. The remaining homogenates were then 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 assay of lipid peroxidation and endogenous antioxidants.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 of different membrane bound enzymes.

3.5 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.

3.5.1 Lipid peroxidation or malondialdehyde (MDA) formation

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 adjusted 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 adjusted to 100ml 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

2.0ml of the tissue homogenate (supernatant) was added to 2.0ml of freshly prepared 10%w/v trichloroacetic acid and the mixture was allowed to stand in an ice bath for 15 minutes. After 15 minutes, the precipitates were separated by centrifugation and 2.0ml of clear supernatant solution was mixed with 2.0ml of freshly prepared thiobarbituric acid. 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 for color development. The absorbance was measured at 532nm against reagent blank.

The values are expressed as nM of MDA formed /gm of tissue.

3.5.2 Assay of Endogenous Antioxidants

3.5.2.1 Superoxide Dismutase (SOD) (Superoxide: Superoxide oxido reductase)

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 500ml of distilled water and the volume was made up to 1000ml with distilled water.
2. *Ehylenediaminetetra acetic acid (EDTA) solution (0.49M)*
   1.82gm of EDTA was dissolved in 200ml of distilled water and the final volume was adjusted to 1000ml with distilled water.
3. *Epinephrine solution (3mM)*
   0.99gm of epinephrine bitartarate was dissolved in 100ml of distilled water and the final volume was adjusted to 1000ml with distilled water.
4. *Superoxide Dismutase standard (SOD) (100 U/L)*
   1mg (1000U/mg) of SOD from bovine liver was dissolved in 100ml of carbonate buffer.

Procedure

0.5ml of tissue homogenate was diluted with 0.5ml of distilled water, to which 0.25 ml 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 2000rpm. To 0.5ml of supernatant, 1.5ml of carbonate buffer and 0.5ml of EDTA solution was 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/gm of tissue. 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.

3.5.2.2 Catalase (CAT) (Hydrogen peroxide oxido reductase)

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

Reagents

1. *Hydrogen Peroxide solution (30mmol/l)*

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

2. *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).Adjust the pH to 7.0 with 1N NaoH.

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 1000U/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/gm of tissue.

3.5.2.3 Reduced Glutathione (GSH)

Reduced glutathione was determined as described by Moran et al. (1979).

Reagents

1. *Trichloroacetic acid (10%w/v)*

   Accurately weighed 10gm trichloroacetic acid was dissolved in sufficient quantity of distilled water and the final volume was adjusted to 100ml 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 100ml with buffer.

4. *Standard Glutathione*

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

Procedure

To 1ml of the tissue homogenate, 1ml of 10% TCA was added. The precipitated fraction was centrifuged and to 0.25ml of the supernatant, 2ml of DTNB reagent was added. The final volume was made up to 3ml with phosphate buffer. The color 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/gm of tissue.

3.5.3 Assay of Membrane Bound Enzymes and Inorganic Phosphorous

3.5.3.1 Sodium-potassium dependent adenosine triphosphatase (Na^+K^+ATPase) (ATP phosphohydrolase)

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

Reagents

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

   11.13gm of Tris-hydrochloride was dissolved in 900ml of distilled water. The pH of solution was adjusted to 7.5 with 2M hydrochloric acid and the volume was made up to 1000ml with distilled water.

2. *Magnesium sulphate solution (5mM)*

   1.232gm of magnesium sulphate was dissolved and made up to 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 up to 1000ml with distilled water.

4. *Sodium chloride solution (60mM)*

   3.231gm of sodium chloride was dissolved in 400ml of distilled water and the final volume was made up to 1000ml with distilled water.

5. *EDTA solution (0.1mM)*

   0.372gm of EDTA was dissolved in 200ml of distilled water and the final volume was made up to 1000ml with distilled water.

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

   0.220gm of ATP was dissolved in 4ml of 0.1N sodium hydroxide and the final volume was made up to 10ml with distilled water.

7. *Trichloroacetic acid (TCA) (10%w/v)*

   10gm of trichloroacetic acid was dissolved in 40ml of distilled water and the final volume was made up to 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 phosphorous content of the supernatant was estimated as described in section 3.3.3.4.The enzyme activity was expressed as mM of inorganic phosphorous liberated/minute/gm of tissue.

3.5.3.2 Calcium dependent adenosine triphosphatase (Ca^{++}-ATPase) (ATP phosphohydrolase)

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

Reagents

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

   15.12gm of Tris-hydrochloride was dissolved in 900ml of distilled water. The pH of solution was adjusted to 7.5 with 2M hydrochloric acid and the volume was made upto1000ml with distilled water.

2. *Calcium chloride solution (50mM)*

   5.55gm of calcium chloride was dissolved in 400ml of distilled water and the final volume was made up to 1000ml with distilled water.

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

   0.551gm of ATP was dissolved in 40 ml of distilled water and the final volume was made up to 100ml with distilled water.

4. *Trichloroacetic acid (TCA) (10%w/v)*

   10gm of trichloroacetic acid was dissolved in 40ml 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, calcium chloride, ATP and homogenate in a test-tube. The mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0ml of TCA (10%), mixed well and centrifuged. The amount of phosphorous liberated was estimated as described in section 3.3.3.4. The

enzyme activity was expressed as mM of inorganic phosphorous liberated /minute/gm of tissue.

3.5.3.3 Magnesium dependent adenosine triphosphatase (Mg^{++}-ATPase) (ATP phosphohydrolase)

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

Reagents

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

   45.24gm Tris-hydrochloride was dissolved in 900ml of distilled water. The pH of the solution was adjusted to 7.6 with 2M hydrochloric acid and finally the volume was made upto 1000ml with distilled water.

2. *Magnesium chloride solution (25mM)*

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

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

   0.551gm of ATP was dissolved in 40ml of distilled water and the final volume was made up to 100ml with distilled water.

4. *Trichloroacetic acid (TCA) (10%w/v)*

   10gm of trichloroacetic acid was dissolved in 40ml 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 reaction mixture was incubated at 37°C for 15 minutes. The reaction was arrested by the addition of 1.0ml of TCA (10%), mixed well and centrifuged. The liberated phosphorous was estimated as described in section 3.3.3.4.

The enzyme activity was expressed as mM of inorganic phosphorous liberated /minute/gm of tissue.

3.5.3.4 Determination of Inorganic phosphorous (Pi)

The inorganic phosphorous was estimated by the method described by Fiske and Subbarow (1925).

Reagents

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

   2.5gm of ammonium molybdate was dissolved up to 100ml with 3M sulphuric acid.

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

   0.25% w/v of ANSA reagent in 15%w/v of sodium metabisulphite and 20%w/v of sodium sulphite. 15gm of sodium metabisulphite was dissolved in 100ml of distilled water, and the solution was used as a vehicle for ANSA reagent. Accurately weighed 20gm of sodium sulphite was dissolved in100ml of distilled water.

   250mg of 1-amino-2-naphthol, 4-sulphonic acid 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 Phosphorous*

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

Procedure

1ml of the supernatant was taken and the volume was made up to 5.0ml with distilled water. To this, 1.0ml of 2.5% ammonium molybdate reagent and 0.5ml of ANSA reagent was added. The color developed within 20 minutes was read using blank containing water instead of sample at 620nm. A standard graph was prepared taking different concentrations of standard phosphorous (16-80µg). The values are expressed as mM of phosphorous liberated/minute/gm of tissue.

3.5.4 Extraction and Estimation of Lipids

The procedure of Folch et al. (1957) as modified by Suzuki (1965) was used for the extraction of lipids from the organs. Suitable quantity (1g) of tissue was homogenised with total 20ml of cold chloroform-methanol (2:1v/v) mixture in the Potter Elvehjem homogeniser with Teflon pestle. The contents were filtered through Whatman filter paper. The residue was re-extracted twice with small volumes of chloroform-methanol (2:1 v/v) mixture and then filtered again. The filtrates were pooled together and 0.3ml of glass distilled water was added, mixed well by vortexing and allowed to stand for phase separation at room temperature. The upper layer was removed with pasture pipette and the lower phase was washed with 0.4ml of Folch's pure upper phase solvent (consists of chloroform-methanol-water in a proportion of 3:48:47) and centrifuged at 3000rpm for 10 minutes. The upper phase was removed. The lower phase was used for the estimation of cholesterol, triglyceride and phospholipid.

3.5.4.1 Cholesterol (CHOL)

Cholesterol in acetic acid gives a red color with ferric chloride and sulphuric acid. The estimation of total cholesterol was carried out by Zlatkis, Zak and Boyle's (1953) method.

Reagents

1. *Ferric chloride solution*
   A 0.05%w/v solution of Ferric chloride, ($FeCl_3.6H_2O$) was prepared in glacial acetic acid (aldehyde free).
2. *Concentrated sulphuric acid*
3. *Glacial acetic acid*
4. *Stock solution of cholesterol*

Accurately weighed 100mg of cholesterol was dissolved in100mlof glacial acetic acid. This stock solution was then used for the preparation of working standard solution.

Procedure

Suitable aliquots of the samples (0.5ml) were transferred accurately into series of 10ml volumetric flasks. To this, 4ml of ferric chloride solution and 4ml of sulphuric acid were added. The solution were made up to 10ml with glacial acetic acid, flasks were closed tightly

with lid and mixed by repeated inversion. Then the lid was loosened and the flasks were left half-open for 20 to 30 minutes. The color developed was read against a reagent blank at 550nm. The values are expressed as mg of cholesterol / gm of wet, fresh tissue (mg /gm).

3.5.4.2 Triglycerides (TGLY)

Triglyceride was estimated by the method described by Foster and Dunn (1973) based on the method of Fletcher (1968).

Reagents

1. *Isopropanol*
2. *Alumina (neutral)*
3. *Potassium hydroxide (1M)*

   56.11gm of potassium hydroxide was dissolved in 1000ml of distilled water.
4. *Sodium meta periodate solution*

   77gm of anhydrous ammonium acetate was dissolved in about 700ml of distilled water. To this solution, 6ml of glacial acetic acid and 65mg of sodium meta periodate were added and the volume was made up to 1000ml with distilled water.
5. *Acetyl acetone reagent*

   7.5ml of acetyl acetone was dissolved in 200ml of isopropanol and the volume was made up to 1000ml with distilled water.
6. *Trieoline standard (10mM/L)*

   0.885ml of trieolein (glycerol trioleate) was dissolved in 60mlof isopropanol and the final volume was made up to 100ml with isopropanol.

Procedure

Aliquots of the lipid extract (0.2ml) were taken in duplicates in test-tubes. Lipid extract, 4ml of isopropanol and 400mg of alumina were mixed by vortexing for 15 minutes, centrifuged and 2ml of supernatant was transferred into separate test-tubes.0.6ml of potassium hydroxide was added to the supernatants and the mixture was incubated at 60-70°C for 1minute.After cooling, 1 ml sodium meta periodate solution was added and mixed in vortex mixer. 0.5ml acetylacetone reagent was added to the vortexed mixture, stoppered incubated at 50°C for 30 minutes. It was allowed to cool and the absorbance was read at

405nm against a reagent blank.A calibration curve of triolein standard (10 mM/L) was prepared in isopropanol in the range of 0-4 mM/L.The values were multiplied by 350 to obtain the triglyceride content. The values are expressed as mg of triglyceride / gm of wet, fresh tissue (mg / gm).

3.5.4.3 Phospholipid (PLIPI)

Phospholipids were estimated by the method described by Stewert (1980).

Reagents

1. *Ammonium ferrothiocyanate solution (0.1 M)*
   27.03gm of ferric chloride hexahydrate and 30.4gm of ammonium thiocyanate was dissolved in distilled water, and the volume was made up to 1000ml with distilled water.
2. *Anhydrous sodium sulphate*
3. *Standard Phospholipid (lecithin) (0.1mg/ml)*
   Stock solution
   50mg of egg lecithin was dissolved in 10ml of chloroform. 2ml of this solution was diluted to 10ml with chloroform.
   Working standard
   1.0ml of diluted stock solution was further diluted to 10ml to give the final concentration of 0.1mg/ml and used for the preparation of calibration curve.

Procedure

0.5ml of lipid extract was mixed thoroughly in a test-tube with 2.0ml of ammonium ferrothiocyanate solution and 2.5ml of chloroform. The contents of tubes were vortexed vigorously using Cyclo Mixer for 5 minutes and centrifuged at 1000 rpm for 15 minutes. The lower layer was removed by using syringe with long needle and retained in another test-tube containing a pinch of anhydrous sodium sulphate. The absorbance of solutions was noted at 485nm using a reagent blank.

The values are for phospholipids expressed as mg of phospholipid/ gm of wet, fresh tissue (mg / gm).

3.6 SERUM ESTIMATIONS

Blood was collected from retro orbital plexus under mild ether anaesthesia. Serum was separated and they were estimated using commercially available kits (Bio Systems Ltd., Spain).

3.6.1 Aspartate aminotransferase (GOT) (L-aspartate: L-aspartate; 2-oxoglutarate aminotransferase)

Quantitative estimation of Serum glutamate oxaloacetate transaminase (GOT) or aspartate aminotransferase (AST) was performed as per instructions provided for diagnostic kit Bio Systems Ltd.,Spain using A 25 auto analyser .Units were expressed as U/L.

3.6.2 Alanine aminotransferase (GPT) (L-alanin: L-alanin; 2-oxoglutarate aminotransferase)

Quantitative estimation of Serum glutamate pyruvate transaminase (GPT) or Alanine aminotransferase (ALT) was performed as per instructions provided for diagnostic kit Bio Systems Ltd.,Spain using A 25 auto analyser.Units were expressed as U/L.

3.6.3 Alkaline phosphatase (Alkp) (Orthophosphoric acid monoester phosphohydrolase)

Quantitative estimation of alkaline phosphatase (Alkp) was performed as per instructions provided for diagnostic kit Bio Systems Ltd., Spain using A 25 auto analyser. Units were expressed as U/L.

3.6.4 Lactate dehydrogenase (LDH) (L-lactate; NAD^+ oxido reductase)

Quantitative estimation of lactate dehydrogenase (LDH) was performed as per instructions provided for diagnostic kit Bio Systems Ltd., Spain using A 25 auto analyser.Units were expressed as U/L.

3.6.5 Total bilirubin (TBIL)

Quantitative estimation of Total bilirubin (TBIL) was performed as per instructions provided for diagnostic kit Bio Systems Ltd., Spain using A 25 auto analyser .Units were expressed as mg/dL.

3.6.6 Total proteins

Quantitative estimation of Total proteins was performed as per instructions provided for diagnostic kit Bio Systems Ltd., Spain using A 25 auto analyser. Units were expressed as gm/dL.

3.6.7 Cholesterol

Quantitative estimation of cholesterol was performed as per instructions provided for diagnostic kit Bio Systems Ltd., Spain using A 25 auto analyser. Units were expressed as mg/dL.

3.6.8 Triglyceride

Quantitative estimation of Triglyceride was performed as per instructions provided for diagnostic kit Bio Systems Ltd., Spain using A 25 auto analyser. Units were expressed as mg/dL.

3.7 DETERMINATION OF LEVELS OF CADMIUM IN TISSUES

The metal (cadmium) levels of the tissues were estimated by using Perkin Elmer-2380 Atomic Absorption Spectrophotometer by nitric acid digestion method (Greenberg et al., 1992).

The suitable aliquots of homogenates were placed in a beaker. To this, 5ml of concentrated Nitric acid was added. It was brought to boil slowly and evaporated on a hot plate to the lowest possible volume (about 3ml) before precipitation. The heating and addition of concentrated nitric acid was continued till the tissue was digested and the solution formed thus, became clear with a light greenish yellow color. After completion of digestion, the solution was allowed to cool and filtered in 50ml volumetric flask through Whatman filter paper No.1. The walls of beaker were washed with distilled water and filtered. Then the final volume was adjusted to 50ml with distilled water. Samples prepared in this way were subjected to Atomic Absorption Spectrophotometry for estimation of cadmium.

The amount of metal was expressed as µg/gm of tissues. The deposition of metal in liver, kidney, lung, heart and brain was investigated in animals exposed to cadmium chloride alone and in combination with Alpha lipoic acid, Selenium and CoenzymeQ10.

3.8 HISTOPATHOLOGICAL STUDIES:

After 30 days of several treatments, the rats were sacrificed and liver, lung, heart and kidney and brain were excised, blotted free of blood and tissue fluids and preserved in 10%v/v formalin 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 hematoxylin-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 observed under Olympus BX40 Photomicroscope and photographed. Expert guidance from the veteran pathologist was sought to determine histopathological changes.

3.9 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) followed by the Bonferroni multiple comparisons test using computer based fitting program (Prism,Graphpad).Differences were considered to be statistically significant when $p<0.05$.