Materials and Methods
2. MATERIALS AND METHODS

2.1 SELECTION OF PATIENTS

The patients for this study were chosen from the Hypertension Clinic at the Sri Ramachandra Medical College Hospital, Chennai - 600 116 and Employees State Insurance (ESI) Corporation Hospital, Chennai – 400 078, India. Normal subjects were selected from staff of Sri Ramachandra Medical College and Research Institute (SRMC & RI).

All chemicals were of analytical grade. Double distilled water was used for making reagents. Totally 70 patients (43 female and 27 male in an age group of 32 – 70 years) showing systolic blood pressure in the range of 140 - 240 mm Hg and diastolic 80 - 120 mm Hg was taken for the study. These patients were under treatment for hypertension for a period of 4 months to 10 years and who fulfilled the criteria of U.S. Joint National Committee. The normal subjects (n = 50) with same age and sex were included in this study. From the hypertensive subject twenty-five female patients were randomly assigned for vitamin E supplementation for 90 days. The study protocol was approved by the Ethics Committee of SRMC and the informed consent was obtained from each subject after the nature of the procedures had been explained. Hypertension was diagnosed on the basis of outpatient blood pressure \( \geq 140/90 \text{ mm Hg} \) on \( \geq 3 \) occasions. Patient who had established essential hypertension and in whom all secondary causes of hypertension was ruled out were only considered for this study.
2.2 EXPERIMENTAL DETAILS

Group 1: Normals (n = 50).

Group 2: Hypertensive patients (n = 70).

Group 3: Twenty-five patients were selected for Vitamin E supplementation for three months.

Group 3a: Hypertensives supplemented with Vitamin E 400 mg (Tocofer - 400, Torrent pharmaceuticals) per day for four weeks (n=25).

Group 3b: Hypertensives supplemented with Vitamin E 400 mg per day for eight weeks (n=20).

Group 3c: Hypertensives supplemented with Vitamin E 400 mg per day for twelve weeks (n=20).

24 h urine samples were collected from groups 1, 2 and 3 and toluene was added as preservative. The fasting blood samples were collected in plain tubes and also in tubes containing heparin as anticoagulant.

2.3 PREPARATION OF HEMOLYSATE

The heparinised samples were centrifuged at 3000 rpm for 15 minutes and the plasma was separated. The red cells were washed four times with 0.9 % saline by centrifugation for 15 minutes at 3000 rpm for each wash. The red cell lysis was carried out by adding equal volume of ice-cold distilled water to a given volume of washed cells. This diluted suspension was allowed to stand for 2 hours at 4°C prior to further analysis.
2.4 PARAMETERS ASSAYED IN HEMOLYSATE, PLASMA, SERUM AND URINE

2.4.1 In hemolysate: Superoxide dismutase (SOD), glutathione peroxidase (GPX) and reduced glutathione (GSH).

2.4.2 In serum: Cholesterol, Triglycerides (TGL), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), Glutamate Oxaloacetate Transaminases (GOT), Glutamate Pyruvate Transaminases (GPT), Alkaline Phosphatase (ALP), Total Protein, Albumin and Total Bilirubin, Calcium, Phosphorus, Uric acid, Magnesium, Gamma Glutamyl Transferase (GGT) and Lactate dehydrogenase (LDH).

2.4.3 In plasma: Glucose, Urea nitrogen, Creatinine, Lipid peroxide (Malondialdehyde, MDA), Vitamins E and C.

2.4.4 In 24 h urine: Urinary stone risk parameters: Calcium, Phosphorus, Uric acid, Magnesium, Oxalate, Citrate, Protein and Creatinine Clearance.

2.4.5 Urinary Enzymes: Alkaline Phosphatase, Lactate Dehydrogenase, Gamma Glutamyl transferase and N-Acetyl Glucosaminidase (NAG).

The above parameters were analyzed by using Hitachi 2001 Spectrophotometer and Ciba Corning 550 Express Plus Auto analyser.
2.5 SOURCES OF REAGENTS, SOLVENTS AND FINE CHEMICALS

Tris hydroxymethyl aminomethane hydrochloride, Pyrogallol, p-nitrophenyl-N-acetyl-β-D-glucosaminide and DL-α-tocopherol acetate were procured from Sigma Chemical Co., St. Louis, USA. Ascorbic acid was obtained from Sisco Research Laboratories, Bombay. 2,4-Dinitrophenyl hydrazine, chromotropic acid, Thiobarbituric acid, Hemoglobin, p-nitrophenol, 2,4- dinitrophenylhydrogen, citric acid, borax, calcium sulphate, diethylene triamine penta acetic acid, ferric chloride, meta phosphoric acid and other chemicals were purchased from Loba-Chemicals, Bombay, India. Reduced Glutathione, and 5, 5’ dithiobis 2-nitrobenzoicacid were obtained from Glaxo Laboratories BDH Division, Poole, England. Petroleum ether, absolute ethanol, banthophenanthroline were from Merck, India.

All available readymade kits were from Pinnacle Marketing Pvt Ltd., Bombay and Boehringer Mannheim, Bombay, India. All other chemicals were of analytical grade and double distilled water was used in all experiments.

2.6 METHODS

2.6.1 ESTIMATION OF HEMOGLOBIN

The hemoglobin was measured by the method of Drabkin and Austin, 1932.

Principle

Hemoglobin is oxidized to methemoglobin, which combines with cyanide to form cyanomethemoglobin. The absorbance of the solution is then measured at 540 nm.
Reagents

1. Drabkins Reagent: This reagent was obtained commercially.
2. Cyanomethaemoglobin: This was obtained commercially and had hemoglobin concentration of 16g/dl.

Procedure

Dilution of 0.02 ml of hemolysate was done with 5.0 ml of Drabkins reagent. The diluted solution was mixed well and allowed to stand for 10 min at room temperature to ensure completion of the reaction. The solution was read at 540 nm together with the standard solution of cyanomethaemoglobin. The reagent blank was used to set the optical density to zero.

The values of hemoglobin was expressed as g %.

2.6.2 OSMOTIC FRAGILITY

The osmotic fragility test was carried out in the blood by the method of Partpart et al, 1974.

Reagents

Stock solution of buffered sodium chloride: 90 g of sodium chloride, 13.65 g of disodium hydrogen phosphate and 2.43 g of sodium dihydrogen phosphate were dissolved in distilled water and made up to one liter. This solution was osmotically equivalent to 10 % sodium chloride. 100 ml of the stock solution was diluted to one liter to prepare 1 % solution. Further dilution giving equivalent to 0.9, 0.85, 0.75, 0.65, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35, 0.3, 0.2, and 0.1 % sodium chloride were prepared and they were stable at 0 °C for two weeks.
Procedure

Exactly 0.05 ml of blood was added to each of the tubes containing 5 ml of the range of hypotonic solution. The contents were thoroughly mixed and allowed to stand at room temperature for 30 minutes. The contents were remixed and centrifuged at 1200 g for five minutes. The supernatant was read at 540 nm using the supernatant obtained from 0.1 % sodiumchloride solution, which represents 100 % hemolysis.

The fragility curve was drawn by plotting % hemolysis versus sodium chloride concentration. The mean corpuscular fragility (MCF) was determined by recording the saline concentration, which would have resulted in 50 % hemolysis.

2.6.3 ESTIMATION OF LIPID PEROXIDATION PRODUCT

The lipid peroxides in the plasma was measured by the method of Okawa et al, 1979.

Principle

The lipids in the cell membranes are highly susceptible to peroxidative damage and are broken down into number of units to form malondialdehyde. This reacts with thiobarbituric acid (TBA) to form thiobarbituric acid reacting substance (TBARS), which has a pink color with absorption maxima at 532 nm.

Reagents

1. TCA: 10 %

2. TBA: 0.6 %
3. Standard malondialdehyde: 0.05 ml of 1,1, 3,3 - tetra ethoxy propane bis (diethyl acetate) was made up to 1.0 ml with 0.9 % saline and 0.03 ml of 6 N HCl was added and this made up to 100 ml with distilled water. This was further diluted 1 in 100 with distilled water to obtain a concentration of 25 nM of MDA/ml

**Procedure**

0.5 ml of plasma is treated with 3 ml of ice cold 10 % TCA. The tubes were mixed well and 2 ml of TBA was added. The tubes were covered with glass marbles and kept in the boiling water bath for 20 minutes. After cooling, the tubes were centrifuged and the absorbance of the supernatant was read at 532 nm.

The MDA content in the plasma was expressed as nM/ml.

2.6.4 ANTIOXIDANTS

2.6.4.1 Ascorbic Acid in Plasma

Ascorbic acid was estimated by the method of Omaye et al, 1979.

**Principle**

Ascorbic acid is oxidised by copper to form dehydro ascorbic acid and diketo gulonic acid. These products reacts with 2, 4, dinitrophenyl hydrazine (DNPH) to form a complex, which in the presence of sulfuric acid undergoes rearrangement to form a product with absorbance maximam at 520 nm. The reaction is carried out in the presence of thiourea to provide a reducing medium to prevent interference from non- ascorbic acid chromogen.
Reagents

1. TCA: 5 %

2. DNPH- thiourea - copper sulphate reagents (DTC): 3 g: DNPH, 0.4 g thiourea and 0.05 g of CuSO₄.5H₂O were dissolved in 9N H₂SO₄ and made upto 100 ml with the same.

3. Sulphuric acid: 65 % ice - cold.

4. Standard ascorbic acid: 10 mg ascorbic acid was dissolved in 100 ml of 5 % TCA.

Procedure

0.5 ml of plasma was treated with 5 % ice - cold TCA to precipitate the proteins. A protein free filtrate is obtained by centrifuging for 20 min at 3500x g. 1.0 ml of the supernatant was mixed with 0.2 ml of DTC and incubated for 3 hr at 37 °C. Then 1.5 ml of 65 % ice-cold H₂SO₄ was added, mixed well and allowed to stand at room temperature for additional period of 30 min and read at 520 nm. Standards were made in 5 % TCA with the concentration ranging form 2 - 20 μg/ml and treated in a similar manner.

The level of ascorbic acid in the plasma was expressed as mg %.

2.6.4.2 Total Reduced Glutathione in Hemolysate

The total reduced glutathione content was determined by the method of Moron et al, 1979.
Principle

Glutathione reacts with dithio bis nitrobenzoic acid (DTNB) to give a compound that absorbs maximally at 412 nm.

Reagents

1. Phosphate Buffer: 0.2 M pH 8.0
2. DTNB: 0.6 mM DTNB in 0.2 M phosphate buffer, pH 8.0.
3. TCA: 5%
4. Glutathione standard: 20 mg in 100 ml distilled water.

Procedure

The proteins were precipitated with 5% TCA. The solution was mixed well and centrifuged. To 0.5 ml of supernatant, 2 ml of phosphate buffer was added followed by 0.5 ml of DTNB reagent. The yellow color developed was read at 412 nm in a spectrophotometer against a blank containing 5% TCA instead of the sample. A series of standards were treated in a similar manner.

The amount of glutathione in the hemolysate was expressed as μg of GSH /mg Hb.

2.6.4.3 Vitamin E

Vitamin E was estimated after extraction by the method of Kayden et al, 1973 followed by the spectrophotometric determination of the liberated vitamin E using bathophenanthroline reagent, according to the modified method of Emmerir and Engle, 1938.
Reagents

1. Absolute ethanol.
2. Petroleum ether.
3. 0.2 % banthophenanthroline.
4. 0.001M, ferric chloride.
5. 0.001M, meta phosphoric acid.
6. DL-Tocopherol acetate-Stock: 100 mg of tocopherol acetate in 100 ml of ethanol.
7. Working tocopherol acetate: 1 ml of the stock diluted to 100 ml with ethanol (10 ug/ml).

Procedure

One ml serum was mixed with 1 ml of ethanol and 4 ml of petroleum ether in test tube. After mixing thoroughly, it was centrifuged. 2 ml from the petroleum ether layer was pipetted out from the test tube and evaporated for dryness. To the dried mass, 0.2 ml of ferric chloride and 0.2 ml of banthophenanthroline were added. After about a minute, 0.2 ml of phosphoric acid was added and mixed well and allowed to stand for five minutes. A series of standards were treated in a similar manner.

The amount of vitamin E in serum was expressed as mg %.
2.6.5 ANTIOXIDANT ENZYMES

2.6.5.1 GLUTATHIONE PEROXIDASE IN HEMOLYSATE

Glutathione peroxidase catalyses the reduction of \( \text{H}_2\text{O}_2 \), in the presence of glutathione, to form water and oxidized glutathione. It also reduces a vast array of other hydroperoxides including fatty acid hydroperoxides. The activity of glutathione peroxidase was determined by the method of Rotruck et al, 1973.

**Principle**

The procedure is based on the reaction between leftover glutathione in the reaction with DTNB to form a complex, which absorbs maximally at 412.

**Reagents**

1. Sodium phosphate buffer: 0.4 M, pH 7.0
2. Sodium azide: 10 M
3. Reduced glutathione: 4 mM
4. \( \text{H}_2\text{O}_2 \): 2.5 mM
5. TCA: 10%
6. Disodium hydrogen phosphate: 0.3 %
7. DTNB Reagent: 40 mg of DTNB in 100 ml of sodium citrate
8. Standard reduced glutathione: 20 mg in 100 ml of distilled water
Procedure

0.4 ml of buffer, 0.1 ml of sodium azide, 0.2 ml of reduced glutathione, an aliquot of the hemolysate, 0.1 ml H₂O₂ and distilled water were taken in a final incubation total volume of 2.0 ml. The tubes were incubated at 37 °C for 10 minutes. The reaction was stopped by adding 0.5 ml of 10 % TCA. To determine the residual glutathione content, the supernatant was removed by centrifugation, 3.0 ml of disodium hydrogen phosphate and 1 ml of DTNB reagent were added. The color developed was read at 412 nm. A blank was treated with only disodium hydrogen phosphate and 1.0 ml of DTNB reagent. Suitable aliquots of standards were also taken and treated similarly.

The activity of glutathione peroxidase was expressed as μg of GSH utilized/min/mg Hb.

2.6.5.2 SUPEROXIDASE DISMUTASE

Superoxide dismutase is a family of metalloproteins that catalyses the dismutation of superoxide radicals. They are present in the cytosol and mitochondria of mammalian cells and provide the first line of defense against free radical damage. The enzyme was assayed according to the method of Markland and Markland, 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. The change in absorbance is measured at 470 nm using a spectrophotometer.
Reagents

1. Tris – HCL and DETAPAAC: 0.1M tris-HCL, pH 8.2 Containing 2mM of diethylene triamine penta acetic acid (DETAPAAC).
2. Tris- HCL buffer: 0.05 M, pH 7.4.
3. Pyrogallol stock: 25.2 mg of pyrogallol was dissolved in 1ml of 0.05 M tris - HCL buffer, pH7.4 in an aluminum foil wrapped, stoppered test tube.
4. Pyrogallol working solution: At the time of assay, 0.5 ml was diluted to 50 ml with 0.05 M tris-HCL buffer, pH 7.4 to give a 2mM solution and shielded from exposure to light.
5. Absolute ethanol.
6. Chloroform.

Procedure

The partially purified superoxide dismutase was prepared as described by McCord and Fridovich, 1969. To an aliquot of the hemolysate, 0.25 ml of absolute ethanol and 0.015 ml of chloroform were added. After 15 min of shaking in a mechanical shaker, the suspension was centrifuged, and the supernatant obtained constituted the enzyme extract. The reaction mixture for auto-oxidation consisted of 2 ml of the buffer containing DETAPAAC, 0.5 ml of 2 mM pyrogallol and 1.5 ml water. Initially, the rate of auto-oxidation of pyrogallol was noted at an interval of one minute for 3 minutes. The assay mixture for the enzyme contained 2 ml of 0.05 M tris-Hcl buffer, 0.5ml of pyrogallol, aliquots of the enzyme preparation and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted.

The enzyme activity was expressed in terms of Units / ml of hemolysate. One Unit corresponded to the amount of enzyme that inhibited the auto-oxidation reaction by 50%.
2.6.6 URINARY MARKERS

2.6.6.1 Estimation of Oxalate

Oxalate was measured according to the method of Hodgkinson and Williams, 1972.

Principle

Oxalate is converted to glycollate, which reacts with chromotropic acid to form a pink coloured compound. This color complex was measured at 570 nm.

Reagents

1. Electrolytic zinc: Electrolytic zinc was cut into short lengths measuring 5.0 mm and weighing approximately 250mg. Immediately before use the zinc was cleaned by immersing in freshly prepared 10N HNO₃. After washing thoroughly in distilled water the zinc was used.

2. 1% chromotropic acid: 1.0 g of 4,5-dihydroxy-naphthalene-2,7-disulphonic acid disodium salt was dissolved in 100 ml of water and stored at 4°C. The solution was prepared fresh once a week.

3. 10 N sulphuric acid.

4. 2N sulphuric acid.

5. Saturated calcium sulphate.

7. Standard oxalic acid: Dissolve 1.0231 g of potassium oxalate monohydrate in 100 ml of water. This solution contained 5 mg of anhydrous oxalic acid per ml.

**Procedure:**

The urine was acidified by adding concentrated HCl (1.0 ml /100 ml of urine) to ensure the solubilisation of any crystals of calcium oxalate. To 2.0 ml of urine add 1.5 ml of water and drop of bromothymol blue indicator were added. The pH was adjusted to 7.0 by the addition of diluted NaOH or acetic acid and 2.0 ml of saturated aqueous solution of calcium sulphate followed by 7 ml of ethanol were added, mixed well and allowed to stand at room temperature at least 3 hours or preferably overnight. It was centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted. The precipitate was taken in 2 ml of 2N sulphuric acid and a piece of freshly cleaned zinc was added and heated in a boiling water bath for 30 min. The tube was left un-stoppered to allow evaporation to occur and the final volume was less than 0.5 ml to ensure full colour development.

Then zinc was removed with a glass rod and 0.5 ml of chromotropic acid was added. The tubes were kept in an ice bath and 5.0 ml of concentrated H₂SO₄ was added with mixing and heated in boiling water bath for 30 minutes. The tubes were cooled, diluted to 15 ml with 10N H₂SO₄ and colour formed was read at 570 nm. The colour was stable for several hours.

Standard curve was prepared using oxalate solution in the concentration of 10 - 50 μg and treating similarly. The oxalate in the urine was expressed as mg / 24 hr urine.

**2.6.6.2 Estimation of Citrate**

Citrate was measured in 24hour urine sample using the method of Rajagopal, 1984.
Reagents

1. 1.10 % TCA
2. 9 N H₂SO₄
3. 40 % Metaphosphoric acid
4. 2 M Potassium bromide
5. 6.5 % Potassium permanganate
6. 6 % Hydrogen peroxide
7. Petroleum ether
8. Thiourea-Borax solution: 4.0 g of thiourea and 2.0 g of borax made up to 100 ml of H₂O.
9. Standard citric acid: 10 mg of anhydrous citric acid dissolve in 100 ml of 1N sulphuric acid containing 100 μg of citric acid per ml.

Procedure

0.5 ml urine was added with 3.5 ml of water and 4.0 ml 10 % TCA. The mixture was centrifuged and 4.0 ml of supernatant was taken in 25 ml of stoppered test tubes and added with 1.0 ml of 9N H₂SO₄, 0.25 ml of 40% metaphosphoric acid, 0.5 ml of 2M potassium bromide and 1.0 ml of 6.5 % potassium permanganate and mixed well. The tubes were kept in ice water bath for 10 minutes, and 6 % H₂O₂ was added to decolourise potassium permanganate. 7.0 ml of petroleum ether was added and shaken well for 2 minute. 4.0 ml of petroleum ether layer was transfered to 15 ml stoppered tubes and 6.0 ml of thioruea borax reagent was added and mixed well. After 10 minute the aqueous layer was aspirated and the color was read at 455 nm.

The urinary excretion of citrate was expressed as mg/day.
2.6.6.3 Blood Glucose

Blood glucose was estimated by the method of Trinder 1969.

The results are expressed as mg/dl.

2.6.6.4 BUN

Blood urea nitrogen (BUN) was estimated by the method of Tiffany et al, (1972).

The results are expressed as mg/dl.

2.6.6.5 Creatinine

Serum and Urinary creatinine was determined by the method of Fabing and Ertinghausen (1971).

The values were expressed as mg/ dl in plasma and mg/day in urine.

2.6.6.6 Cholesterol

Serum cholesterol was estimated by the method of Roeschlaee et al, 1974.

The values values were expressed in mg /dl.
2.6.6.7 Triglyceride

Serum triglyceride was estimated by the method of Fossati and Prencipe, 1982.

The values were expressed as mg / dl.

2.6.6.8 LDL cholesterol

Serum LDL value was calculated from total cholesterol – TGL/5.

The values were expressed as mg / dl.

2.6.6.9 High density lipoprotein (HDL)

High density lipoprotein (HDL) was estimated by the method of Assmann, 1979.

The values were expressed in serum as mg/dl.

2.6.6.10 Calcium

Serum and Urinary calcium was estimated by the method of Ray Sarkar and Chauhan, 1967.

The values were expressed in serum mg / dl and urine mg / day.

2.6.6.11 Phosphorus

Serum and Urinary phosphorus was determined by the method of Day and Ertingshausen, 1972.

Values were expressed as mg / dl in serum and mg / day in urine.
2.6.6.12 Uric acid

Serum and urinary uric acid was determined by the method of Praetorius et al, 1953.

The values were expressed in serum mg/dl and urine mg/day.

2.6.6.13 Total Protein

Serum protein was estimated by the method of Tietz, 1986.

The values were expressed as gm/dl.

2.6.6.14 Albumin

Serum albumin was estimated by the method of Doumas et al, 1971.

The value was expressed in gm/dl.

2.6.6.15 Magnesium

Serum and Urinary magnesium was estimated by the method of Chauman and Sarkar, 1969.

The values were expressed in serum mg/dl and urine mM/day.

2.6.6.16 Aspartate amino transferase/Alanine amino transferase

Serum AST/ALT was determined by the method of IFCC, 1986.

The values were expressed in serum U/L and urine U/g creatinine.

2.6.6.17 Alkaline phosphatase (ALP)

Serum and Urine alkaline phosphatase was determined by the method IFCC, 1983.

The values were expressed in serum U/L and urine U/g creatinine.
2.6.6.18 Gamma glutamyl transferase (γ-GT)

Serum and Urinary gamma glutamyl transferase was estimated by the method of IFCC, 1983.

The values were expressed in serum U/L and urine U/g creatinine.

2.6.6.19 Lactate dehydrogenase (LDH)

Serum and Urinary lactate dehydrogenase was estimated by the method of Sociedad Espanola de Quimica Clinica, 1989.

The values were expressed in serum U/L and urine U/g creatinine.

2.6.6.20 Urinary protein

Urinary protein was estimated by the method of Watanabe, 1986.

The values were expressed as mg/day.

2.6.6.21 Estimation of N-acetyl- β-D-glucosaminidase (NAG-aminidase)

NAG-aminidase was measured according to the method of Moore and Morris, 1982.

**Principle**

The liberation of p-nitrophenol from p-nitrophenol N-acetyl-β-D-glucosamine by NAG-aminidase was measured spectrophotometrically at 410 nm.
Reagents

1. 0.05 M sodium citrate.
2. 5 nM p-nitrophenol N-acetyl-β-D-glucosamine in 0.05 M sodium citrate.
3. 1M NaOH containing 0.5 M glycine
4. p-nitrophenol standard

Procedure

0.5 ml of urine was added to 0.5 ml of the substrate, 5 nM of p-nitrophenol N-acetyl-β-D-glucosamine in 0.05 M sodium citrate buffer, pH 4.5. After 60 minutes of incubation the reaction was stopped by the addition of 1.0 ml of 1.0 M NaOH containing 0.5 M glycine, and the optical density was measured at 410 nm. Blank, in which the glycine/NaOH was added before the substrate, were used. Standard curve was prepared using p-nitrophenol solution in the concentration of 7.2 - 45 nM and treating similarly.

The N-acetyl-β-D-glucosaminidase in the urine was expressed as nM/h/mg of creatinine.

2.7 STATISTICAL ANALYSIS

The data of all the parameters were analyzed by students ‘t’ test for statistical significance. Correlations between the parameters of stone patients were carried out by using Pearson’s correlation coefficient. Statistical significance at the 0.1, 1 and 5 % protection levels were used for comparison.