MATERIALS
&
METHODS
Subjects for the study

Human male and female volunteers, (four groups each group consisting of twenty members) were categorized into four groups viz., group 1-4 included male (group1) and female (group3) controls who were non-alcoholics (teetotalers), male alcoholics (group2) who consumed 70-100 g, female alcoholics (group4) who consume 60-80g alcoholic beverage/day for the past 8-10 years. The beverages consumed by the chosen alcoholics include 80 proof hard liquors such as whisky, rum, zin, and brandy of various brands containing up to 40% ethanol. All the volunteers were well explained about the experimentation and a written consent was obtained from them. Selected subjects consume south Indian vegetarian meal regularly non vegetarian meal once in week. Enough care was taken to prevent the effects of diet, water or sampling time, and daily activities of the subjects. The chosen subjects were not on medication for any known chronic disease or illness. The study was approved by our institutional review and ethical committees. Base line characteristics of the selected subjects are presented in Table 1 (Results).

Chemicals:

Chemicals used were of analytical grade and were obtained from Sigma Chemical Company (St.Louis, MO, USA): TBA, MDA, P- nitroanilide, N-1 naphthyle ethylene diamine, Ethylene diamine tetra acetic acid. Sisco Research Laboratories (Mumbai, India): TEMED, Acrylamide, Bis-Acrylamide, SDS, Tyramine, HEPES, PMSF, AgNo₃, Epinephrine, Guanidine- Hcl, ADP-Na₂.

Blood collection and Experimentation:

Venous blood samples were collected from volunteers into heparinized test tubes after overnight fasting and were used for analysis immediately. Biochemical studies using plasma, erythrocyte and were carried out. The parameters included mainly in plasma, fasting glucose, glycosylated hemoglobin, total cholesterol, lipoprotein patterns (HDL-C, LDL-C, VLDL-C and triglycerides in plama), Plasma lipid peroxidation, nitrites and nitrates. Alcohol levels, activities of gamma-glutamyl transferase (γGT), and the
concentration of testosterone, estradiol, cortisol, T₃, T₄ and thyroid-stimulating hormone (TSH) in plasma. Erythrocyte membrane proteins were separated by using SDS-PAGE, further determination of cholesterol, protein moiety, and lipid peroxidation were carried out.

**Glucose:**

Plasma glucose was estimated by using Monozyme diagnostic kit, which is based on the Glucose oxidase - peroxidase method. Glucose is oxidized by the enzyme glucoseoxidase to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of enzyme peroxidase oxidizes phenol, which combines with 4-aminoantipyrine to produce a red colored quinoneimine dye. To 10μl standard/sample 1ml of enzyme reagent was added and mixed well and incubated at 37°C for 10 min, absorbance was measured at 505nm against the blank.

**Glycosylated hemoglobin:**

Glycosylated hemoglobin was estimated by the method of Eross et al., (1984). To the erythrocytes (0.5 ml) collected from whole EDTA blood, 0.125 ml of distilled water and 0.125 ml of carbon tetra chloride were added, mixed well and centrifuged. The supernatant hemolysate was separated and its hemoglobin concentration was adjusted to 10 % with distilled water. To 2 ml of hemolysate, 1 ml of 0.3N oxalic acid was added in stoppered test tubes and heated at 100°C in a water bath for 60 minutes. After cooling the contents, 1 ml of 40 % TCA was added, shaken well and centrifuged. To 2 ml of supernatant pipetted out into another set of test tubes, 0.5 ml of 0.05M TBA was added and incubated at 37°C for 4 minutes. A blank with 2 ml of distilled water was treated similarly. The resulting yellowish colour was read in a spectrophotometer at 443 nm. Concentration of HbA₁c was calculated on the assumption that 1 % HbA₁c corresponds to an absorbency of 0.029 at 443 nm (experimentally determined millimolar extinction co-efficient for TBA-5 hydroxymethyl furfural adduct is 26 at 443 nm). Hemoglobin in RBC (g %) was estimated by cyanomethemoglobin method.
**Plasma cholesterol:**

Plasma total cholesterol was estimated by enzymatic method using the method developed by Allian *et al.* (1974). Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. Cholesterol Oxidase converts cholesterol to cholest-4-en-3-one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidatively couples with 4-aminoantipyrine and phenol to produce red quinineimine dye which has absorbance maximum at 505 nm. The intensity of the red colour is proportional to the amount of total cholesterol. To 1.0 ml of freshly reconstituted enzyme reagent, 0.01 ml of plasma was added and mixed well and incubated at 37°C for 5 min. After incubation, extinction was measured at 505 nm against water blank. Simultaneously standards were run along with the test under similar conditions. Results were expressed as mg/100 ml.

**HDL- Cholesterol:**

Plasma HDL cholesterol was estimated by enzymatic method developed by Allian *et al.* (1974). Phosphotungstate/Mg$^{2+}$ precipitate chylomicrons, Low-density lipoprotein (LDL) fractions. High density lipoprotein (HDL) fraction remains unaffected in supernatant. To 0.2 ml of serum, 0.3 ml of HDL precipitant reagent (2.4mM phosphotungstic acid and 40 mM magnesium chloride) was added, mixed thoroughly, centrifuged at 4000 rpm for 10 min to obtain a clear supernatant. To 0.1 ml of supernatant, 1.0ml of enzyme reagent was added, incubated for 10 min at 37°C and pink color developed was read at 510 nm against a blank and a standard (50 mg%) was run simultaneously. Values are expressed as mg/dl.

**LDL- cholesterol:**

LDL and VLDL cholesterol were calculated by the formula of Friedewald *et al.*, (1972).

\[
\text{VLDL cholesterol} = \frac{\text{Triglycerides}}{5}
\]

\[
\text{LDL cholesterol} = \text{Total Cholesterol} - \text{VLDL Cholesterol} - \text{HDL Cholesterol}
\]
**Atherogenic index (AD):**

Atherogenic index, the ratio of total cholesterol and HDL cholesterol was calculated by the method given by Kumari and Mathew (1995).

\[ AI = \frac{\text{Total cholesterol}}{\text{HDL Cholesterol}} \]

**Triglycerides:**

Plasma triglycerides were estimated by enzymatic method using Monozyme diagnostic kit, which is based on Fossati and Principe (1982). Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol-3-phosphate, which is oxidized by glycerol phosphate Oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidizes phenolic chromogen to a red colored compound measured at 510 nm. To 0.01 ml of plasma, 1.0 ml of the reagent was added, mixed and incubated at 37°C for 10 min. Triglyceride standard and water blank were also treated in a similar manner. After incubation absorbance was read at 510 nm and values are expressed as mg/dl.

**Assay of Gamma-Glutamyl transferase [\( \gamma \)GT, EC 2.3.2.2]:**

The activity of \( \gamma \)-glutamyl transpeptidase was estimated according to the method of Rosalki and Tarlow (1974). Gamma glutamyl transferase (\( \gamma \)GT) catalyses the transfer of the gamma glutamyl group from gamma glutamyl peptides to suitable acceptors. In the reaction rate analysis method, the artificial substrate \( \gamma \)-glutamyl-p-nitroanilide is used. To 0.05 ml of plasma and 1.0 ml of Tris- glycyglycine buffer (6.96 g tris and 9.11g glycyl glycine in 400 ml water and pH adjusted to 8.5 with 0.5N NaOH and made up to 500 ml with water) is added and equilibrated at 37°C. To this add 100 µl of L-\( \gamma \)-glutamyl-p-nitroanilide (29.5 mg/1.0 ml in 0.5 N HCl) and the amount of p-nitroaniline formed were monitored by following the increase in absorbance at 405 nm. The activity of \( \gamma \)-glutamyl transferase was expressed as IU/L.
Nitrates and nitrites:

Plasma nitrite and nitrate concentrations were determined by the method adopted by Kavitha et al., (2008). Nitrite concentration is determined by using Griess Reagent in which nitrite reacts with Sulfanilamide in phosphoric acid/naphthylethylenediamine forming chromophore which can be read spectrophotometrically at 545 nm. To 100 µl of sample 400 µl carbonate buffer, small amount (0.15g) of activated cadmium filings were added with thorough shaking and tubes were incubated at room temperature for 90 minutes and the reaction was stopped by the addition of 100 µl 0.35M sodium hydroxide followed by 400 µl of 120mM zinc sulphate solution under vortex and the solution was allowed to stand for 10 minutes. The tubes were centrifuged at 4000 g for 10 minutes. To 500 µl of the clear superant 250 µl 1% sulphanamide and 250 µl 0.1% N-naphthyle ethylene diamine was added with shaking. After 10 minutes the absorbance was measured at 545nm against blank. Simultaneously standards were run by using sodium nitrite. For assay of nitrite levels, similar steps were followed except the sum of nitrite and nitrate levels. Nitrate concentrations were obtained by subtracting nitrite concentrations.

Preparation of erythrocyte membrane

Erythrocyte membrane was prepared by using the method adopted by Dodge et al. (1963). Erythrocyte suspension was washed with phosphate buffered saline (pH 7.2). Then cells were lysed with 5 mM phosphate buffer (pH.8.0) and were spun at 15000 g for 30 min. The supernatant was removed carefully and by using the same buffer the latter step was repeated to obtain hemoglobin free ghosts. Membrane protein was estimated by the method of Lowry et al., (1951).

Protein estimation by Folin-lowry method

Membrane protein was estimated by the method of Lowry et al., (1951) Tyrosine and tryptophan present in the proteins reacts with Folin Ciocalteau reagent in the presence of alkaline copper to give coloured complex with a maximum absorbance at 660 nm. To an aliquot of solubilised membrane, 5 ml of copper alkaline reagent (2 % sodium carbonate in 0.4 % sodium hydroxide and 0.5% copper sulphate in 1% sodium potassium tartarate in the
ratio of 1:5 was added and after 10 min of incubation, 0.5 ml of Folin-ciocalteau reagent was added and incubated for 30 min and absorbance was read at 660 nm against reagent blank. Standard concentration ranging from 40 to 200 µg of Bovine serum albumin was used to prepare standard graph.

**Membrane cholesterol**

Total cholesterol content in RBC was estimated by the method Zlatkis (1953). Cholesterol is oxidized by FeCl3-H2SO4. The purple colour thus formed was measured spectrophotometrically at 560 nm. To 0.1 ml of lipid extract 4.9 ml of ferric chloride-acetic acid reagent (0.05% ferric chloride in acetic acid) was added and followed by 3 ml of concentrated sulphuric acid mixed. Standard graph was made by using cholesterol standards ranging from 40-200 mg. Results were expressed as mg cholesterol/mg protein.

**Lipid Peroxidation**

Lipid peroxidation was assessed by the formation of Malondialdehyde (MDA) by using the method of Buege and Aust (1978). Malondialdehyde formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of the peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid (TBA) to give a chromogen absorbing at 535 nm. One milliliter of sample (plasma, erythrocyte membrane, platelet membrane) was taken in a test tube to which 2 ml of reagent (15% w/v TCA, 0.375 % w/v TBA and 0.25 N HCl) was added and kept in boiling water bath for 15 min and the contents were allowed to cool. Then after centrifugation at 1000 g for 10 min the absorbance was measured in the supernatant at 535 nm against reagent blank assuming the molar extinction coefficient 1.56×10⁵ M⁻¹ cm⁻¹. The amount of malondialdehyde formed in the sample is expressed as µmoles/L in plasma and nmoles MDA/mg Hb in erythrocyte.

**Hormones**

The ADVIA Centaur automated immunoassay analyzer were used for measurement of hormone levels. The ADVIA Centaur automated immunoassay for testosterone, estradiol, T3 and T4 is a competitive immunoassay with an analytic sensitivity of 0.1 ng/ml, 70 pg/ml
(25.7 pmol/L), 0.15 nmol/L, 3.9 nmol/L and the ADVIA Centaur automated immunoassay for FSH, LH, Prolactin TSH is a sandwich immunoassay with an analytic sensitivity of 0.3 mIU/ml, 0.07 mIU/L, 0.3 ng/ml, 0.02 mIU/L by the methods ADVIA Centaur Assay Manual.

**SDS-Polyacrylamide Gel Electrophoresis**

The SDS-PAGE was done according to the method of Laemmli (1970). When a mixture of high molecular weight compounds is allowed to traverse by applying electric current through the gel matrix of a polymer of acrylamide, various components get resolved based on the charge. The method has high-resolution capacity, which is further improved by the use of sodium dodecyl sulfate, which in addition allows the resolution on the basis of molecular weights of the proteins. The separating gel (12%) was made of 30% acrylamide, 0.8% N, N'-methylene bis acrylamide, 10% sodium dodecyl sulphate (SDS) and 1.5 M Tris-HCl buffer pH 8.8. The various components were mixed, and polymerization was initiated by adding 10% ammonium persulfate and 0.02% TEMED. The solution was mixed and quickly poured into glass plates and water layered on top. After polymerization was over, stacking gel (5%) containing 30% acrylamide, 0.8% N, N'-methylenobisacrylamide, 10% SDS and 1M Tris pH 6.8 was poured on the separating gel layer and allowed to polymerize after addition of 10% ammonium sulphate and TEMED. The tank buffer contained 0.025M Tris, 0.192 M glycine, 0.2% SDS pH 6.8, 10% Glycerol, 20% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue. The samples were denatured by adding sample buffer and kept in boiling water bath for 5 min cooled and the samples were loaded. The electrophoresis apparatus was connected to power supply. Initially 50 mV was applied in the stacking gel and after reaching dye front in to the running gel the voltage was increased to 150 mV and run the gel until bromophenol blue reached the bottom of the resolving gel.

**Silver Staining**

This was carried out by the method of Okley et al., (1980) with slight modification. After removing the gel it was placed in 5 gel volumes of 30% ethanol and incubated for 30 minutes at room temperature with gentle shaking and this step was repeated 2 times. Later
ethanol was discarded and 10 gel volumes of distilled water was added and incubated for 10 minutes at room temperature and this step was repeated for 3-4 times. After discarding distilled water 5 gel volumes of 0.1 % solution of silver nitrate (freshly prepared) was added and incubated for 30 minutes at room temperature with gentle shaking. Then silver nitrate solution was discarded and both sides of the gels were washed with distilled water. Finally 5 gel volumes of freshly made aqueous solution (2.5 % sodium carbonate, 0.02 % formaldehyde and 0.001 % sodium thiosulphate) was added and incubated with gentle agitation. As soon as bands started appearing glacial acetic acid poured to the run until bubbles were stopped. Then gels were washed 3-4 times with distilled water and stored in a solution containing 6 ml of acetic acid, 2.5 ml methanol in 100 ml distilled water.

**Osmotic fragility of erythrocytes**

The release of hemoglobin from erythrocyte hemolysis was determined by the method adopted by Kanai (1988). The test of osmotic fragility attempts to determine the concentration of solute inside the red cells by placing the cell in different concentration of NaCl i.e., 0.9 % to 0.0 % and observing hemolysis in hypotonic solution. Thus the intracellular solute concentration as reflected by the red cell fragility, can be helpful in establishing the pathologic state of the red cells. NaCl in the concentration range of 0.1 % to 0.9 % was taken in 9 different centrifuge tubes so that the final volume was 10 ml. One ml of 50 % 1:1 diluted red cells suspension was added to each tube and mixed immediately by gently swirling, allowed to stand at room temperature for 30 min and centrifuged at 2000g for 5 min and absorbance of the supernatant was read at 540 nm against blank.

**Statistical analysis**

Data were analyzed for significant difference (P < 0.05) among values of different viz., male and female teetotalers, as well as male and female alcoholic samples using Duncan's Multiple Range (DMR) test.
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