MATERIALS
&
METHODS
Materials and methods

Experimental subjects were selected human male volunteers aged between 25-50 years, of middle class, of Anantapur, categorized into two groups. The 1st group consisted of 12 normal subjects designated as controls or normal group and they had no past or present history of smoking. The other group containing twelve human subjects associated with the habit of smoking. The subjects chosen for the second group were confirmed smokers for the past 7 to 10 years or more. Subjects chosen for the study were used to take a typical local diet with non vegetarian food once or twice a week. Some preliminary information pertaining to age diet socio economic status, clinical complications smoking history use of liquors other psychoactive substances drugs and family history was collected with the help of a questionnaire and physical characters of the study subjects were furnished in table I in the following page. The volunteers were well informed about the experimentation and their prior consent was obtained.

All chemicals used were of analytical grade of SISCO Research Laboratories Private Limited (India.)
<table>
<thead>
<tr>
<th>SI. No</th>
<th>Character</th>
<th>Normal</th>
<th>Experimental group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of volunteers selected for the group</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
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<tr>
<td></td>
<td>Age</td>
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<td>25-45</td>
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<td>Height</td>
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<td>160-175cm</td>
</tr>
<tr>
<td></td>
<td>Weight</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Socio economic status</td>
<td>Middle and lower income</td>
<td>Middle and lower income</td>
</tr>
</tbody>
</table>

**SMOKING HISTORY**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Experimental group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whether the subject is addicted for smoking</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Cigarette consumption history</td>
<td>-</td>
<td>Since 10-15 years</td>
</tr>
<tr>
<td>Number of cigarettes smoked/day</td>
<td>-</td>
<td>10-15</td>
</tr>
<tr>
<td>Frequency of Smoking</td>
<td>-</td>
<td>One cigarette/hour</td>
</tr>
<tr>
<td>Whether the subject smokes the cigarette with tea till last puff</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Whether the subject is an alcoholic</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Whether the subject is suffering from any chronic illness/disease</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Family history whether parents or known elder in the family smoked cigarettes</td>
<td>No</td>
<td>Some of their parents smoked</td>
</tr>
</tbody>
</table>
**Blood glucose**

Blood glucose was estimated by Monozyme kit, which is based on Trinders's method\(^1\)

*Principle:* Glucose is oxidized by the enzyme glucose oxidase to give gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of enzyme peroxidase oxidizes phenol, which combines with 4-Aminoantipyrine to produce a red coloured quinoneimine dye.

*Procedure:* To 10\(\mu\)l of plasma 1ml of enzyme reagent was added mixed and incubated for 10 minutes at 37\(^\circ\)C for 15 minutes at room temperature and read at 505nm. Simultaneously standards were run along with the test under similar conditions. Results were expressed as mg/100ml.

**Plasma cholesterol**

Plasma total cholesterol was estimated by enzymatic kit, which is based on Allian et al method\(^2\).

*Principle:* Cholesterol ester → Cholesterol + Free fatty acids

\[\text{CHE} \rightarrow \text{Cholesterol} + \text{Free fatty acids} + \text{H}_2\text{O}\]

Cholesterol → Cholesterol-3-one + H\(_2\)O\(_2\)

\[\text{CHOD} \rightarrow \text{Cholesterol-3-one} + \text{H}_2\text{O}_2 + \text{O}_2\]

H\(_2\)O\(_2\)+Phenol+Aminoantipyrine → Red Quinoneimine complex +H\(_2\)O

*Procedure:* To 1ml of freshly reconstituted enzyme reagent, 0.01ml of plasma was added and mixed well and incubated at 37\(^\circ\)C for 5 minutes. After incubation, extinction was measured at 505nm against blank. Simultaneously standards were run along with the test under similar conditions. Results were expressed as mg/100ml.
**HDL Cholesterol:**

Plasma HDL cholesterol was estimated by enzymatic kit, which is based on Allian et al method².  

**Principle:** Cholesterol ester $\xrightarrow{\text{CHE}}$ Cholesterol + Free fatty acids  
$+\text{H}_2\text{O}$  
Cholesterol- $\xrightarrow{\text{CHOD}}$ Cholest - ene 3 - one $+\text{H}_2\text{O}_2$  
$+\text{O}_2$  
$\text{H}_2\text{O}_2$+Phenol+Aminoantipyrene $\xrightarrow{\text{POD}}$ Red Quinoneimine complex $+\text{H}_2\text{O}$  

**Procedure:** To 0.2ml of serum, 0.3ml of HDL precipitants reagent (phosphotungstic acid-2.4m mol/L and magnesium chloride-40 m mol/L) was added, mixed thoroughly, centrifuged at 4000 rpm for 10 min to obtain a clear supernatant. To 0.1ml of supernatant, 1ml of enzyme reagent was added, incubated for 10min at 37°C and colour developed was read at 510nm against a blank and a standard (50mg%) was run simultaneously.

$$\text{Absorbance of test HDL cholesterol (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$

**LDL cholesterol**

LDL and VLDL cholesterol were calculated by the formula of Friedewald et al³.  

$$\text{LDL cholesterol} = \frac{\text{Total cholesterol} - \text{Triglycerides}}{5} \times 100$$  

$$\text{VLDL cholesterol} = \frac{\text{Triglycerides}}{5}$$
Phospholipids

*Principle:* The phospholipids were estimated by the method of Connerty, Briggs and Eaton\(^4\). Proteins are precipitated by TCA and the precipitate which contains phosphorus or phospholipids is digested with H\(_2\)SO\(_4\) and perchloric acid reagent and then estimated spectrophotometrically at 680 nm.

*Procedure:* Phospholipids, as precipitate, were obtained in 0.2ml of plasma by addition of 5ml, 5% W/v trichloroacetic acid, 1ml of digestion mixture (50ml of distilled water, 25ml of 70% perchloric acid, 25 ml of concentrated sulphuric acid) and heating gently for about 30 to 45 minutes until the liquid became colourless. It was cooled, 1ml of distilled water was added allowing to boil for 15 seconds to convert pyrophosphate to orthophosphate. One ml of 50% sodium acetate was added and phosphorus was estimated by Fiske Subbarow method\(^5\). To the above digest, 1ml of molybdate II reagent (2.5% ammonium molybdate in 3N H\(_2\)SO\(_4\)), 0.4 ml of amino naphthalphonic acid (ANSA) reagent (0.5 gms of \(\alpha\)-ANSA in 195 ml 15% sodium bisulphite and 5 ml of 20% sodium sulphite) was added and volume was made up to 10ml with distilled water and absorbance was read spectrophotometrically at 680 nm.

Potassium dihydrogen phosphate was used as a standard. To concentration ranging from 4 to 80 \(\mu\)g, 1 ml of molybdate I reagent (2.5% ammonium molybdate in 5N H\(_2\)SO\(_4\)), 0.4 ml of ANSA reagent were added and volume was made up to 10ml. The value obtained has to be multiplied by 25 to get phospholipids. Results were expressed as mg/100ml.
Serum glutamate Oxaloacetate transaminase (SGOT)

Serum glutamate oxaloacetate transaminase (SGOT) was assed by the enzymatic kit method.

**Principle:** SGOT catalyzes the transfer of an amino group between L-Aspartate and 2-oxoglutarate. The oxolacetate formed and reacted with NADH in the malate dehydrogenase. SGOT activity is determined by measuring the rate of oxidation of NADH at 340nm.

**Procedure:** To 100µl of plasma, 1 ml of working reagent {(prepared freshly by mixing reagent 1 (MDH,LDH,NADH, α-ketoglutarate) and reagent 2 (tris buffer pH 7.8, L-aspartate)) was added, mixed and incubated for 1 minute. After incubation change in the optical density was measured per minute. (ΔOD/min) during 3 minutes against reagent blank at 340nm. The activity of serum glutamate oxaloacetate transaminase was expressed as IU/L of plasma.

Serum Glutamate Pyruvate Transaminase (SGPT)

Serum glutamate pyruvate transaminase (SGPT) was assed by enzymatic kit method.

**Principle:** SGPT catalyzes the transfer of an amino group between L-Alanine and α-ketoglutarate. The pyruvate formed is reduced to lactate in the presence of lactate dehydrogenase and NADH. SGPT activity is determined by measuring the rate of oxidation of NADH at 340nm.

**Procedure:** 100µl of plasma, 1ml of working reagent {(prepared freshly by mixing reagent 1 (LDH, NADH, α-ketoglutarate) and reagent 2 (tris buffer pH 7.5, L-alanine)) was added, mixed and incubated for 1 minute. After incubation change in the optical
density was measured per minute. (AOD/min) during 3 minutes against reagent blank at 340nm. The activity of serum glutamate oxaloacetate transaminase was expressed as IU/L of plasma.

**Nitrite and nitrates**

Nitrite & nitrate levels in plasma were estimated as described by the method of Sastry KVH et al\(^8\).

**Principle:** Nitrite concentration is determined by using Gries Reagent in which nitrite reacts with Sulfanilamide in phosphoric acid/naphthalene-ethylene diamine forming chromophore which can be read spectrophotometrically at 545nm.

**Procedure:** Plasma samples were deproteinized by adding 30% ZnSO\(_4\) followed by centrifugation at 10,000g for 5 min. Then 1 ml of plasma supernatant was mixed with 1 ml of Gries reagent (1g/lit sulfanilamide, 25g/lit phosphoric acid and 0.1g/lit N-1 naphthylethylene diamine) and incubated at room temperature for 10 min for colour development. The absorbance was measured at 540nm in Elico spectrophotometer against blank. Simultaneously stands were run by using sodium nitrite concentration ranging between 0-100 µmols/lit and standard graph was drawn. The result were expressed as µmoles/lit of plasma. For determination of nitrates plasma was mixed with activated (cadmium filings were washed 3 times with distilled water and swirled for 1 to 2 min in 5 mM CuSO\(_4\) solution in glycine NaOH buffer. This process was repeated for 3 times and copper coated granules were used within 10 minutes) and then incubated at room temperature for 90 min with thorough shaking. After incubation, cadmium filings were removed and reduced nitrite levels were estimated by using Gries reagent as described earlier. Values obtained by this procedure represent the sum of
nitrite and nitrate levels. Nitrate concentrations were obtained by subtracting nitrite concentrations from the total nitrate + nitrite concentrations. The plasma nitrate and nitrite levels were expressed as μmoles/liter.

**Hemoglobin**

Hemoglobin in the blood was estimated by cyanomethemoglobin method as outlined by Samuel⁹.

*Principle:* In the presence of potassium ferricyanide at alkaline pH, hemoglobin and its derivates are oxidized to methemoglobin. Methemoglobin so formed reacts with potassium cyanide to form cyanmethemoglobin, a red colored complex, which is measured spectrophotometrically at 540nm.

*Procedure:* To 5ml of Drabkin's solution 20 μl of whole blood was added, mixed well and intensity was read at 540 nm. By taking cyanmethemoglobin (65mg/dl) s as standard and treated in the same manner, a graph was plotted from which the concentration of Hb in the blood sample was calculated and expresses as g %.

**Isolation of erythrocytes**

Erythrocytes were isolated according to the method of Beutler to remove WBC and platelets; blood was diluted with saline and then passed through a cellulose column¹⁰. The filtrate collected was centrifuged at 1000 rpm for 10 min. The erythrocytes, settled as pellet, were separated by removing the upper supernatant carefully. This washing step was repeated and the finally obtained erythrocytes were used for the preparation of erythrocyte ghosts.
Ghost preparation

Ghost or erythrocyte membranes were prepared by using the method adopted by Dodge et al. Erythrocyte suspension was washed with phosphate buffered saline (pH 7.2). Then cells were lysed with 5mM phosphate buffer (pH 8) and were spun at 15000g for 30 min. The supernatant was removed carefully and by using the same buffer the latter step was repeated to obtain hemoglobin free ghosts.

Erythrocyte membrane protein

Erythrocyte membrane protein was estimated by the method of Lowry et al.12

Principle: Tyrosine and tryptophan present in the proteins react with Folin-Ciocalteau reagent in the presence of alkali copper to give coloured complex with a maximum absorbance at 750 nm (Lowry, 1951)

Procedure: To an aliquot of plasma or solubilized membrane, 5ml 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 minutes of incubation, 0.5ml of Folin-Ciocalteau reagent was added and incubated for 30 minutes and absorbance was read at 660nm against reagent blank. Standard concentration ranging from 40 to 200 μg of Bovine serum albumin was used to prepare standard graph.

Cholesterol

Membrane cholesterol was estimated by the Zlatkis.13

Principle: Cholesterol is oxidized by FeCl3-H2SO4. The purple colour thus formed is measured spectrophotometrically at 560 nm.
**Procedure:** To 0.1 ml of lipid extract 4.9 ml of ferric chloride-acetic acid reagent (0.05% of ferric chloride acetic acid was added and followed by 3 ml of concentrated sulphuric acid mixed. The contents were cooled and absorbance was read against reagent blank at 560 nm. Standard graph was made by using cholesterol standards ranging from 40-200 mg. Results were expressed as mg cholesterol/mg protein.

**Lipid Peroxidation**

Lipid peroxidative extent was measured by the formation of Malondialdehyde (MDA) by using the method of Buege and Aust\(^14\).

**Principle:** Malondialdehyde formed from the break down 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 TBA to give a chromogen absorbing at 535 nm.

**Procedure:** One ml of sample containing either ghost preparation or plasma 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 minutes and the contents were allowed to cool and then centrifuged at 1000 g for 10 minutes. The supernatant was transferred into a separate test tube and the absorbance of the sample was read at 535 nm in Spectrophotometer against the reagent blank assuming the molar extinction coefficient to be 1.5x10\(^5\). The amount of MDA formed from membrane or plasma sample was expressed per mg protein for which the protein content in membrane preparation or plasma was determined by using Folin-Lowry/Biuret method.
Preparation of hemolysate

Hemolysate of erythrocyte was prepared by the method of Beutler\textsuperscript{10}. Erythrocyte suspension (0.2ml) in saline was added to 1.8ml of \( \beta \)-mercaptoethanol-EDTA stabilizing solution (0.05ml of \( \beta \)-Mercapto ethanol and 10ml of neutralized 10% EDTA to a volume of 1 L with water) to obtain 1:20 hemolysate. The tubes containing hemolysate were stored at 4°C for assay of enzymes within 1 to 2 hrs of preparation unless otherwise stated. Hemoglobin in hemolysate was estimated by the method described by Samuel.

Expression of enzyme activity

Activity of enzyme was expressed as International units/gram hemoglobin (IU/gHb) unless otherwise stated following the method of Beutler as outlined below\textsuperscript{10}.

\[
\text{Enzyme activity (E) in IU/g Hb} = \frac{100}{\text{Hb in grams}}
\]

Where 'A' is the number of the enzyme units per ml and Hb is the concentration of hemoglobin (g/100ml) in the hemolysate. 'A' is calculated as follows.

\[
A = \frac{\Delta \text{ODC} \times V_c}{\text{EC} \times V_H}
\]

\( \Delta \text{OD}\)-Mean change in optical density; VC- Total volume in the cuvette; EC- Extinction coefficient and \( V_H \)-volume of hemolysate.
Catalase:

Activity of Catalase in hemolysate was estimated by the method of Chance.\textsuperscript{15}

**Principle:** Catalase catalyses the breakdown of $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$ and the rate of decomposition of $\text{H}_2\text{O}_2$ was measured spectrophotometrically at 240nm.

**Procedure:** The assay system contained 0.15ml of buffer [Tris HCl (1M) pH 8.0], 2.7 ml of 10mM $\text{H}_2\text{O}_2$ and 0.09ml of distilled water. In the blank, $\text{H}_2\text{O}_2$ was substituted by water and incubated at 37°C for 10 min. The decrease in OD of system was measured against that of the blank for 10 min after the addition of 0.1 ml 1:100 dilution of 1:20 hemolysate containing 20μl of redistilled ethanol per ml of dilute hemolysate. The activity of the enzyme was calculated using the extinction coefficient of $\text{H}_2\text{O}_2$ as 0.071 cm$^{-1}$ mol$^{-1}$ and expressed as IU x $10^4$/g Hb at 37°C.

Reduced glutathione [GSH] in erythrocytes

**Principle:** GSH represents virtually all of non protein –SH groups of red cells: DTNB is a disulfide compound which readily reduced –SH compounds forming a highly coloured anion having maximum absorbance at 412nm.

**Procedure:** Reduced glutathione content was estimated according to the method of Beutler et al.\textsuperscript{16} Cell lysate was prepared by adding 2ml of water to 0.2ml of packed erythrocytes. 0.2ml of lysate was added to 10ml of ferricyanide-cyanide (Drabkin's reagent) reagent for Hb estimation. To the remaining 2ml lysate, 3.0 ml of precipitating reagent (1.67g of glacial metaphosphoric acid, 0.2g of sodium EDTA, 30g of NaCl in 100ml of distilled water) was added, allowed to stand for 5 min and filtered. To 2ml of the filtrate, 8ml of Na$_2$HPO$_4$ (0.3M) was added and optical density was read at 412 nm against blank containing 2.0ml precipitating reagent (diluted 2:5 with H$_2$O) and 8.0 ml
of Na₂HPO₄. After the addition of 1ml of DTNB reagent (in 1% sodium citrate) in the system as well as in blank a second optical density was read. The concentration of reduced glutathione (GSH) was calculated and expressed as μ moles/gHb.

\[
C = \frac{(OD_2 - OD_1) \times E_i \times 101}{Hb}
\]

OD₁ and OD₂ are optical densities measured at 412nm before and after the addition of DTNB solution. E₁ is a derived extinction coefficient (0.542) and Hb=Hemoglobin in grams.

**Superoxide dismutase**

**Principle:** SOD activity was measured based on the ability of the enzyme to inhibit the autoxidation of adrenaline. The superoxide dismutase activity was assayed according to the method described by Mishra and Fridovich¹⁷.

**Procedure:** Hemoglobin from hemolysate was removed according to the method of concetti et al¹⁸. 0.25ml of cold chloroform, 0.5ml of cold ethanol and 0.16ml of cold water were added to 0.5ml of hemolysate. The suspension was submitted to vertex agitation for 10 min at 4°C. The precipitate was separated by centrifugation at 5000rpm at 4°C for 10min and the colourless supernatant was used for assay. The assay medium contained 50mM sodium carbonate-bicarbonate buffer pH 9.8 containing 0.1 mM EDTA, 0.6mM adrenaline and sample in a total volume of 3.0ml. Adrenaline was the last component to be added and the adrenochrome formed in the next four minutes was recorded at 470nm in Elico spectrophotometer. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of adrenaline autoxidation. Super oxide dismutase activity was expressed as Units/mg Hb/min.

Data were analysed for significant difference among values of control, experimental groups by using student t’ test. P values are furnished in tables.
References


