4. MATERIAL AND METHODS

MATERIALS AND METHODS

The present study was conducted in the department of biochemistry of Dr. V. M. Medical College, Solapur and Pad. Dr. D. Y. Patil Medical College; Pimpri, Pune, during the period of August 2004 to May 2009.

A) Selection Of Subjects

In all 380 subjects were enrolled in the present study.

i) Control group: comprising 80 healthy age and sex matched subjects selected from staff of Dr. V. M. Medical College; Solapur and Pad. Dr. D.Y. Patil Medical College, Hospital and Research Centre; Pune. The subject appears to be normal but have history of any disease in past, such as inflammatory diseases, infection, hypertension and the disease known to be associated with oxidative stress, were strictly excluded from control group.

ii) Test group: included freshly clinically diagnosed patients of type 1 and 2 diabetes mellitus. All patients were selected from indoor as well as outdoor department of medicine of Shri Chhatrapati Shivaji Maharaj Sarvopchar Rugnalaya; Solapur and Pad. Dr. D.Y. Patil Medical College, Hospital and Research Centre, Pune. In all 300
patients of type 1 (n=150) and type 2 (n=150) between age group of 20-60 years were enrolled in the study.

World Health Organization Criteria i.e. fasting plasma glucose \( \geq 126 \) mg/dl along with classic symptoms of diabetes mellitus or postprandial plasma glucose \( \geq 200 \) mg/dl at more than one occasion was applied for a diagnosis. No patients had ketoacidosis and had taken any hypolipidemic, antidiabetic, or antioxidant containing drugs. All the patients underwent details history, biochemical investigations which include hemogram, BUL, serum creatinine, lipid profile, electrolytes, Urinary protein etc. For exclusion from the study, retinopathy was confirmed by fundal examination by observing presence of microaneurysm, soft exudates, and intraretinal hemorrhages. Nephropathy was considered to be present, if there was proteinurea 1 gm/L in urine sample. The cardiovasculopathy was diagnosed by ECG findings and history of myocardial infarction.

All the subjects included in the study were volunteered after proper consent and reported for follow-up
at right time. The study was approved by local ethical committee of the institute.

All subjects were categorized into different group as follows and each group includes 30 patients except control group A & B.

**Control A** – Age and sex matched healthy subjects (n=40).

**Group I** – Type 1 DM patients those were only on insulin treatment

**Group II** – Type 1 DM patients those were supplemented vitamin C (Celin 500 mg/day) along with insulin.

**Group III** - Type 1 DM patients those were supplemented vitamin E (Evinal 400 mg/day) along with insulin.

**Group IV** - Type 1 DM patients those were supplemented vitamin (E + C)per day along with insulin.

**Group V** -Type 1 DM patients those were supplemented A-Z tablets per day along with insulin.

**Control B** - Age and sex matched healthy subjects (n=40).

**Group VI** – Type 2 DM patients those were on combination of metformin (500mg) + sulphonylurea(1mg)] 2 tab per day.
Group VII – Type 2 DM patients those were supplemented with vitamin C (Celin 500 mg/day) along with combination of metformin-sulphonylurea.

Group VIII – Type 2 DM patients those were supplemented with vitamin E (Evinal 400 mg/day) along with combination of metformin-sulphonylurea.

Group IX – Type 2 DM patients those were supplemented with vitamin (E+C) per day along with combination of metformin-sulphonylurea.

Group X – Type 2 DM patients those were supplemented with A-Z tablets along with combination of metformin-sulphonylurea.

Contents of A to Z tablets (Alkem Lab Ltd) – Multivitamin-Multimineral Antioxidants- (10 Vitamins+5 minerals)

Vitamin C – 100 mg
Vitamin E acetate - 25 mg
Vitamin A acetate 5000 IU
Niacinamide 50 mg
Calcium Patothenate 12.5 mg
Thiamine Mononitrate 10 mg
Riboflavin 10 mg
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Pyridoxine HCl 3 mg
Folic acid 1 mg
Cyanocobalamine 5 µg
Zinc Oxide equivalents to elemental zinc 15 mg
Cupric oxide equivalents to elemental Cu^{2+} 2.5 mg
Manganese chloride equivalents to elemental Mg^{2+} 1.4 mg
Chromium chloride equivalents to elemental Cr 65 µg
Sodium selenate equivalents to elemental Se 60 µg

b) Collection Of Specimen

After 12 hours fast, 10 ml venous blood sample were collected in different bulbs under aseptic conditions.

1. Fluoride bulb was used for fasting blood glucose estimation by Glucose Oxidase Peroxidase method
2. EDTA bulb was used for glycosylated hemoglobin (HbA1c) estimation by resin binding method.
3. Plain bulb was used for estimations of serum superoxide dismutase (SOD) by Marklund and Marklund, nitric oxide (NO) by Cortas and wakid,
malondialdehyde (MDA) by Satoh K. and Total Antioxidant Status by FRAP method.

4. Acid citrate bulb was used for erythrocyte reduced glutathione (GSH) measurement by method of Beutler et al.

5. Heparin bulb used for estimation of platelet aggregation by ADP induced aggregation method.

6. Erythrocyte ghost preparation was done by Dodge method and used for estimation of membrane proteins (Lowery method), phospholipids (Ethanol ether method) and cholesterol (Zak et al).

Baseline level of all of the above biochemical parameters were measured at the time enrollment in the study for all subjects. But Group I to X was again reassessed for the same parameters after follow up of 90 days. The glycated hemoglobin (HbA1c) levels were used as an index of metabolic control.

**Statistical Analysis**: The sample size was decided in consultation with statistician. The results were expressed as mean ± S.D. Comparison of control and test group was done by unpaired ‘t’ test. The change in parameters before
and after antioxidant supplementation was studied by paired ‘t’ test. Comparison of efficacy of antioxidant supplementation was tested by ANOVA.

**Estimation of Plasma Glucose (FPG)**
*(Glucose Oxidase – Peroxidase method)*

**Principle**
Glucose in plasma oxidized by glucose oxidase in presence of oxygen to form gluconic acid and hydrogen peroxide which further degraded by peroxidase enzyme into nascent oxygen which form pink colored quinoneimine dye in presence of 4 aminoantipyrine and 4 hydroxybenzoic acid. The colour intensity was measured at 505nm on semi autianalyzer i.e. Erba Chem Pro.

**Reagents**
1. Glucose Reagent - Glucose oxidase - 20000 IU/L
   Peroxidase - 3250 IU/L
   4-Aminoantipyrine - 0.52 mmol/L
   4-Hydroxybenzoic acid - 10 mmol/L
   Phosphate buffer - 110 mmol/L

2. Glucose Standard – 100 mg/dl
Working reagents – Each vial is dissolved in glucose diluent with special clearing agents and made the volume 200ml and stored in amber colored bottle.

**Procedure**

<table>
<thead>
<tr>
<th>Reagents(µl)</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagents</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>10</td>
<td>--</td>
</tr>
<tr>
<td>Test</td>
<td>--</td>
<td>--</td>
<td>10</td>
</tr>
</tbody>
</table>

Mixed well and incubated for 15 minutes at 37°C. Read the absorbance of standard and each test tube against reagent blank at 505nm.

**Calculation**

\[
\text{Glucose (mg/dl)} = \frac{\text{Absorbance of Tests}}{\text{Absorbance of Std}} \times \frac{\text{Conc. of std}}{100}
\]
4. MATERIAL AND METHODS

Estimation Of Glycated Hemoglobin (HbA1c)  
(Resin Binding Method\textsuperscript{231})

**Principle**

A hemolyzed preparation of the whole blood was mixed continuously for five minutes with a weak binding cation-exchange resin. During this time, nonglycosylated (HbA0) binds to the resin. After the mixing period, a filter was used to separate the supernatant containing the glycohemoglobin from the resin. The percent glycohemoglobin was determined by measuring the absorbance at 415nm of the glycohemoglobin fraction and the total hemoglobin fraction on semi auto analyzer i.e. Erba Chem Pro. The ratio of the two absorbances gives the percent glycohemoglobin.

**Reagents**

1. Resin reagents -8mg/dl cation exchange resin buffered at pH 6.9.
2. Lysing reagent – 10mM potassium cyanide, surfactant added.
3. Glycohemoglobin standard – 10% glycohemoglobin
4. Serum separators
4. MATERIAL AND METHODS

**Procedure**

**A. Hemolysate Preparation** - Prepared two tubes labeled as test & Standard

<table>
<thead>
<tr>
<th>Reagents (µl)</th>
<th>Test</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysing reagents</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Blood sample</td>
<td>100</td>
<td>--</td>
</tr>
<tr>
<td>Std</td>
<td>--</td>
<td>100</td>
</tr>
</tbody>
</table>

Allowed to stand for 5 minutes it forms hemolysate.

**B. Glycohemoglobin Preparation**

1. Dispensed 3.0ml of glycohemoglobin cation exchange resin into 13 × 100 mm glass tube labeled as test and standard.
2. Added 100 µl of the hemolysate from above tubes.
3. Position the filter separators in the tubes so that the rubber sleeve was approximately 1 cm above the liquid level.
4. Placed the tubes on the rocker or rotator and mixed continuously for 5 minutes.
5. Removed the tubes from the rocker or rotator.
6. Pushed the filter separator into the tubes until the resin was firmly packed.

7. The supernatant poured into another tube for absorbance measurement.

8. Adjusted the instrument to zero absorbance at 415 nm with deionized water as the blank.

9. Absorbance of test and standard tubes were taken and it was glycohemoglobin.

C. Total Hemoglobin fraction

1. Dispensed 5.0 ml deionized water into tubes of test and standard.

2. Added 20 µl of the hemolysate into the appropriately labeled tube and mixed.

3. Adjusted the instrument to zero absorbance at 415 nm with deionized water as the blank.

4. Read and recorded the absorbance values of test and standard.

Calculations

\[
R \text{ (unknown)} = \frac{\text{Abs. of Glyco (unknown)}}{\text{Abs. of total Hb (unknown)}}
\]
4. MATERIAL AND METHODS

\[ R \text{ (standard)} = \frac{\text{Abs. of Glyco (standard)}}{\text{Abs. of total Hb (standard)}} \]

\[ \% \text{GHb (unknown)} = \frac{R \text{ (unknown)}}{R \text{ (standard)}} \times \text{Std conc.} \]

Glycated Hb was calculated as follows

\[
\% \text{HbA1c} = \frac{\% \text{GHb} + 1.76}{1.49}
\]

**Estimation Of Serum Malondialdehyde [MDA]**
*(Modified Satoh K. Method \(^{[232]}\))*

**Principle**

Serum sample treated with trichloroacetic acid (TCA) for lipoprotein precipitation and then treated with 0.67% thiobarbituric acid (TBA). The mixture is heated for 10 minutes in boiling water bath. One molecule of MDA reacts with two molecules of TBA. The resulting chromogen is centrifuged and intensity of colour developed in supernatant is measured colorimetrically at 530nm on spectrophotometer.
4. MATERIAL AND METHODS

Reagents

1. 40% TCA (Trichloroacetic acid) - 40gm of TCA powder dissolved in distilled water and made the volume 100ml.

2. 0.67% TBA (thiobarbituric acid) - 670mg of triobarbituric acid was dissolved in 100ml of distilled water in boiling water bath. Reagent prepared freshly.

Procedure

<table>
<thead>
<tr>
<th>Reagents (ml)</th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Dist. water</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>40% TCA</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>0.67%TBA</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Mixed well and kept the tubes in boiling water bath for 10 minutes. Cooled at room temp and centrifuged at 3500 r.p.m. for 20 minutes. Supernatant were taken and read at 530nm.

Calculation

\[
\text{Conc. of serum total (abs. of test – abs. of blank)} \times 3846 = \text{MDA (nmol/100ml)}
\]
Factor was arrived using the molar extension/5. The values were expressed as nmol/100ml of serum was converted to nmol/ml.

**Estimation Of Serum Nitric Oxide (NO i.e. Nitrate + Nitrite) (Cadmium reduction method)**

**Principle**

Nitric oxide is highly unstable gas and undergoes rapid oxidative degradation to the stable breakdown products, nitrite and nitrate. Nitrite is difficult to measure in blood because it is unstable, being rapidly oxidized to nitrate. This nitrite and nitrate in serum was assayed by a modified cadmium reduction method. The samples were deproteinized with somogyi reagent and reduction of nitrite by copper coated cadmium in glycine NaOH buffer at pH 9.7. It was then diazotized with sulphanilaminde and it is then coupled to naphthylethylenediamine to get red azo compound. Intensity of this colored complex was measured at 545 nm spectrophotometrically.

**Reagents**

1. Cadmium granules
2. 0.1M H$_2$SO$_4$ – 0.98ml concentrated sulphuric acid diluted by deionized water making volume 100ml.

3. 75mmol/L ZnSO$_4$ – 2.15gms of zinc sulphate dissolved in 100ml deionized water.

4. 55mmol/L NaOH- 2200 mgs of sodium hydroxide pallets dissolved in 1liter of deionized water.

5. Glycine NaOH buffer – 15 gms of glycine is dissolved in deionized water and made the volume 1liter. 9.7 pH was adjusted by 2 mol/L NaOH. Stable for 1 month at 2-8°C.

6. 5 mmol/L CuSO$_4$ – 124.8 mgs of copper sulphate dissolved in 100ml of glycine NaOH buffer.

7. Sulfanilamide - 1 gms of Sulfanilamide is dissolved in 3mol/L HCL. Reagent is stable for one year.

8. N-naphthylethylene diamine – 50mgs of N-naphthylethylene diamine powder was dissolved in 250 ml of deionized water. Stable for 2 months at 2-8°C.

9. Stock Standard (0.1 mol/L) - 690 mgs of NaNO$_2$ was dissolved in 100ml of 10 mmol/L sodium borate solution.
10. Working standard (10 µmol/L) – 10 µl of stock was diluted to 100 ml with 10 mmol/L solution of sodium borate.

Procedure

I) Deproteinization – Added 0.5 ml of serum to 2.0 ml of ZnSO₄ (75mmol/L) solution. Then added with mixing 2.5ml of NaOH (55 mmol/L) reagent. The final pH should be between 7.0-7.5. kept it for 10 minutes and then centrifuged.

II) Activation of cadmium granules. – Rinse the acid of copper coated cadmium granules three times with deionized water. Swirled the granules for 1-2 minutes in a 5mmol/L CuSO₄ solution in glycine NaOH buffer. Drain and rinse CuSO₄ three times with glycine NaOH buffer. Use Cu- coated cadmium granules within 10 min. Prolonged exposure of granules to air diminishes their reductive ability. After use rinse granules and store them in 0.1M H₂SO₄.

III) Preparation of Flasks – Took three flasks and labeled as blank test and standard
4. MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank</th>
<th>Test</th>
<th>Std</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine NaOH buffer</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Deproteinized sample</td>
<td>--</td>
<td>1.0</td>
<td>--</td>
</tr>
<tr>
<td>Std NaNO₂</td>
<td>--</td>
<td>--</td>
<td>0.5</td>
</tr>
<tr>
<td>Deionized water</td>
<td>3.0</td>
<td>3.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Reaction was started by adding the above solution in the labeled tubes to the corresponding Erlenmeyer’s flask containing 2.5 gms of activated granules and stirred once by swirling. Exactly 90 mins later transferred 2.0ml of above solution into the following tubes.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Blank</th>
<th>Test</th>
<th>Std</th>
</tr>
</thead>
<tbody>
<tr>
<td>Above mixture</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>N-napthylethylenediamine</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The test tubes were mixed well and then absorbance was taken against the blank at 545nm after 20-60min.
**Calculation**

<table>
<thead>
<tr>
<th>Nitric Oxide (µmol/L)</th>
<th>Tests - Blank</th>
<th>Conc. of std</th>
<th>Std - Blank</th>
<th>Effect. serum used</th>
<th>× 100</th>
</tr>
</thead>
</table>

**Estimation Of Serum Superoxide Dismutase (SOD)**
*(Marklund and Marklund Method* *^{234}\)*)

**Principle**

Pyrogallol autooxidises rapidly in aqueous or alkaline solution and this has been employed for the estimation of SOD. SOD inhibits the autooxidation of pyrogallol.

**Reagents**

1. 1N HCl – 8.9 ml of HCL was poured carefully into distilled water made the volume upto 100 ml.
2. 50mM Tris HCl - 0.785 gm tris dissolved in1N HCL and made volume upto 100ml.
3. 10 mM HCl- Took concentrated 0.5 ml HCl and diluted it upto 500 ml distilled water.
4. EDTA free acid 1 mM - Add 0.0292 gm of EDTA free acid to 0.785 gm of HCl and dissolve the crystals in glass distilled water.
and make volume upto 100 ml and adjust the pH to 8.2 by IN HCl or NaOH.

5. Pyrogallol 20 mM - 0.0252 gm of pyrogallol is dissolved in 10 ml of 10 mM HCl.

**Procedure**

Take following quantities in a curette to start the reaction.

<table>
<thead>
<tr>
<th>Reagents (ml)</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer</td>
<td>3.0</td>
<td>2.95</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Serum</td>
<td>--</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Mixed and immediately measured the absorbance continuously for 4 min. at 420 nm at 30 seconds interval on spectrophotometer.

**Calculations**

Absorbance reading to be taken for calculation was reaching at 3 ½ minute minus reading of 1 ½ minute (1 ½ min are required for the assay mixture to stabilize initially).
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One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxidation as per 3.0ml of assay mixture.

Absorbance reading of control was A and absorbance reading of test was B.

\[
\text{Serum SOD in (unit/ml)} = \frac{\text{Control } A - \text{ Test } B}{A \times 50} \times \frac{100}{0.05}
\]

**Estimation of Erythrocyte Reduced Glutathione**

(Beutler et al \(^{235}\))

**Principle**

Virtually all the non-protein sulphhydryl groups of red blood cells are in the form of reduced GSH. 5.5’ DTNB (Dithiobis – 2 nitrobenzoic acid) is a disulphide compound which is readily reduced by sulphhydryl compounds to an intensely yellow compound. The absorbance of the reduced chromogen was measured at 412 nm on spectrophotometer.

**Reagents**

1. Precipitating solution – 1.67 gms glacial metaphosphoric acid + 0.20 gms disodium or dipotassium EDTA +30 gms
4. MATERIAL AND METHODS

NaCl per 100 ml of distilled water. Solution is stable for approx 3 wk. at 4°C).

2. Phosphate solution- 42.59 gm of Na₂HPO₄ in 1000 ml distilled water stable at 4°C.

3. DTNB reagent (5 5’ Dithiobis – 2 nitrobenzoic acid) - 40 mgs of DTNB per 100 ml of 1% sodium citrate solution. Stored in deep freeze stable for 13 weeks at 4°C.

4. GSH Calibrators - 100 mgs GSH in a 100 ml volumetric flask and bring to volume with reagent grade water. Invert repeatedly until GSH is completely dissolved.

   **a) Prepare 50 and 10 mg/dl calibrators.**

   50 mg/dl – 5 ml of stock solution diluted upto 100ml by dist H₂O.

   10 mg/dl – 5 ml of stock solution diluted upto 45 ml by dist H₂O.

   GSH calibrations were not stable so freshly prepared.

5. ACD (Preparation of acid citrate Dextrose) for sample collection - 2.20 gms trisodium citrate + 0.80 gms citric acid + 2.45 gms dextrose was dissolved in 100ml distilled water. For 2 ml blood collection- 0.2 ml ACD
Procedure

0.2 ml of whole blood was taken in test tube. Then 1.8 ml of distilled water was added and mixed to haemolyse. Promptly added 3.0 ml of precipitating solution and mixed. Allowed to stand 5 minute at room temperature and filtered through coarse grade filter paper.

Prepared three cuvetts as follows

<table>
<thead>
<tr>
<th>Reagents (ml)</th>
<th>Blank</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrate</td>
<td>--</td>
<td>2.0</td>
</tr>
<tr>
<td>Precipitating reagent</td>
<td>1.2</td>
<td>--</td>
</tr>
<tr>
<td>Dist Water</td>
<td>0.8</td>
<td>--</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>DTNB</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Caped Cuvetts and inverted three times to mix.

2) Read absorbance at 412 nm within the 4 minute of preparing cuvetts.

3) Obtained Hb (gm/dl) of original whole blood specimen.
4) Assayed GSH calibrators, omitting filtration step.

5) Prepared a calibrator curve. Determined the GSH conc. of whole blood specimen form the graph.

6) Calculated the GSH concentration.

**Calculations**

Reduced Glutathione (mg/dl of RBC) = \( \frac{\text{GSH conc (calibration curve)}}{\text{Hemoglobin}} \)

= \( X \ \mu\text{mol/gm of Hb} \)

**Estimation of Serum Total Antioxidant Capacity**

*(Ferric Reducing Ability Of Plasma)*

**Principle**

Antioxidant power reduces ferric tripyridyltriazine (Fe\(^{III}\) TPTZ) to ferrous(\(^{II}\)) ion at low pH and it forms intense blue colored ferrous tripyridyltriazine complex. FRAP values are obtained by comparing the absorbance change at 593nm in mixture with those containing ferrous ion in known concentration on spectrophotometer.

**Reagents**
1. 20 mmol/L FeCl₃·6H₂O - 324 mgs FeCl₃·6H₂O was dissolved in distilled water to make volume up to 100ml.

2. Acetate Buffer (pH 3.6) - 3.1gms of sodium acetate was dissolved in 500ml of distilled water to this 16ml of glacial acetic acid was added and pH of the solution was adjusted upto 3.6 by 1% sodium carbonate and finally diluted with distilled water to make volume upto 1000ml.

3. 10ml Ferrozine or TPTZ - 49 mgs of ferrozine was dissolved in 0.04M HCL to make volume up to 10ml.

4. 1000µmol/lit ferrous Std (FeSO₄·7H₂O) - 278mgs FeSO₄·7H₂O was dissolved in 500ml of distilled water and final volume was made upto 1000 ml with distilled water.

**Procedure**

About 300 µl freshly prepared FRAP reagent was warmed to 37°C and a reagent blank reading was taken (M1) at 593 nm; 10 µl of sample was then added, along with 30 µl of H₂O; final dilution of sample in reaction mixture was, therefore, 1/34. Absorbance (A) reading was taken after 0.5sec and every 15sec thereafter during the monitoring period. The change in absorbance (ΔA) between the final rending selected and the M1 reading was calculated for
each sample and related to $\Delta A$ of Fe$^{II}$ standard solution tested in parallel.

**Calculations**

Different concentration of std i.e. 200,400,600,800 µmol/lit were prepared from stock std (1000µmol/lit) and tested as test and plotted the graph each time and unknown concentration was determined from graph.

**Estimation of platelet aggregation**

*(ADP induced Method)*

**Principle**

Absorbance of platelet rich plasma (PRP) is directly proportional to the concentration of platelet present in it. When ADP is added to PRP with constant stirring, it induces aggregation and form clumps which settle down to the bottom of the test tube.

**Reagent**

1. 200 micro g / ml ADP.

2. Heparin- 0.2 mg / ml of blood.

**Procedure**

All blood samples were collected in tubes containing heparin and platelet aggregation was assessed with PRP by
spectrophotometric method. PRP was obtained as a supernatant after centrifugation of heparinized blood at 600 rpm for 10 minutes. The isolated PRP was kept at 37° C for 20 minutes before use. 2ml PRP was taken in plastic cuvette and optical density was read on spectrophotometer at 550 nm (A1). 0.1 ml ADP solution was then added and the contents were mixed by stirring and absorption was measured exactly after 20 seconds. (A2)

More is the absorption of platelet rich plasma more the platelet aggregation. Extent of platelet aggregation is expressed as % of platelet aggregability.

Change in optical density at the end of 20 seconds was taken as change in platelet aggregation.

**Calculation**

\[
\text{Platelet Aggregation Percentage (\%)} = \text{A1} - \text{A2 (ADP)}
\]

**Assay of Membrane Markers**

*Isolation of Erythrocyte Membrane*  
(Dodge Method (238))
Red cell membrane ‘ghosts’ free of hemoglobin were prepared by hypotonic lysis of red cells. The procedure described by Dodge et al as followed.

**Reagents**

1. Isotonic phosphate buffer – 2.148 gm of NaH$_2$PO$_4$.2H$_2$O was dissolved and diluted to 100 ml with distilled water (solution A). 3.689 gm of NaHPO$_4$.12H$_2$O was dissolved and diluted to 100 ml with distilled water (solution B). 100 ml of solution B and 19.2ml of solution A were mixed to get a pH of 7.6. This buffer was 310 ideal milliosmolar (imOsm).

2. 20 ml of the above isotonic phosphate buffer (pH 7.4) was added to 290 ml of distilled water.

**Procedure**

One volume of whole blood was mixed with an equal quantity of cold isotonic phosphate buffer (310 ideal milliosmolar, pH 7.4) and the suspension centrifuged for 10 minutes at 4°C at 5000 rpm. The supernatant and buffy coat was aspirated and the packed cells washed three times in 30 volumes of the same isotonic phosphate buffer. The buffy coat was aspirated after each wash. Finally the packed cells were lysed by mixing one volume of packed
cells with 50 volumes of cold hypotonic phosphate buffer pH 7.6 (20 imOsm).

The hemolysate was centrifuged for 30 min at 10,000 rpm at 4°C. The deep red supernatant was discarded leaving reddish colored pellets at the bottom of the tube. Two additional washings under similar conditions resulted in a cream colored homogenous pellet as all the hemoglobin was removed. Membrane thus isolated was analyzed further on the same day without storage.

The above pellets were suspended in 2ml 20 imOsm phosphate buffer.

**Estimation Of Membrane Proteins**

(Lowery Method \(^{(239)}\))

**Principle**

Proteins are reacted with Cu\(^{++}\) alkaline solution to form copper peptide bound protein complexes when the folin ciocalteu reagent is added, the copper protein complexes join with tyrosine and tryptophan residues in the reduction process and form the blue color. Colored intensity is measured at 650nm on spectrophotometer.

**Reagents**
1. 2% sodium carbonate solution containing 0.4% NaOH in dist water.

2. 1% copper sulphate solution in distilled water.

3. 2% potassium sodium tartarate solution in distilled water.

4. 0.1% deoxycholate in distilled water.

5. Folin Ciocalteu working solution – 0.5 ml of copper sulphate solution and 0.5ml of potassium sodium tartarate solution were mixed and diluted to 50ml with 2% sodium carbonate solution containing 0.4% NaOH. This reagent was prepared fresh every time.

6. Phenol reagent – The normality of commercially available phenol was determined by titrating with 0.1N NaOH using phenolphthalein as indicator. This phenol was then diluted to 1N and stored in a dark bottle at room temperature.

7. Protein standard – 100 mg of pure bovine serum albumin was dissolved and diluted to 100 ml with normal saline.

**Procedure**

0.02 ml of membrane suspension was dissolved in 1.0ml of 0.1% deoxycholate and 2.0ml of fresh folin working
solution added to it. The contents were mixed and allowed to stand for 10 minutes at room temperature. 0.2ml of 1N phenol was added to the tubes by allowing it to flow down the sides of the test tube. A blank and standard were run parallel to the test by substituting 0.02ml of distilled water and 0.02ml of protein standard for the membrane suspension. The resultant colour developed was read at 578 nm on a spectrophotometer against blank.

**Calculation**

\[
\text{Membrane Proteins (mg/ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of std}}
\]

After estimation of membrane protein content the samples were further analyzed for membrane cholesterol, membrane phospholipids and membrane triglycerides.

**Estimation Of Membrane Cholesterol**

( Zak et al Method (240) )

**Principle**

Cholesterol was extracted in ethanol-ether mixture and dissolved in chloroform which is further dehydrated and oxidized by concentrated sulfuric acid in presence of ferric chloride and glacial acetic acid. It form red colored
complex. The colour intensity is measured at 510nm on spectrophotometer.

Reagents

1. Ethanol : Ether mixture 3:1      2. Acetic acid
3. Ferric chloride – 0.05% solution of FeCl$_3$ $6\text{H}_2\text{O}$ in glacial acetic acid.
4. Concentrated reagent grade sulphuric acid.
5. Cholesterol standard – 10 mg of cholesterol powder dissolved in 10ml glacial acetic acid.

Procedure

Step I – Extraction of membrane cholesterol in a stoppered centrifuge – 0.2ml membrane suspension + 10 ml ethanol-ether mixture. Tubes were stoppered and shaken vigorously and kept horizontally for 30 mins then centrifuged to get firm deposit. Supernatant was completely decanted into suitable test tubes, contents were then evaporated to dryness in a water bath. A residue was dissolved in 5ml chloroform.

Step II -

0.1ml dissolved cholesterol was added to 5.0ml ferric chloride reagent and shaken vigorously. It was then
allowed to stand for 15 minutes at room temperature. The tubes were then cooled to room temperature and the optical density measured at 560 nm against reagent blank. A blank and standard were run parallel to the test by substituting 0.1ml of distilled water and 0.1ml of cholesterol standard for the membrane suspension.

**Calculation**

\[
\text{Membrane Cholesterol (μg/mg of proteins)} = \frac{\text{Abs of test}}{\text{Abs of Std}} \times \frac{1}{X} \times 386.67
\]

Where \( X = \) mg protein / ml of membrane suspension. 386.67 = molecular weight of cholesterol

**Estimation Of Membrane Phospholipids (Extraction by ethanol:ether mixture)**

**Principle**

Lipids were extracted using an ethanol:ether mixture and further digested with sulphuric acid perchloric acid reagent. The phosphorus now present as phosphate was determined colorimetrically by estimation of inorganic phosphorus.

**Reagents**

1. Ethanol:Ether mixture – 3:1 (V/V) mixture
2. Digestion mixture – One volume of 85% sulphuric acid mixed with an equal volume of 70% perchloric acid.

**Procedure**

In 5.0ml of ethanol:ether mixture 0.1ml if isolated ghost suspension drop by drop was added. The mixture was then boiled in a boiling water bath, cooled and centrifuged. The supernatant was transferred to another test tube and incubated at 80-100°C till the solvents evaporated completely. 0.5ml of digestion mixture was then added to the residue and digested for 3 hours till the solution turned colourless. The tubes were then cooled at room temperature and 0.25ml glass distilled water added. The contents were again boiled to convert all the pyrophosphates to orthophosphates. The inorganic phosphate content was then processed and the total phospholipids content expressed as µg/mg of protein.

**Estimation of Inorganic Phosphate**

*(Fiske & Subbarow)*

**Principle**

Inorganic phosphate is reacted with ammonium molybdate in presence of reducing agent 1,2,4-amino naphthol sulphonic acid( ANSA) and form yellow colored ammonium
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phosphor-molybdate. The color intensity was measured at 625nm on spectrophotometer.

Reagents

1. Ammonium molybdate – 2.5% in distilled water
2. 1,2,4-amino naphthol sulphonic acid (ANSA) – 2.72 gm of sodium bisulphate and 0.2gm of sodium sulphite were dissolved in distilled water. 0.1gm ANSA was added to the above solution and the volume was made upto 100ml with distilled water.
3. Standard phosphorus (5 mg/dl)- 0.292 gm of potassium dihydrogen phosphate dissolved and diluted to 1 liter with distilled water.

Procedure

To 3.5ml of sample, 1.0ml of ammonium molybdate and 0.5ml of ANSA was added. The tubes were boiled for 5 minutes and the colour developed was read at 625 nm against blank after cooling the tubes. A standard solution was also run alongside.

Calculation

<table>
<thead>
<tr>
<th>Membrane phospholipids =</th>
<th>Abs of test</th>
<th>10</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>(μg/mg of proteins)</td>
<td>Abs of Std</td>
<td>X</td>
<td>Y</td>
</tr>
</tbody>
</table>
Where $X = \text{mg protein / ml of membrane suspension.}$

$Y = 774.5$ (molecular weight of Lecithin)