CHAPTER II

Methodology

A detail account of “Methodology” is presented in this chapter. The cancer patients with various malignancies were treated as Test groups. The healthy normal subjects were treated as Control group for comparison. The study related to the patients with Cervix Cancer, Breast Cancer and Ovarian tumor is included in Chapter III, further subdivided as IIHA, IIIB and IIIC respectively. Chapter IV deals with oral and oral related cancers. These included cancer of cervical nodes and secondaries in neck, pyriform fossa, maxilla, parotid tumours, larynx and oesophagus. The parameters studied were common in all malignancies.

Study Design:

The study deals with the biochemical estimations of various parameters namely Kidney function tests, Liver function tests, levels of serum Antioxidants and that of oxidative stress indicators. The Kidney function tests included estimations of blood urea, serum creatinine and serum uric acid. The serum bilirubin, serum alkaline phosphatase, serum aspartate transaminase (AST), serum alanine transaminase (ALT), serum total proteins and serum albumin were determined as Liver function tests. The serum reduced glutathione and vitamin A were determined for assessment of antioxidant status. The lipid peroxidation (malondialdehyde MDA or Thiobarbituric Acid
Reactive Substances-TBARS), and Sensitivity of Erythrocytes to Peroxide Haemolysis (SEPH) were determined for evaluation of Oxidative Stress.

**Selection of Control Subjects:**

For comparison with test group, healthy control subjects without any diseased conditions were treated as Control.

**Subject Population:**

The subject population was divided into two groups i.e. fifty cases of normal healthy subjects were treated as control group and fifty cases of each type of malignancy were treated as test group.

**Sample Collection and Analysis:**

The present study was carried out on blood samples of the patients from the Cobalt Unit of Government Medical College and Hospital, Aurangabad. It was made sure that the patient is not subjected to any treatment of chemotherapy or radiotherapy so that changes in biochemical parameters can be studied.

For the analysis of blood urea and reduced Glutathione, blood sample was collected in a oxalate bulb, that is, oxalated blood sample was obtained. For evaluation of sensitivity of erythrocytes to peroxide haemolysis, sample was collected in EDTA bulb. For rest of the parameters investigated viz. liver function test, serum creatinine, serum uric acid and antioxidants parameters, the sample was collected in clean dry plain bulb to
obtain serum from the blood. Nearly 10 ml of venous blood was drawn from the patients.

The availability of the control group subject along with the test group was made from the Government Medical College and Hospital, Aurangabad.

The biochemical estimations of the following various parameters were performed on taken blood samples of test and control group.

**Studied Parameters:**

1) **Kidney Function Test:**

   i.  Blood urea

   ii. Serum Creatinine

   iii. Serum Uric Acid

2) **Liver Function Test:**

   i.  Serum Bilirubin

   ii. Serum Alkaline Phosphatase

   iii. Serum Aspartate Transaminase (AST/SGOT)

   iv.  Serum Alanine Transaminase (ALT/SGPT)

   v.  Serum Total Proteins

   vi.  Serum Albumin
3) Oxidative Stress (Lipid peroxidation):

Lipid Peroxidation: Malondialdehyde (MDA) or Thiobarbituric Acid Reactive Substances (TBARS) Sensitivity of Erythrocytes to Peroxide Haemolysis (SEPH)

4) Serum Antioxidants:

   i. Vitamin - A

   ii. Reduced Glutathione

1. Kidney Function Test:

   i. Estimation of Blood Urea:

Method :- Diacetyl Monoxime (DAM)

Principle: Under acidic condition and in presence of ferric ions and thiosemicarbazide (Acid Reagent), urea present in plasma sample reacts with Diacetyl Monoxime to form a pink coloured complex, a diamine derivative which is measured calorimetrically at 530 nm. The intensity of the colour is directly proportional to the concentration of urea present in the blood.\(^{(96)}\)

Reagents:

1. Acid Reagent

2. Diacetyl Monoxime Reagent (DAM Reagent)

3. Urea Standard Solution (Concentration 0.03 mg/ml)

Procedure:

In two test tubes labeled as Standard ‘S’ and Test ‘T’, following addition were made.
<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>01</td>
<td>D/W</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>02</td>
<td>Plasma</td>
<td>--</td>
</tr>
<tr>
<td>03</td>
<td>Urea Standard</td>
<td>20µltr</td>
</tr>
<tr>
<td>04</td>
<td>Acid Reagent</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>05</td>
<td>DAM Reagent</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

Place the tubes in Boiling water bath (BWB) for 20 minutes. Cool them at room temperature. Take readings on calorimeter at 530 nm.

Calculations :-

Concentration of Urea = \( \frac{OD(T) \times \text{Conc. std.} \times \text{Vol of std.} \times 100}{OD(S) \times \text{Vol of test}} \)

= \( \frac{OD(T) \times 0.3 \times 0.02 \times 100}{OD(S) \times 0.02} \)

= \( \frac{OD(T)}{OD(S)} \times 30 \text{ mg%} \)

Normal Range : 15 to 40 mg% (mg/dl)

ii. Estimation of Serum Creatinine:-

Method : By Jaffe’s Method.\(^{(97)}\).

Introduction :- Creatinine is an anhydride of Creatine. It is synthesized from amino acids lysine, arginine and methionine and
is excreted in urine. It is present as Creatinine phosphate in body, out of it 98% is present in muscles.

**Principle:** Creatinine present in protein free filtrate precipitates with Alkaline picrate to from an orange coloured complex of creatinine picrate, the intensity of which is measured calorimetrically at 530 nm. A standard Creatinine (concentration = 0.06 mg/ml) is similarly treated and the colour intensities are compared.

**Reagents:**

1. 10% Sodium tungstate,
2. 2/3 N Sulphuric Acid (H₂SO₄)
3. Alkaline picrate.
4. Standard Creatinine (concentration = 0.06 mg/ml)

**Preparation of Protein free filtrate (PFF):**

To 7.0 ml of distilled water (d/w) 1.0 ml of serum was added. Further 1.0 ml of 10% sodium tungstate was added following with 1.0 ml 2/3 N H₂SO₄. This was mixed properly to precipitate. This precipitate was filtered and a protein free filtrate [PFF] was obtained. To the three test tubes labelled ‘B’, ‘S’ and ‘T’, following additions were made.
<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>PFF</td>
<td>B: --</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S: --</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T: 5.0</td>
</tr>
<tr>
<td>02</td>
<td>D/W</td>
<td>B: 5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S: --</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T: --</td>
</tr>
<tr>
<td>03</td>
<td>Standard Creatinine</td>
<td>B: --</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S: 5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T: --</td>
</tr>
<tr>
<td>04</td>
<td>Alkaline Picrate</td>
<td>B: 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S: 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T: 2.5</td>
</tr>
</tbody>
</table>

Mix well and keep for 20 minutes at room temperature. Take readings (optical density) on green filter (520 nm) against d/w.

**Calculations:**

Concentration of Creatinine =

\[
\frac{T - B}{S - B} \times \frac{\text{Std. Conc} \times \text{Vol. of Std.}}{\text{Vol. of Test}} \times 100
\]

\[
\frac{T - B}{S - B} \times 0.006 \times 5.0 \times 100
\]

\[
\frac{T - B}{S - B} \times 6 \text{ mg%}
\]

**Normal Range** - 0.6 to 1.2 mg% (mg/dl).

**iii. Estimation of Serum Uric Acid:**

**Method** - Caraway’s (98)

**Introduction** :- Uric acid is an end product of purine catabolism formed in liver from where it is transported to kidneys and excreted (500 - 700 mg / day)
Principle:- Proteins in serum are precipitated by Tungstic acid. Uric acid present in protein free filtrate (PFF) reduces the phosphotungstic acid in the presence of sodium carbonate to form a blue coloured phosphotungstous complex. The colour intensity of the complex is measured calorimetrically at 620 nm and compared with a uric acid standard treated similarly. The colour intensity is directly proportional to the concentration of uric acid.

Reagents:

1. Tungstic Acid
2. Phosphotungstic Acid
3. 10% Sodium carbonate
4. Uric Acid standard (concentration = 0.01 mg/ml)
Procedure:

Preparation of Protein free filtrate (PFF) :- to 0.6 ml of serum in a test tube, add 5.4 ml of Tungstic acid. Shake well and then filter it. Take 3 test tubes labelled as B, S and T, Further additions are done as per the table below :-

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>PFF</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>02</td>
<td>Uric acid standard</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0 ml</td>
</tr>
<tr>
<td>03</td>
<td>D/W</td>
<td>3.0 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>--</td>
</tr>
<tr>
<td>04</td>
<td>10% Sodium carbonate</td>
<td>0.6 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6 ml</td>
</tr>
<tr>
<td>05</td>
<td>Phosphotungstic Acid</td>
<td>0.6 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.6 ml</td>
</tr>
</tbody>
</table>

Mix well and keep in “DARK” for 15 minutes

Take readings (optical density) on red filter (620 nm) against d/w.
Calculations:

Concentration of Uric Acid = \[
\frac{\text{OD}(T) - \text{OD}(B)}{\text{OD}(S) - \text{OD}(B)} \times \frac{\text{Conc. of Std.} \times \text{Vol. of Std.}}{\text{Vol. of Test}} \times 100
\]

\[
= \frac{T - B}{S - B} \times \frac{0.01 \times 3.0}{0.3} \times 100
\]

\[
= \frac{T - B}{S - B} \times 10 \text{ mg}\%'
\]

Normal Range - 3.5 to 5.5 mg% (mg/dl)

2. Liver Function Tests:

i. Estimation of Serum Bilirubin:

Method: Method of Powell\(^{(99)}\)

Introduction:

Bilirubin is synthesized by degradation of heme in cells of reticuloendothelial system that is spleen, bone marrow and liver. In association with albumin it enters the liver and conjugates with glucoronic acid. Conjugated bilirubin is excreted as bile into intestine. It is not soluble in water. It is reduced by bacterial action into stercobilinogen and urobilinogen. Stercobilinogen is excreted in faeces while urobilinogen is reabsorbed into total circulation and carried to the liver [enterohepatic circulation] while remaining urobilinogen is then excreted in the urine. Unconjugated bilirubin is water soluble.
Principle:

Serum Bilirubin is present in two forms - Conjugated i.e. direct Bilirubin and unconjugated i.e. indirect bilirubin. Total bilirubin is sum of both. Bilirubin in serum couples with diazotized sulphanilic acid to form a pink coloured compound azobilirubin i.e. Bilirubin glucoronide (direct bilirubin). This
azobilirubin’s colour intensity is measured calorimetrically at 530 nm.

**Reagents:**

1. Diazo ‘A’ Reagent - Sulphanilic acid and hydrochloric acid.
2. Diazo ‘B’ Reagent - Sodium nitrite
3. Diazo Mixture - is mixture of Diazo ‘A’ & Diazo ‘B’ [9.7 ml + 0.3 ml]
4. Sodium Benzoate-Urea Mixture -
5. Bilirubin Standard - Methyl Red

**Procedure:**

Take two clean dry test tubes labeled as “T” for Test and “S” for Standard and make the following additions as per the table below.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Serum</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>02</td>
<td>Diazo-mixture</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>03</td>
<td>Sodium Benzoate-Urea mixture</td>
<td>1.7 ml</td>
</tr>
<tr>
<td>04</td>
<td>Methyl Red Standard</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Mix and keep at room temperature for 10 minutes. Then read the corresponding optical density on calorimeter at 520 nm.
Calculations:

Concentration of Bilirubin =
\[
\frac{T \times \text{Conc of Std.} \times \text{Vol. of Std.}}{S \times \text{Vol. of Test}} \times 100 \text{ mg%}
\]
\[
= \frac{T \times 0.04 \times 0.2}{S \times 0.2} \times 100 \text{ mg%}
\]
\[
= \frac{T}{S} \times 4 \text{ mg%}
\]

Normal Range : 0.1 to 1.0 mg% (mg/dl)

ii. Estimation of Serum Alkaline Phosphatase (ALP. EC 3.1.3.1) Activity

Method - King and Armstrong\(^{100}\)

Principle:- Phosphatases are enzymes which hydrolyze monophosphoric esters to liberate phosphoric acid. Enzyme alkaline phosphatase hydrolyzes the organic phosphates like disodium phenyl phosphate under defined conditions of time, temperature and pH.

Chemical Reaction:

\[
\text{Alkaline Phosphatase} \rightarrow \text{Disodium phenyl} \xrightarrow{\text{Phosphoric acid}} \text{Phenol} + \text{Phosphoric acid Phosphate} \quad (\text{pH10, Temp.37}^\circ\text{C, time 15 mins})
\]

The hydrolysis product, phenol is condensed with 4-Amino antipyrine and then oxidised with alkaline potassium ferricyanide to give red colour complex which is measured colorimetrically on green filter.
Alkaline phosphatase is found in bone, liver, intestine, placenta and kidney.

**Reagents:**

1. Carbonate Bicarbonate Buffer (0.1 M, pH 10).
2. Disodium Phenyl Phosphate (0.02 M) - Substrate.
3. 0.5 N NaOH (Sodium Hydroxide).
4. Sodium Bicarbonate (0.5 M).
5. 4 - Amino antipyrine (0.6%).
6. Potassium ferricyanide.
7. Stock Phenol Standard (1 mg/ml).
8. Working Standard Phenol (0.01 mg/ml).
**Procedure:**

Take four clean dry test tubes labeled as blank ‘B’ standard ‘S’, control ‘C’ and test ‘T’ and make further additions as per the table below.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Carbonate-Bicarbonate buffer</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>02</td>
<td>Substrate (Disodium phenyl phosphate)</td>
<td>--</td>
</tr>
<tr>
<td>03</td>
<td>Serum</td>
<td>--</td>
</tr>
<tr>
<td>04</td>
<td>Working standard</td>
<td>--</td>
</tr>
<tr>
<td>05</td>
<td>D/W</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Mix well and incubate at 37°C for 15 minutes. Then make the following additions.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>06</td>
<td>0.5 N Sodium hydroxide</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>07</td>
<td>0.5 M Sodium bicarbonate</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>08</td>
<td>Serum</td>
<td>--</td>
</tr>
<tr>
<td>09</td>
<td>4-Amino-antipyrine</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>10</td>
<td>Potassium ferricyanide</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Take readings (optical density) on green filter.
Calculations:

Concentration of Alkaline Phosphatase in serum =

\[
\frac{T - C \times \text{Conc of Std.} \times \text{Vol. of Std.}}{S - B \times \text{Vol. of Test}} \times 100
\]

\[
\frac{T - C}{S - B} \times 0.01 \times 1.0 \times 100
\]

\[
\frac{T - C}{S - B} \times 10 \text{ KA Units.}
\]

1 KA unit is the amount of enzyme which sets free 1 mg of phenol in 15 minutes at pH10. Conversion Factor is 7.1 to convert into International Units.

**Normal Range**: 3 - 13 KA Units or 30 – 305 IU/L

**iii and iv. Estimation of Serum AST and ALT:**

**Method** - Reitman and Frankel\(^{(101,102)}\)

**Introduction**:- Transamination is reversible reaction in which \(\alpha\)-amino group of one amino acid is transferred to \(\alpha\)-keto acid resulting in formation of new keto and amino acid. Enzymes concerned with transamination are transminases or amino-transferases. In serum two types of transaminases are present -

1] Serum Glutamate oxaloacetate transaminase SGOT or AST i.e. Aspartate transaminase and

2] Serum Glutamate pyruvate transaminase SGPT or ALT i.e. Alanine transaminase.
Aspartate transaminase (AST) - catalyses the following reaction.

\[
\text{L-aspartate + } \alpha\text{-Ketoglutarate} \xrightleftharpoons{\text{B6}} \text{Oxaloacetate + Glutamate} \quad \text{AST}
\]

Alanine transaminase (ALT) - catalyses the following reaction -

\[
\text{L-alanine + } \alpha\text{-Ketoglutarate} \xrightleftharpoons{\text{B6}} \text{Pyruvate + Glutamate} \quad \text{AST}
\]

iii. **Estimation of SGOT (AST) Serum Aspartate transaminase**

**Reagents:**

1. SGOT/ SGPT substrate
2. Phosphate Buffer [pH 7.4]
3. 2, 4 - Dinitrophenyl hydrazine [2, 4 - DNPH]
4. 0.4 N Sodium hydroxide (NaOH)
5. Standard for SGOT and SGPT - Sodium pyruvate
Procedure:

Take 4 test tubes labelled ‘T’, ‘C’, ‘S’ and ‘B’ and add reagents as per the table below.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>01</td>
<td>AST Substrate</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>02</td>
<td>Phosphate buffer</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>03</td>
<td>Serum</td>
<td>--</td>
</tr>
<tr>
<td>04</td>
<td>Standard Pyruvate</td>
<td>--</td>
</tr>
<tr>
<td>05</td>
<td>D/W</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

Incubate at 37°C for 60 minutes

|        |                         |        |        |        |        |
| 06     | Serum                   | --     | --     | 0.1 ml | --     |
| 07     | 2, 4 – DNPH             | 1.0 ml | 1.0 ml | 1.0 ml | 1.0 ml |

Incubate at room temperature for 20 minutes

| 08     | 0.4 N NaOH              | 5.0 ml | 5.0 ml | 5.0 ml | 5.0 ml |

Take readings (optical density) on green filter.
Calculations:

Concentration of AST
\[
\frac{\text{Conc of Std.}}{\text{Vol. of Std.}} \times \frac{\text{Vol. of Test}}{\text{Time}} = \frac{T - C}{S - B} \times 4.0 \times 0.1 \times 1000
\]
\[
\frac{S - B}{0.1 \times 60}
\]
\[
= \frac{T - C}{S - B} \times 66.67 \, \mu\text{mol} / \text{min} / \text{ltr}
\]
\[
= \frac{T - C}{S - B} \times 66.67 \, \text{IU} / \text{L}
\]

Normal range: 2 - 20 IU/L

Unit transaminase activity is International Unit per litre (IU/L). One IU is the number of micromoles of respective ketoacid formed per minute per litre serum.

iv. Estimation of SGPT (ALT)/Serum Alanine transaminase

Principle:- ALT present in serum acts on Alanine, when incubated at 37°C in buffer (pH 7.4) to form Pyruvate which reacts with 2,4 Dinitro-phenyl hydrazine [2, 4 DNPH] in alkaline medium to give brown coloured complex. Intensity of the colour developed is directly proportional to serum ALT activity.

Reagents:- As above.
**Procedure:**

Take 4 test tubes labelled ‘T’, ‘C’, ‘S’ and ‘B’ and add reagents as per the table below.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>01</td>
<td>ALT Substrate</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>02</td>
<td>Phosphate buffer</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>03</td>
<td>Serum</td>
<td>--</td>
</tr>
<tr>
<td>04</td>
<td>Standard Pyruvate</td>
<td>--</td>
</tr>
<tr>
<td>05</td>
<td>D/W</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

Incubate at 37°C for 30 minutes

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>06</td>
<td>Serum</td>
<td>--</td>
</tr>
<tr>
<td>07</td>
<td>2,4 – DNPH</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Incubate at room temperature for 20 minutes

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4 N NaOH</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

Take readings optical density on green filter.

**Calculations**: -

Concentration of ALT

\[
\text{Concentration of ALT} = \frac{T - C \times \text{Conc of Std.} \times \text{Vol. of Std.} \times 1000}{S - B \times \text{Vol. of Test} \times \text{Time}}
\]
= \frac{T - C}{S - B} \times 4.0 \times 0.1 \times 1000

= \frac{T - C}{S - B} \times 133.3 \text{ µmol} / \text{min} / \text{ltr}

= \frac{T - C}{S - B} \times 133.3 \text{ IU} / \text{L}

**Normal range:** 3 - 17 IU/L

Unit for Transaminase activity is International Unit per liter [IU/L]

One IU is the number of micromoles of respective Ketoacid formed per minute per litre serum.

**v. Estimation of Total Serum Proteins:**

**Method** - Biuret Method\(^{(103)}\)

**Principle:** In the alkaline medium, copper ions form co-ordinate linkages with peptide bonds of nitrogen of protein to give a violet coloured complex compound. The intensity of this colour is directly proportional to the concentration of protein in the serum. The colour is compared with the Standard (Bovine albumin) treated similarly.

**Reagents:**

1. Biuret Reagent
2. Protein standard solution (concentration: 0.005 g/ml)
Procedure:

Three test tubes taken were marked as ‘B’ (Blank), ‘S’ (Standard) and ‘T’ (Test). Further additions were made as shown in the table below.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B</td>
</tr>
<tr>
<td>01</td>
<td>D/W</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>02</td>
<td>Standard protein solution</td>
<td>--</td>
</tr>
<tr>
<td>03</td>
<td>Serum</td>
<td>--</td>
</tr>
<tr>
<td>04</td>
<td>Biuret Reagent</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

These tubes were kept at 37°C in incubator for 15 minutes. Then readings (optical density) was taken at 520nm on calorimeter.

Calculations:

Concentration of total serum proteins

\[
\text{Concentration} = \frac{T - B}{S - B} \times \frac{\text{Conc. of Std.} \times \text{Vol. of Std.}}{\text{Vol. of Test}} \times 100
\]

\[
= \frac{T - B}{S - B} \times \frac{0.005 \times 3.0}{0.2} \times 100
\]

\[
= \frac{T - B}{S - B} \times 7.5
\]

Normal Range: 6 - 8 g% (mg/dl)
vi. Estimation of Serum Albumin:

Method: - Bromo Cresol Green Method (BCG Method) \(^{(104)}\)

Principle: - Albumin binds with Bromocresol green dye (BCG) at pH 3.8 to give an emerald green coloured complex which is compared with standard treated similarly and read on red filter i.e. 660 nm.

Reagents:

1. Bromocresol Green dye (BCG dye)
2. Albumin Standard (concentration 0.005 g/ml)

Procedure:

Three test tubes taken were labeled as ‘B’, ‘S’ and ‘T’. Additions to these tubes were made as per the table followed.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>S</td>
</tr>
<tr>
<td>01</td>
<td>BCG dye</td>
<td>4.0 ml</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>02</td>
<td>D/W</td>
<td>0.2 ml</td>
<td>--</td>
</tr>
<tr>
<td>03</td>
<td>Standard Albumin</td>
<td>--</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>04</td>
<td>Serum</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

The readings of the blank (B), standard (S) and test (T) were taken on calorimeter against d/w using red filter (660 nm). Calculated results were obtained in gm% (mg/dl).
Calculations:

Concentration of albumin in given serum =

\[ \frac{T - B \times \text{Conc. of Std.} \times \text{Vol. of Std.}}{S - B \times \text{Vol. of Test}} \times 100 \]

\[ = \frac{T - B \times 0.005 \times 0.2 \times 100}{S - B \times 0.02} \]

\[ = \frac{T - B}{S - B} \times 5\text{g%}. \]

Normal Range: 3.5 to 5.0 gm% (mg/dl).

3) Oxidative Stress Indicators:

i. Lipid Peroxidation: Malondialdehyde (MDA) or Thiobarbituric Acid Reactive Substances (TBARS)

Estimation of Lipid Peroxidation: (MDA) or (TBARS):

Method: Satho, 1978\(^{(105)}\)

Principle:- Serum containing lipid peroxide is treated with thiobarbituric acid in presence of 20% trichloroacetic acid. After heating in boiling water bath for 15-20 minutes the resulting chromogen is extracted with n-butyl alcohol and measured at 530nm on calorimeter. Malondialdehyde (MDA) is used as standard. The lipid peroxide is expressed in terms of nmoles per ml.
Reagents:

1. 20% Trichloroacetic acid in d/w
2. 0.05 M Sulphuric Acid
3. 0.2% Thiobarbituric acid reagent
4. n-Butyl alcohol
5. 2 M Sodium Sulphate Solution
6. Std-Solution of Malondialdehyde [1,1,3,3 Tetramethoxy Propane]

Procedure:

0.5 ml serum was taken in a centrifuge tube and 2.5ml 20% Trichloroacetic Acid (TCA) was added. The tube was kept for 10 minutes at room temperature and then centrifuged at 3500 rpm for 10 minutes. Supernatant was discarded and precipitate was washed with 0.05 M Sulphuric Acid. Then the following additions were made.

i. 0.05 MH$_2$SO$_4$..................................................2.5ml.

ii. 0.2% Thiobarbituric acid reagent......................3.0ml.

Above contents were mixed and heated in a boiling water bath (BWB) for 15-20 minutes. Then it was kept in cold water and 4 ml n-Butyl alcohol was added and mixed vigorously to extract the chromogen.

Next it was centrifuged at 3000 rpm for 10 minutes. The absorbance of organic phase was determined at 530nm. From standard curve, test values were calculated. From standard curve, values were calculated. For standard curve graph different
dilutions were prepared from 1,1,3,3 teramethoxypropane and readings were obtained using above procedure and graph was plotted of concentrations in nmoles against optical density. Working Standard solution used was having concentration of 10 nmoles per ml.

**Procedure for Standardization:**

Take 7 test tubes and level as S1, S2, S3, S4, S5, S6, and B for standards and blank respectively. to prepare various standard concentrations, make additions as follows:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Std. No.</th>
<th>Std. (10 nmol) ml</th>
<th>D/w Ml</th>
<th>total ml</th>
<th>O.D.</th>
<th>Conc. of Std. nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S1,</td>
<td>0.5</td>
<td>2.5</td>
<td>3.0</td>
<td>0.04</td>
<td>1.67</td>
</tr>
<tr>
<td>2</td>
<td>S2,</td>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>0.07</td>
<td>3.34</td>
</tr>
<tr>
<td>3</td>
<td>S3,</td>
<td>1.5</td>
<td>1.5</td>
<td>3.0</td>
<td>0.09</td>
<td>5.01</td>
</tr>
<tr>
<td>4</td>
<td>S4,</td>
<td>2.0</td>
<td>1.0</td>
<td>3.0</td>
<td>0.12</td>
<td>6.68</td>
</tr>
<tr>
<td>5</td>
<td>S5,</td>
<td>2.5</td>
<td>0.5</td>
<td>3.0</td>
<td>0.13</td>
<td>8.35</td>
</tr>
<tr>
<td>6</td>
<td>S6,</td>
<td>3.0</td>
<td>-</td>
<td>3.0</td>
<td>0.14</td>
<td>10.00</td>
</tr>
<tr>
<td>7</td>
<td>B</td>
<td>-</td>
<td>3.0</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Then to each tube 2.5 ml of 0.05M Sulphuric acid was added. Then three ml Thiobarbituric acid was added, mixed and heated in boiling water bath for 15-20 minutes Then kept in cold water and 4ml n-butyl alcohol was added and mixed.
vigorously to extract the chromogen and its absorbance at 530 nm was determined.

The graph of absorbance against Malondialdehyde (MDA) standard concentration was plotted.

ii. **Estimation of Sensitivity of Erythrocytes to Peroxide Haemolysis (SEPH):**

Standardization of the method for determination of the sensitivity of erythrocytes to peroxide hemolysis.\(^{(106)}\)

**Reagents:**

1. Phosphate buffer : 1M, pH 7.4 (Prepared as per reference)
2. Stock Sodium chloride solution : 170 g/l in double distilled water.
3. Working Sodium chloride solution : 25 ml of phosphate buffer mixed with 25 ml of stock sodium chloride solution, mixture diluted upto 100 ml by d/w. Prior to use, the solution is shaken vigorously (to be saturated with atmospheric oxygen).

**Procedure:**

1. 0.1 ml of whole blood was added to 7.5 ml of working sodium chloride solution to prepare the erythrocyte suspension. The mixture is mixed on vortex to allow complete haemolysis.
2. The suspension is centrifuged at 3000 rpm for 10 minutes. The supernatant is drawn off and erythrocytes precipitated and the earlier procedure is repeated.
3. 1 ml of suspension is transferred to each of the three tubes labeled 1, 2 and 3.
4. 4 ml of working Nacl is added to tube ‘1’ and ‘2’ and 4 ml of d/w to tube ‘3’ (to achieve complete haemolysis).
5. The contents were stirred and incubated at 370C for two hours.
6. At the end of incubation period, the contents were mixed and centrifuged at 3000 rpm for 10 minutes.
7. The supernatant was decanted into 3 tubes and the absorbance measured on calorimeter against a d/w at 530 nm.

Calculations:

The following equation is used to determine the percentage of erythrocyte sensitivity to peroxide hemolysis.

\[ X = \frac{[E1 + E2] \times 100}{2} \times E3 \]

\[ = [E1 + E2] \times 50 \times E3 \]

Where, \( X \) = degree of hemolysis in %

\( E1 \) = Absorbance of tube ‘1’

\( E2 \) = Absorbance of tube ‘2’

\( E3 \) = Absorbance of tube ‘3’

The results are expressed as percentage of the erythrocytes in the given sample, which are sensitive to peroxide hemolysis.
4. Serum Antioxidants:

i. Estimation of Vitamin “A”.

Method: Singh R A et al \(^{(107)}\)

Reagents:

1. Toluene
2. Acid Mixture
   Concentrated \(H_2SO_4 : CH_3COOH\)
   \[1 : 4\]
3. Standard - Vitamin ‘A’ Acetate

Procedure:

- Take 0.2 ml serum
- Add 0.8 ml d/w to it.
- To this above mixture add 2.0 ml of toluene.
- Shake vigorously and add to it 7.0 ml of acid mixture.
- Wait for 30 mins.
- Pink colour supernatant is seen.
- Take optical density of this supernatant on green filter of calorimeter.

Calculations:

Conversion factor of Vitamin “A” is 0.50 OD = 50 mg Vitamin A.

Normal Range: Conventional Unit: 20 - 80 mg/dl.
ii. **Estimation of Reduced Glutathione in blood:**

**Method:** Satoh K. (1978) ; Patterson and Lazarow.\(^{(108,109)}\)

**Reagents:**

1. Metaphosphoric acid - Stock 25% solution.
2. Metaphosphoric acid (working) - 5% solution.
   
   Preparation: Stock: d/w.
   
   
   \[
   \text{1 : 5}
   \]

3. Alloxan solution (0.1M) - 1.5 g%.
4. Equivalent Sodium hydroxide (NaOH)
5. Phosphate Buffer (0.5 M, pH 7.5)
6. 1N Sodium hydroxide
7. Glutathione Standard - 50 mg /ml.

**Normal value:** 28 - 34 mg%.(mg/dl).
It has been reported that the red blood cell Glutathione (GSH) levels are inversely related to the haematocrit levels.

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>Reagents</th>
<th>Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Oxalated blood</td>
<td>-- -- -- 1 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+2 ml 25% Metaphosphoric acid</td>
</tr>
<tr>
<td>02</td>
<td>d/w</td>
<td>-- -- -- 7 ml</td>
</tr>
<tr>
<td>03</td>
<td>Filtrate of above</td>
<td>-- -- -- 1 ml 1 ml</td>
</tr>
<tr>
<td>04</td>
<td>5% Metaphosphoric acid</td>
<td>1 ml 1 ml -- -- --</td>
</tr>
<tr>
<td>05</td>
<td>Glutathione Standard</td>
<td>-- -- 1 ml 1 ml --</td>
</tr>
<tr>
<td>06</td>
<td>0.1 M Alloxan</td>
<td>1 ml -- 1 ml -- 1 ml</td>
</tr>
<tr>
<td>07</td>
<td>Distilledwater (d/w)</td>
<td>1 ml 1 ml 1 ml 1 ml</td>
</tr>
<tr>
<td>08</td>
<td>0.5 M Phosphate buffer</td>
<td>1 ml 1 ml 1 ml 1 ml</td>
</tr>
<tr>
<td>09</td>
<td>*Immediately add equivalent NaOH</td>
<td>1 ml 1 ml 1 ml 1 ml</td>
</tr>
<tr>
<td>10</td>
<td>After 6 mins add 1N NaOH</td>
<td>1 ml 1 ml 1 ml 1 ml</td>
</tr>
</tbody>
</table>

Take readings at 305 nm on Spectrophotometer.

**Normal Range:** 28 - 34 mg% (mg/dl).
Calculations:
Concentration of Reduced Glutathione = \frac{A_x-A_b \times 0.05 \times 100}{A_s - A_b} = A_x - A_b \times 50 \text{ mg%}

Statistical Analysis:

Data obtained from the present study has been statistically analysed. Mean and standard deviations were calculated. Students t’ values were calculated to draw the probabilities to find out the significance and non-significance of each parameter. Statistical comparison was made between test subjects and healthy control subjects. The values given in the tables and the figures are mean + SD. The probability value of P < 0.05 was taken to be significant while probability P < 0.01 was termed as highly significant.