MATERIAL AND METHODS

The study was carried out in G.R. Medical College, Gwalior and Chhattisgarh Institute of Medical Sciences, Bilaspur (C.G.). Since the Eastern region of central India is prone to Sickle Cell Anemia. Hence samples preferably collected from this area.

The study was conducted on 300 human subjects. out of which 100 were suffering from Sickle cell disease, 100 were Sickle Cell Trait and 100 were taken as control group (Healthy age matched, having no blood disorder)

Inclusion Criteria: we have selected those SCD patients who were not taking medicine for at least a month before taking blood sample i.e. free from acute illness

Age > 10 years All the subjects/Patients of Sickle cell Anemia

Exclusion Criteria: we have excluded SCD patients having history of recent blood transfusion, on medication severe illness and were suffering from other diseases like HTN, Diabetes, CVD, Thelasmia, HIV etc.

The family history, clinical manifestations pertaining to disease was collected in the study Performa. The written consent were also obtained before starting the study. The study was approved by the Institutional Ethical committee of G.R. Medical College, Gwalior for Ph D degree.
Sample collection:
A Blood sample for the estimation of all the parameters was collected from the subjects after overnight fasting. Eight ml (08 ml) Fasting blood samples were freshly withdrawn from the anticubital vein and collected. One part is distributed into anticoagulant (EDTA) tube and other part was kept for clotting from both patients and volunteer and immediately transferred to laboratory. Each sample was centrifuged at 3000 rpm and the serum was kept at -40°C for further estimation but hemoglobin indices were taken to measure immediately.

Parameters: Hematological: Hemoglobin Indices, DLC, Solubility test
Hemoglobin Electrophoresis (Alkaline)

Endogenous Antioxidants
Superoxide Dismutase, Glutathione Peroxidase, Glutathione Reductase, Catalase
Glutathione.

Exogenous Antioxidants
Vitamin A, Vitamin E, Vitamin C

Antioxidants activity
Total antioxidant capacity

Oxidative stress marker
Plasma Malondialdehyde (MDA)

Inflammatory marker: C - reactive protein (CRP)
Separation of serum:

Blood sample was collected in plain vial and incubated at 37°C for 30 minutes. After incubation, clot was removed and remaining sample was taken in centrifuge test tube. Samples were centrifuged at 3000 rpm for 10 to 20 minutes. Supernatant collected in clean and dry serum test tube for analysis.

PREPARATION OF HAEMOLYSATE (Donal and Ronrlod -1959):

The blood samples collected in anticoagulant vial centrifuged at 3000 rpm for 15 minutes. Plasma was removed from Packed Cell Volume (PCV). Packed Cell Volume was washed 3 times with normal saline. Cell were lysed by adding 1ml of D.W. Mixture was refrigerated for 10 minutes and then vigorously shaken in vertex for 2 minute, then 0.5ml chloroform was added as a preservative. Mixture was centrifuged at 3000 rpm for 20 minutes. The whole mixture was clearly separated into three layers, lower most layers was chloroform, middle layer was cell stroma (mucous) and upper most layer was clear haemolysate solution. This haemolysate solution was pipetted out and collected in microcentrifuge tube, which was finally utilized for the estimation.

Biochemical Investigations:

Biochemical parameters, like Albumin, Uric acid, Bilirubin, CRP were estimated by fully automated analyser in a commercially available kit (Transasia EM 360) according to manufacturer instructions.
Antioxidants profile by manual method using double beam spectrophotometer Systronic 2202.

**Haematological Investigations:**

Hematological parameter, hemoglobin indices were estimated by Hematological using hematology analyzer make Orphee, France and solubility test by commercially available kit (HIMEDIA). Hemoglobin Electrophoresis (Alkaline) by GENIO S, The assay was performed exactly as recommended by the manufacturer.

**Instrument used:**

**DOUBLE BEAM SPECTROPHOTOMETER SYSTRONIC 2202 –**
Material & Methods

HAEMATOLOGY ANALYSER MAKE ORPHEE, FRANCE -

GENIO FULLY AUTOMATED SYSTEM:
Haematological Investigations:

Hematological parameter, hemoglobin indices were estimated by Hematological using hematology analyzer make Orphee, France.

**Hb Estimation:**

It is measured directly done in the WBC chamber by spectrophotometry at 555 nm Hb is dictated by formation of a chromogen cyanomethemoglobin type for lytic solution with cyanide and oxyhemoglobin for lytic solution without cyanide. A measurement of the blank of HB is done for each analytic cycle and driving up rising step. An automatic circuit for the LED 555nm allow to maintain the blank level at the same range .it is not necessary to adjust this range with potentiometer.

**Erythrocyte analysis:** The erythrocyte analysis is done by impedance metery in the RBC counting chamber

- RBC Directly
- HGB Directly
- HCT Directly
- MCV Calculation

Hematocrit is measured by integration volume the cell of the red blood cell which flow in the RBC counting chamber aperture
Leukocyte analysis:

The leukocyte analysis is done by impedancemetry in the WBC counting chamber.

Granulocyte

Lymphocyte

Monocyte

Differential leukocyte count:

Blood smear is stained with Leishman's stain (Methylene blue and Eosin) and different leukocytes are identified and counted under microscope.

SOLUBILITY TEST

Principle:
This test is based on the solubility difference between HbS and HbA in solubility test reagent. When red cells are introduced into such a solution, they lyse immediately. The hemoglobin released from the lysed red cells is reduced by reagent. This reaction causes precipitation of HbS leading to turbidity of the reaction mixture. However, hb A as well as other haemoglobins are soluble leading to clarity in the reaction mixture.

**Storage:**

Store the reagent mix in a cool and dry place. Avoid direct exposure to sunlight. The reagents in the solubility kit have a shelf life one year.

**Procedure:**

1. Add molecular grade water to the tube contain pre weighed reagent mix. Dispense the solubility test reagent with the help of a dropper in 5 reaction tubes till the water level in the reaction tubes reaches the uppermost black line marked on the test tube stand

2. Gently add 1 drop of freshly collected whole blood sample to each reaction tube with the help of a dropper

3. Gently mix the tubes for 10-15 seconds

4. Allow the tubes to stand for 10 minutes at room temp.

5. Place the tube in the test tube stand and read for turbidity.

**Interpretation:**
Material & Methods

**Negative** – if solution is clear and black lines visible

**Positive** – if solution is turbid and black lines not visible

<table>
<thead>
<tr>
<th>s.no.</th>
<th>Turbidity</th>
<th>Clarity</th>
<th>Visibility of black lines through the tubes</th>
<th>interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
<td>Normal</td>
</tr>
<tr>
<td>II</td>
<td>YES</td>
<td>NO</td>
<td>NO</td>
<td>Sickle cell</td>
</tr>
</tbody>
</table>

**HB ELECTROPHORESIS**

**BY GENIO FULLY AUTOMATED SYSTEM:**

**Principle:**
All Hemoglobin molecules have a negative charge, and migrate towards the anode proportional to their net negative charge. Amino acid substitutions in hemoglobin variants alter net charge and mobility.
1- Sickle cell disease (Hb SS)
2- Normal (HbAA)
3- Sickle cell Trait (Hb AS)
4- Sickle cell Trait (Hb AS)
TOTAL ANTIOXIDANT CAPACITY
BY D. KORACEVIC ET AL (2001):
PRINCIPLE:

A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by Fenton type reaction, leading to the formation of hydroxyl radicals (OH’). These ROS degrade benzoate, resulting in the release of TBARS. Antioxidants from the added sample of human fluid cause suppression of production of TBARS. This reaction can be measured spectrophotometrically at 532 nm and the inhibition of colour development defined as the AOA.

REAGENTS:

Sodium phosphate buffer: 100 mmol/L : pH 7.4
Sodium benzoate : 10 mmol/L
NaOH : 50 mmol/L
EDTA (acidum aethylendiamin tetraacetic ): 2 mmol/litre (solution 1)
Fe (NH₄)SO₄ : 2 mmol/L
Fe-EDTA complex (prepared freshly by mixing equal volume of solution 4 and 5 left to stand 60 minutes at room temperature).
H₂O₂: 10 mmol/litre
Acetic acid: 20 %
Thiobarbituric acid (TBA): 0.8 % (wt/vol) in 50 mmol/litre NaOH
Uric acid : 1 mmol/L in 5 mmol/l NaOH
Solution 4-9 should be prepared immediately before use. The solution phosphate buffer and sodium benzoate should be kept in refrigerator (0-4°C) and the uric acid solution in deep freeze.

**PROCEDURE:**

Each sample ($A_1$) should have its own control ($A_0$, sample blank) in which the Fe-EDTA mixture and $H_2O_2$ should be added after 20% acetic acid for each series of analysis of negative control ($K_1$ and $K_0$) should be prepared (at least triplicate), containing the same reagents as $A_1$ or $A_0$, except the plasma is replaced with phosphate buffer. Standard containing 1 mmol/L uric acid (Uric acid $U_{A1}$ and $U_{A0}$) is used for calibration.

<table>
<thead>
<tr>
<th>Addition Sequence</th>
<th>$A_1$</th>
<th>$A_0$</th>
<th>$K_1$</th>
<th>$K_0$</th>
<th>$U_{A1}$</th>
<th>$U_{A0}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>0.01</td>
<td>0.01</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Uric acid</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0.01</td>
<td>0.01ml</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.49</td>
<td>0.49</td>
<td>0.50</td>
<td>0.50</td>
<td>0.49</td>
<td>0.49</td>
</tr>
<tr>
<td>Na benzoate</td>
<td>0.50 ml</td>
<td>0.50 ml</td>
<td>0.50 ml</td>
<td>0.50 ml</td>
<td>0.50 ml</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>--</td>
<td>1.0</td>
<td>--</td>
<td>1.0</td>
<td>--</td>
<td>1.0</td>
</tr>
<tr>
<td>Fe- EDTA</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Incubate for 60 minutes at 37 ºC then add

<table>
<thead>
<tr>
<th>Addition</th>
<th>$A_1$</th>
<th>$A_0$</th>
<th>$K_1$</th>
<th>$K_0$</th>
<th>$U_{A1}$</th>
<th>$U_{A0}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>1.00</td>
<td>---</td>
<td>1.0</td>
<td>---</td>
<td>1.0</td>
<td>---</td>
</tr>
<tr>
<td>TBA</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Incubate for 10 minutes at 100 °C (in boiling water bath) then cooled in an ice bath, measure absorbance at 532 nm against deionised water.

**CALCULATION:**

Antioxidants activity should be calculated as follows

\[
\text{AOA (mmol)} = \frac{(C_{UA}) \times (K - A)}{K - UA}
\]

Where,

\[K = \text{absorbance of control} (K_1 - K_0)\]
\[A = \text{absorbance of sample} (A_1 - A_0)\]
\[UA = \text{absorbance of uric acid solution} (UA_1 - UA_0)\]
\[C_{UA} = \text{concentration of uric acid} \text{ (in mmol/litre)}\]

**Normal Range:**  1.2 – 3 mmol/l
PLASMA MALONDIALDEHYDE (P-MDA)
BY JEAN C.D. ET AL (1983):

REAGENTS:

Sodium Hydroxide Solution (M.W. 40) in DW: 1 N

\[
\text{N} = \frac{\text{Weight of Solute}}{\text{Atomic No.}} \times \frac{\text{Valence}}{\text{Volume of Solution}}
\]

\[
\text{N} = \frac{40}{20} \times \frac{1}{1000}
\]

= 20 gm/1000ml

= 2 gm/100ml

Perchloric acid liquid PCA: 7%

Thiobarbituric acid solution (TBA) pH 7.4 : 0.8 % (stock solution):

Thiobarbituric acid : 800 mg

NaOH : 100 ml

Mix well vigorously then neutralized with 7 % perchloric acid until pH become up to 7.4

Thiobarbituric acid solution (TBA) : (working solution)

It should be used freshly prepared – by mixing 2 volume of TBA (stock solution) with 1 volume of 7% perchloric acid.

n- Butanol: Supplied by qualigens , galxo india pvt.ltd

NOTE: TBA working solution should be used freshly prepared.
PROCEDURE:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>Blank</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Perchloric acid (7%)</td>
<td>.5 ml</td>
<td>.5 ml</td>
</tr>
<tr>
<td>Mix well</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1 ml</td>
<td>---</td>
</tr>
<tr>
<td>Plasma</td>
<td>---</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Mix well and heat the mixture in a boiling water bath for 30 mins. Then cool it

| n-butanol             | 3 ml      | 3 ml     |

Mix by shaking and centrifuge at 3000 rpm for 15 mins

Take supernatant and read absorbance of the test against blank at 531 nm by using spectrophotometer. Colour is stable for at least 7 hours after cooling.

CALCULATION:

\[
\text{Conc. Of Plasma MDA} = \frac{V \times \text{OD of test}}{\text{OD of Std.}}
\]

Where; \( V \) is dilution factor 5.5 (Total volume used in test sample analysis)

Reading of standard is 0.152.

\[
\begin{align*}
\text{Conc. Of Plasma MDA} &= \frac{5.5 \times \text{OD of test}}{0.152} \\
&= 36.18 \times \text{OD of sample}
\end{align*}
\]

**Normal Range:** 2 – 4 nmol/ml or 2 – 4 µmol/L
SUPER OXIDE DISMUTASE (SOD)
BY MARKLUND AND MARKLUND (1974):

PRINCIPLE:

Superoxide anion is involved in auto oxidation of pyrogallol at alkaline pH (8.5). The SOD inhibits auto oxidation of pyrogallol, which can be determined as an increase in absorbance per two minutes at 420 nm on a spectrophotometer.

REAGENTS:

Tris buffer (50 mM of tris and 1 mM of EDTA)

500 ml of tris – EDTA buffer was prepared by taking 3.0275 gms of tris buffer (50 mM) + 0.186 gms of EDTA (1 mM) and to this 50 mM HCL was added to adjust the pH 8.5 and volume was made upto 500 ml.

Pyrogallol (20 mM) – 25 mgs of pyrogallol was dissolved in 10 ml of deionised water.

(note: dilute pyrogallol solution, which shows changes in absorbance A 0.02 to 0.023 in min)

PROCEDURE:

Preparation of haemolysate –

Heparinised blood was centrifuged and plasma was removed then RBCs were washed by 0.9% NaCl for 2 to 3 times. The washed NaCl was removed completely. 10 volume of cold DW was added to 1 volume of RBCs and mixed the content and then whole mixture was centrifuged for 10 mins at
3000 rpm. The cell debris was removed and the clear haemolysate was collected (concentrated hemolysate). The concentrated haemolysate was diluted to 1:10 with 0.9% and used for the assay

**ASSAY PROCEDURE:**

Two test tube were taken and labeled as test T and control C. Addition of reagents / solution in ml, were made as per the following protocol

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer</td>
<td>2.85</td>
<td>2.9</td>
</tr>
<tr>
<td>Hemolysate</td>
<td>0.05</td>
<td>--</td>
</tr>
<tr>
<td>Pyragallol</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The content of the tubes was mixed and absorbance was taken at 420 nm exactly after 1 minutes 30 seconds and 3 minutes 30 seconds. The absorbance per two absorbance per two minutes was recorded.

**CALCULATION:**

SOD units /ml of hemolysate = \( \frac{C-T}{C} \times 50 \times 100 \times \frac{1}{0.05} \)

\[ = \frac{C-T}{C} \times 50 \times 2000 \]

Then result were expressed as SOD units / gms Hb

Definition of Units: One units of SOD is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation.

Normal Range: 4.2 – 7.6 U/g of Hb
CATALASE (CAT)


Catalase (E.C. 1.11.1.6) is a tetrameric enzyme consisting of four identical tetrahedrally arranged subunits of 60 kda that contains single ferriprotoporphyrin group per subunit and has a molecular mass of about 240 kda.

\[ 2\text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{O}_2 \]

Also oxidation of H donors e.g. Methanol, ethanol, formic acid or phenols.

\[ \text{ROOH} + \text{AH}_2 \rightarrow \text{Catalase} \rightarrow \text{H}_2\text{O} + \text{ROH} + \text{A} \]

PRINCIPLE:

Catalase decomposes the \( \text{H}_2\text{O}_2 \) and form water and molecular oxygen in the ultraviolet range \( \text{H}_2\text{O}_2 \) shows a continual increase in the absorption with decreasing wavelength at 420 nm \( \text{H}_2\text{O}_2 \) absorb maximum light. When \( \text{H}_2\text{O}_2 \) was decomposed by Catalase then the absorbance was decreased. The decreased absorbance was measured at 240 nm for every 15 seconds interval up to 1 min and the difference in absorbance per unit time is a measure of the Catalase activity.
REAGENTS:

Phosphate buffer (60 mM) pH 7.4 – The 4.0827 gms of KH₂PO₄ was dissolved in 500 ml DW (solution A). Then the 10.68 gms of Na₂HPO₄. 2H₂O was dissolved in 1 lit (solution B). These solution A and B were taken in the proportion 1:1.8 respectively and pH was adjust to 7.4

Hydrogen peroxide (30mM) – The 0.34 ml of 30% H₂O₂ was diluted to 100 ml with phosphate buffer.

PROCEDURE:

The Haemolysate preparation procedure was as per the SOD. Stock Haemolysate prepared (5 gm Hb/100 ml) by the addition of DW. A 1: 500 dilution of this concentrated Haemolysate was prepared with phosphate buffer prior to the assay.

Two test tube were taken and labeled as Teat (T) and blank (B). Addition of reagents / solution was made as per the following protocol.

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
<tr>
<td>Haemolysate</td>
<td>10 µl</td>
<td>--</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

After addition of H₂O₂ the solution was mixed well and the initial absorbance was measure immediately at 420 nm then the decrease in
absorbance was measured at a 15 sec. interval up to 1 min on UV-VIS spectrophotometer.

The unit of Catalase activity was expressed as mM of H$_2$O$_2$ decomposition / mg Hb/ min.

**CALCULATION:**

\[
\text{Catalase activity} = \frac{\Delta A/\text{min}/0.07 \times 3}{0.01\text{ml}}
\]

\[
= \frac{\Delta A/\text{min}/0.07}{300}
\]

Where $\Delta A =$ difference in the absorbance reading of initial time 7 at the end of 1 min.

0.071 = molar extinction coefficient of H$_2$O$_2$

Unit: U/gm Hb.

Normal Range: 5.1 – 8.7 U/g of Hb
GLUTATHIONE PEROXIDASE (GPx)
BY HALFMASN AND D.G. (1974):

REAGENTS: 5

DTNB (5-5' di thiobis -2- nitro benzoic acid) (C_{14}H_{8}N_{2}O_{8}S_{2}) reagents:

DTNB (0.040gm% w/v)

Sodium citrate (C_{6}H_{5}Na_{2}O_{7}.2H_{2}O) 01gm% w/v

Distilled water

Storage: at 2 to 8 °C for 13 weeks

Sodium phosphate buffer (0.4 M, pH 7) in distilled water:

Sodium phosphate monobasic di hydrated (NaH_{2}PO_{4}.2H_{2}O: 0.4 M, pH 07 MW 156.01)

01 M = 156.01 g/1000 ml

04 M = 624.04 g/1000 ml

0.4 M = 62.404 g/1000 ml

0.4 M = 15.6 g/250 ml

Sodium phosphate di basic anhydrous (Na_{2}HPO_{4}: pH 07 , MW 141.96)

01 M = 141.96 g/1000 ml

04 M = 567.8 g/1000 ml

0.4 M = 56.78 g/1000 ml

0.4 M = 14.20 g/250 ml

Sodium azaide (NaN_{3} : 0.01 M , MW 65) in DW

01M = 65 gm/1000ml
Material & Methods

\[ M = 6.5 \text{ gm/1000 ml} \]

\[ 0.01 \text{ M} = 0.65 \text{ gm/1000 ml} \]

\[ 0.01 \text{ M} = 0.065 \text{ gm/100 ml} \]

Hydrogen peroxide (\( \text{H}_2\text{O}_2 \): 1.25 mM MW 34) in DW

\[ M = 34 \text{ gm/1000 ml} \]

\[ 0.01 \text{ mM} = 0.001 \text{ M} \]

\[ 0.001 \text{M} = 34 \times 0.001 \text{ gm/1000} \]

\[ 0.1 \text{ mM} = 0.034 \text{ gm/1000 ml} \]

\[ 0.125 \text{ mM} = 0.034 \times 0.125 \text{ gm/1000 ml} \]

\[ = 0.0425 \text{ gm/1000 ml} \]

\[ = 0.00425 \text{ gm/100 ml} \]

GSH (glutathione) (\( \text{C}_{10}\text{H}_{17}\text{N}_3\text{O}_6\text{S} \): 2 mM, MW 307.32) in DW

\[ 0.01 \text{ M} = 307.32 \text{ gm/1000 ml} \]

\[ 0.01 \text{ mM} = 0.001 \text{ M} \]

\[ 0.01 \text{ mM} = 307.32 \times 0.001 \text{ gm/1000 ml} \]

\[ 0.1 \text{ mM} = 0.30732 \text{ gm/1000 ml} \]

\[ \text{mM} = 3.0732 \times 2 \text{ gm/1000 ml} \]

\[ = 0.61464 \text{ gm/1000 ml} \]

\[ = 0.0615 \text{ gm/100 ml} \]

Meta – phosphoric acid (precipitating agent)

meta- phosphoric acid (A mixture of HPO\(_3\) and NaPO\(_3\)) 1.63 gm
Di sodium or di potassium (EDTA) 0.2 gm
Sodium Chloride 30 gm
Distilled water 100 ml

Storage: this solution stable for approx. three week at 4 °C

Potassium chloride (KCl: 0.15 M, MW 74.55) in DW

01 M = 74.55 gm/1000 ml
M = 74.55 × 0.150 gm/1000 ml
M = 11.1825 /1000 ml
= 1.118 gm/100 ml

Sodium phosphate dibasic anhydrous (Na$_2$HPO$_4$: 0.4M, MW 141.96) in DW

01M = 141.96 gm/1000 ml
M = 141.96 ×0.1 /1000 ml
0.1 M = 14.196 /1000 ml
0.4 M = 14.196 × 4 /1000 ml
= 56.8 gm/1000 ml
PROCEDURE:

Pipette into three clean and dry test tubes labeled test (T), standard (S), and blank (B) (ml)

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>Test</th>
<th>Std.</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysate</td>
<td>0.03</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DW/ KCl</td>
<td>---</td>
<td>---</td>
<td>01</td>
</tr>
<tr>
<td>Na-phosphate buffer</td>
<td>01</td>
<td>01</td>
<td>01</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>GSH</td>
<td>01</td>
<td>01</td>
<td>---</td>
</tr>
<tr>
<td>DW</td>
<td>1.47</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>To take total volume</td>
<td>04</td>
<td>04</td>
<td>04</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>01</td>
<td>01</td>
<td>01</td>
</tr>
</tbody>
</table>

Keep for three min

Take 01 ml of aliquots of incubated mixture from above each three test tubes which used for further adding sequence steps in part II

| Meta-phosphoric acid    | 04   | 04   | 04    |
| Partlaliquots mixture   | 01   | 01   | 01    |

Filter it by using whatman no. 1 to collect filtrate

| Take filtrate           | 02   | 02   | 02    |
| Na$_2$HPO$_4$ solution  | 02   | 02   | 02    |
| DTNB                    | 01   | 01   | 01    |

Read absorbance of test and standard against blank at 412 nm within 2 mins.
CALCULATION:

\[
GSH - Px = \frac{\text{OD of test}}{\text{OD of Std.}} \times \frac{20 \text{ enzyme unit}}{\text{Conc. Of Hb in gm%}}
\]

Normal Range: 6.6 to 10.4 u/mg% of Hb
**GLUTATHIONE REDUCTASE (GR)**

HORN HD (1963)

**Principle:**

Glutathione reductase catalyses the reduction of GSSG in the presence of NADHP which is oxidized to NADP⁺.

The decrease in absorbance unit/ml is measured at 340 nm.

\[
2\text{NADPH} + \text{H}^+ + \text{GSSG} \rightarrow \text{NADP}^+ + 2\text{GSH}
\]

**Reagents:**

**Phosphate buffer:**

\[
\text{Na}_2\text{HPO}_4 \text{ MW } 141.96 \text{ (0.05M)} - 3.459 \text{ gm } \text{Na}_2\text{HPO}_4 \text{ dissolved in } 500 \text{ ml DW} \\
\text{KH}_2\text{PO}_4 \text{ MW } 136.99 \text{ (0.05M)} - 3.40225 \text{ gm } \text{KH}_2\text{PO}_4 \text{ dissolved in } 500 \text{ ml DW} \\
5000 \text{ ml } \text{Na}_2\text{HPO}_4 + 450 \text{ ml } \text{KH}_2\text{PO}_4 \text{ pH adjust with } 1 \text{ N NaOH or phosphoric acid}
\]

**GSSG (Oxidized glutathione)** - 0.2mM (1.2) mg/10ml MW 612.63

1.22526 mg GSSG dissolved in 10 ml DW

**NADPH (.12mM)** - 1 mg /10 ml (MW 833.35)

1 mg NADPH dissolved in 10 ml DW

**Procedure:**

Preparation of haemolysate

- Aspirate the plasma from anticoagulated blood sample
- Washed the RBCs with phosphate buffer six times
- Taken 0.1 ml RBC and add 0.9 ml DW i.e. 1:10 dilution
Then take

Phosphate buffer - 1.4 ml
GSSG - 0.2 ml
Haemolysate - 0.2 ml
NADPH - 0.2 ml

Reaction start, taken OD at 340 nm in decreasing order on time scale 7 times after 30 seconds

Wave length - 340 nm
Cuvette 1 cm path length
Temprature - 37 °C

Calculation:

Catalytic activity of GR

\[
\frac{\Delta A}{\text{Min}} \times \frac{\text{ml, reaction mixture}}{\text{ml, reaction mixture}} \times 106
\]

6.3 \times 103

\[
\frac{\Delta A}{\text{Min}} \times \frac{\text{ml, reaction mixture}}{\text{ml, reaction mixture}} \times 1000
\]

6.3

Normal value: 18.0 – 20.8 Units / gm protein
C- REACTIVE PROTEIN (CRP)

Principle:

Latex particle coated with specific human anti-CRP are agglutination when mixed with sample containing CRP. The agglutination causes an absorbance change, depending upon the CRP content of the patient's sample, that can be quantified by comparison from a calibrator of known CRP concentration.

REAGENT COMPOSITION:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Reagent</th>
<th>Composition</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CRP Turbilatex R1</td>
<td>Diluent, Tris buffer (pH 8.2), Sodium Azide</td>
<td>20 mmol/L, 0.95 g/L</td>
</tr>
<tr>
<td>2.</td>
<td>CRP Turbilatex R1</td>
<td>CRP Latex, Suspension of latex particle coated with anti human CRP, Sodium azide</td>
<td>0.95 g/L</td>
</tr>
<tr>
<td>3.</td>
<td>CRP calibrator</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Assay Parameters:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode</td>
<td>Fixed time</td>
</tr>
<tr>
<td>Slope of reaction</td>
<td>Increasing</td>
</tr>
<tr>
<td>Wavelength</td>
<td>546 nm (530-550 nm)</td>
</tr>
<tr>
<td>Flow-cell temperature</td>
<td>37 °C</td>
</tr>
<tr>
<td>Optical path length</td>
<td>1 cm</td>
</tr>
<tr>
<td>Blanking</td>
<td>Distilled water blank</td>
</tr>
<tr>
<td>Sample Volume</td>
<td>5 µl</td>
</tr>
<tr>
<td>Reagent volume</td>
<td>1000 µl</td>
</tr>
<tr>
<td>Delay time</td>
<td>5 sec</td>
</tr>
<tr>
<td>Interval</td>
<td>120 sec</td>
</tr>
<tr>
<td>Concentration of Calibrator</td>
<td>----</td>
</tr>
<tr>
<td>linearity</td>
<td>150 m/dl</td>
</tr>
<tr>
<td>Units</td>
<td>mg/L</td>
</tr>
</tbody>
</table>

**CALCULATION:**

\[
\text{CRP Conc}^n(\text{mg/L}) = \frac{(A_2 - A_1) \text{ Sample}}{(A_2 - A_1) \text{ Calibrator}} \times \text{Conc. of Cal.}
\]

**Normal Range:** Serum up to 6 mg/L
VITAMIN C
BY A KYAW 1978 METHOD:

PRINCIPLE:

Ascorbic acid reduces phophotungstic acid in acidic medium to blue chromogens, which has absorption maximum at 700 nm. The PTA serve not only as protein precipitant and ascorbic acid extractant but also as colour developing agent.

REAGENTS:

Phosphotustic acid - colour developing solution

Solution A: 20 gms of sodium Tungstate Na₂WO₄·H₂O and 10 gms of disodium hydrogen phosphate (Na₂HPO₄·2H₂O) was suspended in 30 ml of water warmed to dissolve.

Solution B: to 15 ml of distilled water, 5 ml of sulphuric acid was added

Solution B was poured in warm solution A and the content was boiled gently for 2 hrs under reflux. The resulting solution was then cooled to room temp. By allowing it to stand on the table.

0.5% Oxalic acid solution: 0.5% gms of oxalic acid dissolved in DW and value made up to 100 ml.

Stock standard ascorbic acid: 50 mg L- ascorbic acid is dissolve din 100 ml of 0.5 % (w/v) oxalic acid.
Working standard: For working standard solution of 1 mg/100 ml stock solution is diluted 50 times with 0.5% oxalic acid

**STANDARDIZATION:**

5 ml of stock solution was diluted to 100 ml with 0.5% oxalic acid the concentration of this standard solution is 2.5 mg/100ml. These standard & serial dilution of the resulting standards were treated with reagents as shown in the table (ml)

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>B</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std. solution</td>
<td>--</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
<td>1.2</td>
<td>1.4</td>
<td>1.6</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>D.W.</td>
<td>2.0</td>
<td>1.8</td>
<td>1.6</td>
<td>1.4</td>
<td>1.2</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>--</td>
</tr>
<tr>
<td>Colour reagents</td>
<td>2.0</td>
<td>1.8</td>
<td>1.6</td>
<td>1.4</td>
<td>1.2</td>
<td>1.0</td>
<td>0.8</td>
<td>0.6</td>
<td>0.4</td>
<td>0.2</td>
<td>--</td>
</tr>
<tr>
<td>Conc. Of std.</td>
<td>--</td>
<td>0.25</td>
<td>0.5</td>
<td>0.75</td>
<td>1.0</td>
<td>1.25</td>
<td>1.5</td>
<td>1.75</td>
<td>2.0</td>
<td>2.25</td>
<td>2.5</td>
</tr>
<tr>
<td>OD</td>
<td>--</td>
<td>0.07</td>
<td>0.13</td>
<td>0.18</td>
<td>0.23</td>
<td>0.28</td>
<td>0.32</td>
<td>0.38</td>
<td>0.45</td>
<td>0.50</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Mixed thoroughly and allowed to stand for 30 min. at RT. After this, the blue colour developed was read at 660 nm. Graph was plotted using absorbance concentration.

Normal Range: 0.5 – 1.6 mg/dl
TEST PROCEDURE:

The serum ascorbic acid levels was estimated as fellow

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>Test (T)</th>
<th>Standard (S)</th>
<th>Blank (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>2 ml</td>
<td>---</td>
<td>--</td>
</tr>
<tr>
<td>Std. solution (1 mg/dl)</td>
<td>--</td>
<td>2 ml</td>
<td>--</td>
</tr>
<tr>
<td>DW</td>
<td>--</td>
<td>--</td>
<td>2 ml</td>
</tr>
<tr>
<td>Colour reagents</td>
<td>2 ml</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

The tube were mixed thoroughly and allowed to stand for 30 min. at RT. Reaction is completed and colour is stable. The tubes were centrifuged at 300 rpm for 10 min. The clear supernatant was taken into the cuvettes with out disturbing the precipitate and the blue colour developed was read at 660 nm against blank. The unknown test conc. was extrapolated from the standard graph using their respective OD.
VITAMIN E
BY BAKER AND FRANK, 1968M:

PRINCIPLE:

Serum Tocopherol can be measured by their reduction of ferric to ferrous ions which can then forms a red complex with α-α’ dipyridyl. Tocopherols and carotenes are first extracted into xylene and the absorbance was read at 460 nm to measure the carotene. A correction for the carotenes was made after adding ferric chloride and reading at 520 nm.

REAGENTS:

Absolute alcohol, aldehyde free (ethanol).

Xylene

α-α’ dipyridyl: 1.20 g/ml in propanol.

Ferric chloride solution: 1.20 g FeCl₃, 6 H₂O/L in ethanol (Keep in brown bottle)

Standard solution of dL- α tocopherol: 10 mg/l in ethanol.

PROCEDURE:

<table>
<thead>
<tr>
<th>Addition sequence</th>
<th>Test (ml)</th>
<th>Standard (ml)</th>
<th>Blank (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1.5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Std. vit E (10mg/l in ethanol)</td>
<td>--</td>
<td>1.5</td>
<td>--</td>
</tr>
<tr>
<td>DW</td>
<td>--</td>
<td>--</td>
<td>1.5</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.5</td>
<td>--</td>
<td>1.5</td>
</tr>
<tr>
<td>DW</td>
<td>--</td>
<td>1.5</td>
<td>--</td>
</tr>
</tbody>
</table>
Material & Methods

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>xylene</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tubes are stoppered, mixed well and centrifuged for 10 minutes.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene layer</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Dipridyl reagent</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mixed well all the tube take three cuvettes and pipette 1.5 ml mix from each and take OD of td and test against blank at 460 nm.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric chloride solution</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The readings are taken at 520 nm after 1.5 minutes of final addition.

**CALCULATION:**

\[
\text{Serum Tocopherol (mg/L)} = \frac{\text{Reading Of Unkn at 520 nm} - \text{reading at 460 nm} \times 0.29}{\text{Reading of standard at 520 nm}} \times 10
\]

Since the std. contains 10 mg/L

**Normal range:** 0.45 – 1.7 mg/dl
**Material & Methods**

**Vitamin A**

**BRADLEY and HORNBECK, 1973**

**Principle:**

Proteins are precipitated with alcohol and retinol and carotenes extracted into light petroleum. After reading the intensity of the yellow colour due to carotenes the light petroleum is evaporated and residue dissolved in chloroform before carrying out the colour reaction. Allowance is made for the carotene contribution to the reaction.

**Reagents:**

1. Ethanol 950 ml analytical reagent grade
2. Light petroleum analytical grade b.p. 40 to 60 °C
3. Chloroform, anhydrous, analytical grade
4. TFA reagents, mix 1 volume TFA analytical grade with 2 volumes chloroform just before use the reagents is stable for 4 hrs at 25 °C
5. Retinol stock standard 160 ml /L. transfer 18.35 mg retinyl acetate (all trans,) to a 100 ml volumetric flask and dilute to volume with chloroform.
6. Retinol working standard dilute 10.0 l stock standard to 100 ml with chloroform. Then dilute 2.5, 5.0, 7.5 ml amounts of this solution to 100ml with chloroform to obtain working standards with
concentrations 0.4, 0.8, 1.20, and 1.60 mg/L. These standards are stable for one week at 4 to 8°C in the dark.

7. β-carotene stock standard 200 mg/L. Dissolve 20.0 mg synthetic crystalline β-carotene in about 4 ml chloroform and dilute to 100 ml with light petroleum.

8. β-carotene working standard. Dilute 10.0 ml stock standard to 100 ml with light petroleum. Then dilute 2.5, 5.0, 10.0, 15.0, and 20.0 ml amounts of this solution to 100 ml with light petroleum to obtain working standards with concentrations of 0.5, 1.0, 2.0, 3.0, and 4.0 mg/L. Working standards are stable for only a few hours at 25°C and should be made freshly for each analysis.

Procedure:

Pipette 1.0 ml serum into a 15 ml glass stoppered centrifuge tube, and add 2.0 ml ethanol, stopper and mix well with a vortex mixer.

Add 3.0 ml light petroleum and place the stoppered tubes in a mechanical shaker for 10 min to extract the retinol and carotenoids into petroleum phase. Centrifuge the tubes for 10 min at 2500g. Carefully transfer 2.0 ml of the upper, light petroleum phase or 2.0 ml of each carotene working standard into a dry cuvette, 10 mm optical path length and read absorbance (A_{450}) at 450 nm against a light petroleum blank. Do this without delay to prevent solvent evaporation and destruction of carotenoids by light.
Evaporate the contents of the cuvette to dryness in 50 °C water bath with the aid of a fine stream of nitrogen. Remove the cuvette, drying each carefully to avoid marking them, add 100 µl chloroform to each mixing briefly with a vortex mixer. Also prepare cuvettes containing 100 µl of each retinol working standard. Add 1.0 ml TFA reagent to a blank cuvette containing 100 µl chloroform mix and use it to zero the spectrophotometer at 620nm. Add forcefully to facilitate immediate mixing, 1.0 ml TFA reagents to each of the other cuvette in turn, recording the absorbance \( A_{620} \) at exactly 2s after adding the reagent. As TFA is a strong acid with an irritant vapour take care to avoid spilling or splashing pipette linked to a recording spectrophotometer. This allow the absorbance to be read at the peak of infection point after initial surging peak caused by the introduction of the TFA reagent.

**Calculation:**

\[
A_{450} \text{ of unknown} \]

\[
\text{Serum Carotene (mg/L) = } \frac{A_{450}}{\text{of standard}} \times C_{\text{onc}} \times 3
\]

\[
A'_{620} \text{ of unknown}
\]

\[
\text{Serum Carotene (mg/L) = } \frac{A'_{620}}{\text{of standard}} \times C_{\text{onc}} \times \frac{3}{2}
\]

Where \( A'_{620} = A_{620} - F \). \( A_{450} \) is determined as follows

For F: 2.0 ml of each of the carotene working standards in the same way as the 2.0 ml of the light petroleum extract in the full method and measure \( A_{620} \).
for each. Determine $F$ as $\frac{A_{620}}{A_{450}}$ for each working standard and calculate the mean value

Normal range:

Carotene: 0.5 – 2.0 mg/L
Retinol: 0.5 – 0.65 mg/L
GLUTATHIONE

BY BEUTLER E, DURON O & KELLY B.M. METHOD

Reagent:

Precipitating solution: 1.67 gm glacial metaphosphoric acid (mixture of $\text{H}_3\text{PO}_3$ & $\text{NaPO}_3$), 0.2 gm disodium or dipotassium ethylediamine tetra acetic acid (EDAT) & 30 gm sodium chloride per 100 ml of DW. This solution is stable for approximately 3 weeks at 4°C.

Phosphate solution: 5.33 gm of NaHPO$_4$ dissolved in 100 ml DW. Thios solution is stable indefinitely forms.

DTNB reagents: 40 mg 5-5' dithio bis – 2 nitrobenzoic acid in 100 ml of 1% sodium citrate. Care should be taken to prevent prolonged period of light exposure and extremes in pH. In this condition this solution is stable for 3 months.

Procedure: Prepare 2 tubes as Test (T) & Blank (B)

Test

Take 0.1 ml of whole blood, which is collected in heparinized vial and add 0.9 ml of DW

Add 1.5 ml of precipitating solution

Shake well than filter, take 1 ml of filtrate

Add 4 ml of phosphate solution add 0.5 ml of DTNB reagent. Yellow colour developed O.D. was read immediately at 412 nm within minute.
Material & Methods

Blank

1 ml of diluted precipitating solution (0.75 ml precipitating reagent and .25 ml DW) was taken in a test tube.

Add 4 ml of phosphate solution.

Then add 0.5 ml DTNB reagent.

Calculation:

Mg % of glutathione in blood in three Coleman junior instruments identical E valve were found that is 0.542

\[
\text{Mg % of glutathione in blood} = 0.542 \times \text{O.D} \times 310.4
\]

\[
= 168.2 \times \text{O.D}
\]

\[
= \text{mg% of glutathione in blood}
\]

Normal values: 20-40 mg %
Material & Methods

Statistical analysis:

Differences between continuous variables were tested with analyses of variance (one-way ANOVA) utilizing Dunnet T₃ test. One Way ANOVA was used to estimate differences between ages matched controls/ group I, group II and in group III. The comparisons between two groups were analyzed by Student’s t-test. All parameters were given as mean± standard deviation. The criterion for significance was $P < 0.05$. Pearson’s correlation was used to evaluate the correlations between the variables.

Data analyses were performed with the Statistical Package for the Social Sciences, version 16.0 (SPSS, Chicago, Illinois, USA).