CHAPTER 3: METHODOLOGY

3.1. Introduction

Oxidative stress has been implicated in a growing list of human diseases, such as cardiovascular, inflammatory, metabolic and neurogenerative diseases, as well as cancer and the ageing process. A diet rich in antioxidants has been identified as a potentially non-invasive means controlling oxidative stress (Roberts et al., 2005; Herrera et al., 2009). Antioxidant supplementation has received much attention because of its capacity to support the endogenous defence by scavenging additional ROS and therefore, by reducing oxidative damage (Alessio et al., 1997; Goldfarb et al., 2005; Meydani et al., 1993).

In view of the relevant studies of the preceding chapter and to comply with the objectivity of this study, the modalities of objective measurement were planned, oriented and executed in this chapter.

To attain the objectives of the study the investigator has attempted to evaluate the effect of certain treatment that involved the intricate physiological function of the organism. This study involves the tests, measurements and conducting the experiments with suitable tools and the modus operandi of collecting data in this experimental study in the field of sports.

Some researchers injected subjects with α-tocopherol and some had subjects intake in tablet form thus their is no standard doses that is used to determine the effect of vitamin E upon work or fatigue (Sacheck et al., 2003). Choose to monitor vitamin E intake levels rather than have subjects supplement with exogenous vitamin E. No difference was found between the subjects samples with high fat and low fat diet.
Fig. 1: Vitamin-C+E Group

Fig. 2: Vitamin-C Group

Fig. 3: Vitamin-E Group
3.2. Subjects

Thirty male football players with the age of 18 through 23 years volunteered as subjects for this study. The subjects were regular football players who had represented their respective districts. The subjects were equally divided into three experimental groups A, B and C. The subjects were certified by a registered physician and declared 'fit' to undergo rigorous training.

3.3. Design of the Study

The study was a quasi-experimental study of trained male footballers, volunteered as subjects. The subjects were regular football players with a training age of at least three years, participating in 'District level competitions'. The subjects were divided equally into three experimental groups 'A', 'B' and 'C'. The age, height and body mass of the subjects were recorded. The subjects were certified by a registered physician and declared 'fit' to undergo rigorous muscular task.

Initially, blood samples were drawn from each subject for estimation of free radical content. After resting of at least half an hour each subject's Resting Heart Rate (RHR) was recorded. After proper warming up a training load volume of 50% at 75% intensity for weight training exercises, were estimated by 1 RM for each subject. The circuit training programme was comprising of eight exercises.

The same procedure was followed at 15 days interval with 80% and 85% intensity respectively.

3.4. Criterion Measure

i) Superoxide Dismutase (SOD)

ii) Glutathion peroxidase (GPx)

iii) Catalase (CAT)

iv) Uric Acid

3.4.1. Preparation of Blood Sample

Non anti coagulated blood yields serum. The non-cellular fluid part of the blood serum is used for estimation of free radicals of the blood through biochemical analysis.
The blood was collected by disposable syringe (DISPOVAN) and preserved in the vials. The blood centrifuge vials were put into the cold centrifuge with 2500 rpm for 10 minutes within two hours after the sample is collected.

3.4.2. Enzymatic Assay of Superoxide Dismutase (SOD)

Superoxide Dismutase (SOD) catalyzes the reaction:

\[
2 \text{O}_2 + 2\text{H} \xrightarrow{\text{SOD}} \text{O}_2 + \text{H}_2\text{O}_2
\]

Superoxide dismutase activity is measured as the inhibition of the rate of reduction of cytochrome C by the superoxide radical, observed at 550 nm.

Cytochrome C (Oxidized) + O\(_2\) → Cytochrome C (reduced) + O\(_2\)

The superoxide radical is produced cyzynamically by the reaction:

\[
\text{Xanthine} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{XOD}} \text{Uric acid} + \text{O}_2^- + \text{H}^+
\]

Method : Continuous Spectrophotometric Rate Determination.

3.4.2.1. Reagents

A. 216 mM potassium phosphate

Buffer, pH 7.8 at 25°C

(Prepare 50 ml. in deionized water using potassium phosphate, Monobasic, Anhydrous, Sigma Prod. No. P - 5379 Adjust to pH 7.8 at 25°C 1 M KOH).

B. 10.7 mM Ethylenediaminetetra acetic acid solution (EDTA)

(Prepare 50 ml. in deionized water using Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate, Sigma Stock No. ED 2SS)

C. 1.1 mM cytochrome C solution (Cyt C)

(Prepare 2 ml. in deionized water using cytochrome C, from Horse Heart, Sigma prod.)

Enzymatic Assay of SOD

EC (1.151.1)
D. 0.108 mM Xanthine Solution (Xanthine)

(Prepare 500 ml. in deionized water using Xanthine, Sigma prod no. X-0626 Titrate into solution with 1 M (KOH).

E. Xanthine Oxidase Enzyme Solution (XOD)

(Immediately before use, prepare a solution containing 0.05 unit / ml. of Xanthine Oxidase, Sigma prod. No. X - 1875, in cold deionized water).

F. Superoxide Dismutase Enzyme Solution

(Immediately before use, prepare a solution containing 10 units / ml. of superoxide Dismutase in cold deionized water).

3.4.2.2. Procedure

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into as suitable container.

<table>
<thead>
<tr>
<th>Deionized water</th>
<th>23.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A (Buffer)</td>
<td>25.0</td>
</tr>
<tr>
<td>Reagent B (EDTA)</td>
<td>1.0</td>
</tr>
<tr>
<td>Reagent C (Cyt C)</td>
<td>1.0</td>
</tr>
<tr>
<td>Reagent D (Xanthine)</td>
<td>50.0</td>
</tr>
</tbody>
</table>

Mix and adjust to pH 7.8 at 25ºC, if necessary with 1 M HCl or 1 M KOH. Pipette (in milliliters) the following reagents into suitable cuvettes:

<table>
<thead>
<tr>
<th>Test 1</th>
<th>Test 2</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninhibited</td>
<td>Inhibited</td>
<td></td>
</tr>
<tr>
<td>Reaction Cocktail</td>
<td>2.80</td>
<td>2.80</td>
</tr>
</tbody>
</table>
Equilibrate to 25°C. Monitor the absorbance, $A_{550\text{ nm}}$, until constant, using a suitably thermostatted spectrophotometer. Then add

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Amount</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized water</td>
<td>0.10</td>
<td>–</td>
<td>0.20</td>
</tr>
<tr>
<td>Reagent E (XOD)</td>
<td>0.10</td>
<td>0.10</td>
<td>–</td>
</tr>
<tr>
<td>Reagent F (Enzyme Solution)</td>
<td>–</td>
<td>0.10</td>
<td>–</td>
</tr>
</tbody>
</table>

Immediately mix by inversion and record the increase in $A_{550\text{ nm}}$ for approximately 5 minutes. Obtain the $\Delta A_{550\text{ nm}}$ / minute by using the maximum, linear rate for both the uninhibited (Test 1), inhibited (Test 2) and Blank.

Enzymatic Assay of Superoxide Dismutase (EC 1.15.1.1)

Calculations

$$% \text{ inhibition} = \frac{\Delta A_{550\text{ nm}} / \text{ min uninhibited} - \Delta A_{550\text{ nm}} / \text{ min inhibited}}{\Delta A_{550\text{ nm}} / \text{ min uninhibited} - \Delta A_{550\text{ nm}} / \text{ min blank}}$$

Units / ml enzyme = % inhibition (df)

$$(50\%) \ (0.1)$$

$df =$ Dilution factor

$0.1 =$ Volume (in milliliter) of enzyme used

$50\% =$ inhibition of the rate of cytochrome C reduction as per the unit definition.

Units / mg solid = \(\frac{\text{units / ml enzyme}}{\text{mg solid / ml enzyme}}\)

Units / mg protein = \(\frac{\text{units / ml enzyme}}{\text{mg protein / ml enzyme}}\)

3.4.2.3. Unit Definitions

One unit will inhibit the rate of reduction of cytochrome c by 50% in a coupled system, using Xanthine and Xanthine Oxidase at pH 7.8 at 25°C in a 3.0 ml. reaction volume. The Xanthine Oxidase concentration should produced an (uninhibited).
3.4.2.4. Final Assay Concentration

In a 3.00 ml reaction mix, the final concentrations are 50 mm potassium phosphate, 0.1 mm ethylenediaminetetra acetic acid 0.01, mm cytochrome C, 0.05 mm xanthine, 0.005 unit xanthine oxidase and 1 unit superoxide dismutase.

3.4.3. Enzymatic Assay of Glutathion peroxide

Principle:

\[
2 \text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{Glutathione Peroxidase}} \text{GSSG} + 2\text{H}_2\text{O}
\]

\[
\text{GSSG} + \text{β-NADPH} \xrightarrow{\text{Glutathione Reductase}} \text{β-NADP} + 2\text{GSH}
\]

Conditions: \( T = 25^\circ\text{C} \), \( \text{pH} = 7.0 \), \( A_{340\text{NA}} \)

Light path 1 cm.

Method: Continuous spectrophotometric Rat Determination

3.4.3.1. Reagents

A. 50 mm Sodium phosphate Buffer with 0.40 Ethylenediaminetetraacetic Acid (EDTA), pH 7.0 at 25ºC.

(Prepare 100 ml. in deionized water using sodium phosphate, Monobasic, Anhydrous Sigma prod. No. S-0751 and Ethylenediamine tetra acetic acid, Tetra Sodium Salt, Sigma Stock No. ED4SS, Adjust to pH 7.0 at 25ºC with 1 M NaOH)

B. 1.0 mM Sodium Azide solution (Bufferw / Azide)

(Prepare 50 ml. in Reagent A using Sodium Azide, Sigma prod. No. S-2002)

C. β-Nicotinamide Adenine Dinucleo tide phosphate, Reduced Form (β-NADPH)

(Use 1.0 mg vial of β-Nicotinamide Adenine Dinucleotide phosphate, Reduced form, Tetra Sodium Salt, Sigma Stock No. 201–201).
D. Glutathione Reductase Enzyme Solution (GR)

(Immediately before use, prepare a solution containing 100 units / ml. of Glutathione Reductase, Sigma Prod. No. G-3664, in cold deionized water).

E. 200 mM Glutathione, Reduced (GSH)

(Prepare 5 ml. in deionized water using Glutathione, Free Acid, Reduced form, Sigma Prod. No. 6-4251).

F. 10.0 mM Sodium phosphate Buffer with 1.0 mM Dithiothreitol, pH 7.0 at 25 EC (Buffer W / DTT)


Adjust to pH 7.0 at 25°C with 1 M NaOH)

G. Glutathione peroxidase Enzyme Solution.

(immediately before use, prepare a solution containing 1.5–3.0 units / ml. of Glutathione peroxidase in cold reagent F.)

H. 0.042% (W/W) Hydrogen peroxide (H₂O₂)

(Prepare 5 ml. in deinoized water using Hydrogen peroxide 30% (w/w) solution, Sigma Prod. No. H–1009³ Prepare fresh).

3.4.3.2. Procedure

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into reagent C (□-NADP vial)

Reagent B (Buffer W / Azide) 9.20
Reagent D (GR) 0.10
Reagent E (GSH) 0.05

Mix by inversion and adjust to pH 7.0 at 25°C with 1 M HCl or 1 M NaOH, if necessary. Pipette (in milliliters) the following reagents into suitable cuvettes:
Reaction cocktail  | Test | 3.00 | Blank | 3.00
Reagent F (Buffer W / DTT) | – | – | – |
Reagent G (Glutathione peroxidase) | 0.05 | – | – |

Mix by inversion equilibrate to 25°C. Monitor the $A_{340\text{nm}}$ until constant, using a suitable thermo stat ted spectrophotometr. Then add:

| Test | 0.05 | Blank | 0.05 |
Reagent H (H$_2$O$_2$) | |

Immediately mix by inversion and record the decrease in $A_{340\text{nm}}$ for approximately 5 minutes. Obtain the $\Delta A_{340\text{nm}} \text{ / minute}$ using the maximum linear rate for both the Test and Blank.

**Calculations**

$$\text{Units / ml enzyme} = \frac{(\Delta A_{340\text{nm}} \text{ / min Test} - \Delta A_{340\text{nm}} \text{ / min Blank}) (2) (3.1) (df)}{(6.22) (0.05)}$$

$2 = 2 \mu$ moles of GSH produced per $\mu$ mole of $\beta$-NADPH Oxidized

$3.1 = \text{Total volume (in milliliters) of assay.}$

$\text{df} = \text{Dilution factor}$

$6.22 = \text{Millimolar extinction coefficient of } \beta\text{-NADPH at 340 nm.}$

$0.05 = \text{Volume (in milliliters) of enzyme used.}$

$$\frac{\text{Units / mg solid}}{\text{mg solid / ml enzyme}} = \frac{\text{units / ml enzyme}}{\text{mg solid / ml enzyme}}$$

$$\frac{\text{Units / mg protein}}{\text{mg protein / ml enzyme}} = \frac{\text{units/ml enzyme}}{\text{mg protein / ml enzyme}}$$

**3.4.3.3. Unit Definition**

One unit will catalyze the oxidation by H$_2$O$_2$ of 1.0 $\mu$ mole of reduced glutathione to oxidized glutathione per minute at pH 7.0 at 25°C.
3.4.3.4. Final Assay Concentration

In a 3.05 ml reaction mix, final concentrations are 48 mM Sodium phosphate, 0.38 mM ethylenediaminetetra acetic acid, 0.12 mM β-nicotinamide, adenine dinucleotide phosphate, reduced from 0.95 mM sodium azide, 3.2 units of glutathione reductase, 1 mM glutathione, 0.02 mM DL–dithiothreitol, 0.0007% (w/w) hydrogen peroxide and 0.075–0.15 unit of glutathione peroxidase.

3.4.4. Photometric Determination of Catalase Activity (CAT)

Catalase activity may be measured quantitatively by the method of Von Euler and Josephson (1) by allowing the enzyme solution to react with hydrogen peroxide for varying periods of time and measuring the excess peroxide remaining by titration with potassium permanganate. The similarity of this titration procedure to the assay of ascorbic acid by titration with 2: 6-dichlorophenolindophenol suggested to the authors the possibility of measuring Catalase activity photometrically (in a manner similar to the photometric estimation of vitamin C) by addition of an excess quantity of potassium permanganate and subsequent photometric measurement of the color. This procedure has been found to be practical and rapid. It gives reproducible results and, by photometric means, obviates the human error in reading the end-point of the potassium permanganate. As both hydrogen peroxide and potassium permanganate in the present procedure are used in the same concentrations as in the conventional von Euler and Josephson method, compounds that inhibit Catalase such as HCN should have no different effect in this procedure from that in the conventional one.

3.4.4.1. Apparatus and Reagents

The photometric determination of Catalase activity may be carried out with any standard colorimeter or spectrophotometer.

The reagents required for this measurement are as follows:

Hydrogen peroxide (approximately 0.01 N) in phosphate buffer at pH 6.8. Use 5.67 ml of 3 percent hydrogen peroxide per litre.

Potassium permanganate (approximately 0.005 N); 0.158 gm, made to 1 litre with distilled water.

A solution of diluted Catalase enzyme. For our study, a 1: 2500 Catalase–Sarett solution was used.
Sulfuric acid (5 N); 142 ml. of concentrated sulfuric acid (sp. gr. 1.84) made to 1 liter with distilled water.

**3.4.4.2. Preparation of Standard Curve**

In a volumetric flask, 10 ml. of hydrogen peroxide (3 percent by volume) are diluted to 100 ml. with distilled water and mixed. Then into consecutive 100 ml. volumetric flasks are pipetted 6, 5, 4, 3, 2 and 1 ml., respectively, of the 0.3 percent hydrogen peroxide solution. The contents of the flasks are made to volume with phosphate buffer (0.0067 M) and mixed. From each flask, a 5 ml. amount is pipetted into six reaction test-tubes containing 2 ml. of 5 N Sulfuric acid. The photometer is set to transmit maximally at 515 m\(\mu\) and is adjusted to 100 percent transmission with a reference tube which contains 2 ml. of 5 N sulfuric acid plus 15 ml. of distilled water into one of the six reaction tubes, 10 ml. of the 0.005 N permanganate solution are blown. The contents are quickly mixed, and the transmission is read at once. This is repeated for all six tubes.

A Seventh tube is prepared with 2 ml. of Sulfuric acid, 5 ml. of water and 10 ml. of permanganate. The transmission of this mixture is read and serves as the “blank”.

**Table 1: Typical Reading obtained with Coleman Universal Spectrophotometer (Standard Curve)**

<table>
<thead>
<tr>
<th>Contents of tube 17 ml.</th>
<th>Tube 1</th>
<th>Tube 2</th>
<th>Tube 3</th>
<th>Tube 4</th>
<th>Tube 5</th>
<th>Tube 6</th>
<th>Blank</th>
<th>Reference Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_2)O(_2) per 100 ml.</td>
<td>0.018</td>
<td>0.015</td>
<td>0.012</td>
<td>0.009</td>
<td>0.006</td>
<td>0.003</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>% of H(_2)O(_2)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>H(_2)SO(_4), ml.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>KMNO(_4)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transmission / Optical density</th>
<th>90(^0)</th>
<th>83(^1)</th>
<th>75(^2)</th>
<th>71(^3)</th>
<th>66(^0)</th>
<th>61(^1)</th>
<th>57(^1)</th>
<th>100(^0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dbl - Ds</td>
<td>0.196</td>
<td>0.162</td>
<td>0.121</td>
<td>0.093</td>
<td>0.061</td>
<td>0.027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.032</td>
<td>0.032</td>
<td>0.030</td>
<td>0.031</td>
<td>0.030</td>
<td>0.027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>average slope</td>
<td>0.0309</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 lists the contents of each of the tubes and presents a typical set of readings obtained with the use of a Coleman Universal Spectrophotometer.
The optical densities which correspond to the transmissions, expressed in percent, are calculated and can be plotted with reference to the relative concentrations of hydrogen peroxide in the test-tubes to obtain the “Standard curve”. As the relationship between optical density and percentage of concentration is linear in this case, it is actually unnecessary to plot the standard curve. All that is necessary is to calculate the amount of decrease in optical density below that of the blank for each increase of 1 percent in relative concentration. This is done by subtraction of the value for the optical density of each concentration from that of the blank, and division of the result by the relative concentration. The resulting quotients or “slopes” are averaged to obtain the mean slope of the standard curve.

3.4.4.3. Measurement of Catalase Activity

The necessary dilutions of a preparation of Catalase are made and placed in an ice-water bath. In this work, a 1:2500 solution of Catalase–Sarett was used. To 50 ml. of 0.01 N hydrogen peroxide containing 0.07 M phosphate buffer at a pH of 6.8 (which has been allowed to sit in a beaker of ice water for 5 minutes) add 1 ml. of properly diluted enzyme. Mix, and immediately pipette 5 ml. of this solution into a reaction tube containing 2 ml. of the 5 N sulfuric acid. Mix, and add 10 ml. of the permanganate solution. Mix, and read in the colorimeter against the reference tube with a 515 m\(\mu\) filter. Repeat again after 3, 6, 9 and 12 minutes. Then calculate the \(K_0\) value and the Kat. f. value (2).

A blank reading is made with a mixture containing 2 ml. of acid, 5 ml. of distilled water, and 10 ml. of the permanganate solution.

The volume of permanganate remaining after the reaction with hydrogen peroxide is calculated according to the following formula:

\[
\frac{D_{\text{bl}} - D_s}{\text{Slope}} = \text{ml. permanganate used.}
\]

where \(D_{\text{bl}}\) is the optical density of the blank reading, \(D_s\) is the optical density of the Sample reading and “Slope” is the average slope of the standard curve calculated as described above.
3.4.4.4. Comparison of Measurements of Catalase Activity by Photometric Method by Conventional Permanganate Titration Method

For comparative purposes the activity of a sample of “Catalase-Sarett” was determined by the photometric method described above and by the conventional permanganate titration method. The results are summarized in Table II. As can be seen from the date, the Kat. f. Values are in good agreement.

Several precautions should be emphasized.

The time between pipetting of the permanganate solution into the tube and the photometric reading should be not more than 1 minute. After a period of from 1 to 1½ minutes, a colloidal suspension forms in the tubes, which is especially pronounced if the quantity of hydrogen peroxide remaining is significant.

\[
\frac{D_{bl} - D_s}{\text{Slope}}
\]

\[
K_s = \frac{1}{T} \log_{10} \left( \frac{a}{a - K} \right), \text{ where } K_s \text{ is the reaction rate, } T \text{ time, a initial KMnO}_4 \text{ concentration, and } a-x \text{ is the KMnO}_4 \text{ remaining. The } K_s \text{ value for } T = 0 \text{ obtained by extrapolation.}
\]

\[
\text{Kat.f} = \frac{K_s(0 \text{ time})}{\text{gm. enzyme (dry weight)}}. \text{ See Summer and Somers (2).}
\]

Enzyme dilution used was 1: 2500 in the titration method; the enzyme content was 0.0271 gm (dry weight) per ml. instances.

One standard curve, if properly obtained with good duplicate checks and small deviations in slopes between the individual points, suffices for the particular instrument, wave-length and permanganate pipette. A 10 ml. graduated pipette with a wide tip to facilitate rapid flowing is recommended.

Increasing or decreasing the permanganate concentration or increasing or decreasing the peroxide concentration has no effect on the slope of the curve, which is constant. Hence for such a determination, one has only to ascertain the blank one has only to ascertain the blank reading of permanganate, acid and water and the reading of the unknown solution containing the reaction products of Catalase activity.
Table 2: Comparison of Measurements of Catalase Activity by Photometric and Permanganate Titration Method

<table>
<thead>
<tr>
<th>Reaction Time</th>
<th>Transmission Percent</th>
<th>Photometric Method</th>
<th>Titration Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Optical Density</td>
<td>KMnO₄ used (ml.)</td>
</tr>
<tr>
<td>min</td>
<td>percent</td>
<td>0.064</td>
<td>5.95</td>
</tr>
<tr>
<td>0</td>
<td>86¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>81⁰</td>
<td>0.092</td>
<td>5.06</td>
</tr>
<tr>
<td>6</td>
<td>77¹</td>
<td>0.112</td>
<td>4.39</td>
</tr>
<tr>
<td>9</td>
<td>74¹</td>
<td>0.129</td>
<td>3.84</td>
</tr>
<tr>
<td>12</td>
<td>70³</td>
<td>0.150</td>
<td>3.16</td>
</tr>
<tr>
<td>Blank</td>
<td>56²</td>
<td>0.248</td>
<td>–</td>
</tr>
<tr>
<td>Kat. f.</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Summary:

A technique is described for measuring Catalase activity photometrically with the use of a Coleman spectrophotometer or any colorimeter.

A properly diluted enzyme solution is allowed to react with hydrogen peroxide for a specified period of time. The reaction is then stopped by use of a sulfuric acid solution. Potassium permanganate in excess is next added to the mixture and allowed to react with the peroxide not decomposed by the Catalase. Within one minute after addition of the permanganate, the excess in potassium permanganate is determined photometrically.

3.4.5. Enzymatic Assay of Uric Acid

Uric acid is the end product of Purine metabolism. Uric Acid is excreted to a large degree by the kidneys and to a smaller degree in the intestinal tract by microbial degradation. Increased levels are found in Gout, Arthritis, impaired renal functions and starvation. Decrease levels are found in Wilson’s disease, fanconis syndrome and yellow atrophy of the liver.

Uric acid in plasma and muscle is an important antioxidants with direct effects on single oxygen, HoCl, Peoxyl radical, peroxynitrite or ozone (Ames et al., 1981; Grootveld et al., 1987; Kean et al., 2000).
Uric acid helps to protect cell membrane and DNA from radical oxidation. Its ability to form stable complexes with iron ions, inhibits Fe$^{3+}$, catalysed Vitamin C oxidation and lipid peroxidation (Davis, et al., 1986; Sevanian, et al., 1991) are the other important property of antioxidant of uric acid.

**Principle:**

Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenolic compound and 4 aminoantipyrine by the catalyse action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.

\[
\text{Uric acid} + \text{H}_2\text{O} \xrightarrow{\text{uricase}} \text{Allantoin} + \text{H}_2\text{O}
\]

\[
\text{H}_2\text{O}_2 + 4 \text{Aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{Red}
\]

Quinoneimine dye + H$_2$O + phenolic compound

**3.4.5.1. Reagent preparation**

Working reagent:

Pour the contents of 1 bottle of (Enzyme reagent) into 1 bottle of Li (Buffer reagent). This working reagent is stable for at least four weeks when stored at 2–8°C. Upon storage the working reagent may develop a slight pink colour however this does not affect the performance of the reagent.

Alternatively for flexibility as much of working reagent may be made as and when desired by mixing together 4 parts of L$_1$ (Buffer reagent) and 1 part of L$_2$ (Enzyme Reagent).

Alternatively 0.8 ml. of L$_1$ and 0.2 ml. of L$_2$ may also be used instead of 1 ml. of the working reagent directly during the assay.

**3.4.5.2. Calculations**

\[
\text{Uric acid in mg / dl} = \frac{\text{Abs.T} \times 8}{\text{Abs.S}}
\]
3.4.6. Exercise Protocol

To execute the plan for giving specific load intensity during circuit exercise program, all the subjects were given uniform loading procedure to have uniform action. Three different specific training intensity were selected to find out the effectiveness as adopted in the objective of the study.

The athletes were prepared, the training intensity for each individual subject and frequency were obtained through methodical procedure at 50% individual load.

3.4.6.1. Treatment Plan

After a thorough warm up all the subjects were given final instruction along with the required set of equipment in the gymnasium. Each subject was instructed to perform the exercises from Station No. 1 to No. 8 in a sequence with specified recovery. The circuit training program consisted of eight different stations comprising eight different exercises. The sequence of exercises was as follows:

Fig. 4: Two hand biceps curl
Fig. 5: Half Squat

Fig. 6: Front Press
Fig. 7: Stepping up

Fig. 8: Back Press
Fig. 9: Bench Press

Fig. 10: Leg Press
Table 3: The Sequence of Exercise

<table>
<thead>
<tr>
<th>Station No.</th>
<th>Name of Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Two hand biceps curl</td>
</tr>
<tr>
<td>2</td>
<td>Half squat</td>
</tr>
<tr>
<td>3</td>
<td>Front press</td>
</tr>
<tr>
<td>4</td>
<td>Stepping up</td>
</tr>
<tr>
<td>5</td>
<td>Back press</td>
</tr>
<tr>
<td>6</td>
<td>Bench press</td>
</tr>
<tr>
<td>7</td>
<td>Leg press</td>
</tr>
<tr>
<td>8</td>
<td>Abdominal curl</td>
</tr>
</tbody>
</table>

Before the commencement of warm up exercise blood samples were collected from each subject who had not performed any physical activity for previous 48 hours. After collection of resting blood samples for estimation of selected free radical
components, the subjects were allowed to warm-up for the forthcoming circuit at their respective load.

After thorough warm up each subject performed the selected exercise of the protocol circuit training one by one. The researcher, who is also conversant with weight training program, had personally supervised the proper execution of each exercise. There was 30 second recovery period during transition between the stations (Uppal, 2001).

The subjects sincerely completed three circuits with full recovery in between circuits.

![Collection of Blood Sample](image)

**Fig. 12: Collection of Blood Sample**

Blood samples were collected after three minutes of the completion of three circuits by each subject for biochemical estimation of free radicals.

Antioxidant supplementation (vitamin–C, vitamin–E & vitamin C + E) were made within six minutes of the completion of circuit exercise. After a complete rest of 75 minutes, again blood samples were drawn from each subject to estimate the free radical concentration. The quantum of vitamins was selected as referred by Jackman and Maxwell (1993) were 250 mg. Vitamin–C and 400 mg vitamin–E and combined administration. According to the said application it is assumed that supplementation of antioxidant will reduce the oxidant produced during oxidative stress at three different intensive metabolic function.
The administration of antioxidants was made as follows:

Table 4: Antioxidant supplementation protocol at post circuit exercise program at 75% – 80% – 85% intensity

<table>
<thead>
<tr>
<th>% Load intensity</th>
<th>Day-1</th>
<th>Day-2</th>
<th>Day-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>75%</td>
<td>vitamin–C</td>
<td>vitamin–E</td>
<td>vitamin–C + E</td>
</tr>
<tr>
<td></td>
<td>250 mg</td>
<td>400 mg</td>
<td>250 + 400 mg</td>
</tr>
<tr>
<td>80%</td>
<td>vitamin–C</td>
<td>vitamin–E</td>
<td>vitamin–C + E</td>
</tr>
<tr>
<td></td>
<td>250 mg</td>
<td>400 mg</td>
<td>250 + 400 mg</td>
</tr>
<tr>
<td>85%</td>
<td>vitamin–C</td>
<td>vitamin–E</td>
<td>vitamin–C + E</td>
</tr>
<tr>
<td></td>
<td>250 mg</td>
<td>400 mg</td>
<td>250 + 400 mg</td>
</tr>
</tbody>
</table>

3.4.7. Statistical Treatment

1. Mean.

2. SD.

3. ANOVA (Two–tailed).

4. Post Hoc Bonferroni multiple comparison.

All statistical treatments were done by SPSS version 20 by univariate tests. The level of significance was chosen at 0.05 level.