CHAPTER III

STUDY PLAN & PROCEDURE

Effect of Yoga on Endothelial Function, Vascular Compliance and Sympathetic Tone in Elderly Subjects with Increased Pulse Pressure: A Randomized Clinical Study
1. **STUDY DESIGN**

An open parallel-group randomized controlled study was conducted on elderly subjects between 60 and 75 years with increased PP (˃ 60mmHg) over a period of 3 months. Volunteers were screened at Visit 1-3. At visit 4, the baseline examination and recordings followed by randomization of selected subjects to either yoga group (Yoga intervention) or control group (walking intervention) was done. Post-intervention examination and recordings were made at Visit 5. No intervention was given on the day of investigation. Persons handling data analysis were kept blinded.

![Figure 8: Study protocol](image-url)
2. STUDY POPULATION

2.1. Participants

The study participants were elderly volunteers with increased PP, who were recruited from the health camp for elderly and Geriatric clinic of Shri B.M.Patil Medical College, and volunteers enrolled through advertisement.

2.2. Sample size

The sample size was calculated on the basis of the difference in means and standard deviation of PP obtained from the pilot study (Patil SG et al., 2014). A total of 60 elderly subjects included in the study. The probability was 80% that study will detect a treatment difference at a two-sided 0.05 significant level, if the true difference between treatments was 4 units and standard deviation of the outcome variable was 5.11.
3. INCLUSION AND EXCLUSION CRITERIA

3.1. Inclusion criteria

Subjects who have fulfilled the following criteria were enrolled at Visit 3:

- Either sex between 60 and 75 years with pulse pressure > 60mmHg.
- The subject was expected to stay within driving distance of study for at least 5 months.
- No significant diseases or clinically significant abnormal laboratory values during screening.

3.2. Exclusion criteria

Any of the following was regarded as a criterion for exclusion from the study:

- Subjects with SBP > 159mmHg and DBP > 99mmHg.
- Subjects with secondary hypertension
- Subjects on any regular medical treatment.
- Subjects with CV risk factors such as diabetes mellitus, hypercholesterolemia and high triglyceride level.
- 12-lead ECG with any significant abnormalities
- Subjects with neuromuscular disorders.
- Subjects with joint pains.
- History of alcoholism (alcohol abuse) and cannot refrain from alcohol consumption during the study period.
- History of Smoking and cannot refrain from smoking during the study period.
- Subjects who do regularly yoga practice.

3.3. Justification for inclusion & exclusion criteria

The inclusion and exclusion criteria for selection of elderly subjects for life-style changes intervention for three months were as per the 2007 guidelines of the task force for the management of arterial hypertension of the European Society of Hypertension and of the European Society of Cardiology (Task force for the management of arterial hypertension of the ESH and ESC., 2007).
4. CRITERIA FOR DISCONTINUATION

- Participant refusal
- Participant who could not sustain the intervention
- Participant who’s SBP raised above 165 mmHg and DBP raised above 100 mmHg
- Participant who develops any adverse affect
5. ETHICS

5.1. Informed consent
Informed written consent was obtained for participation in the study (Appendix I).

5.2. Institutional approval
The study was approved by the institutional ethical committee of Shri B.M.Patil Medical College, Hospital and Research Centre, BLDE University, India, as per the guidelines (2006) of Indian Council of Medical Research (ICMR ethical guidelines for biomedical research on human participants., 2006).

5.3. Declaration of Helsinki & ICMR guidelines
We followed the declaration of Helsinki during the entire study.

5.4. Study Registration
The study was registered retrospectively in the Clinical Trial Registry-India (CTRI/2011/10/002077).

5.5. CONSORT statement
The study was reported as per the recommendations of the CONSORT group (Schulz KF., 2010).
6. STUDY SUBJECTS SELECTION PROCEDURE

The subjects were screened (n=242) from the health camp for elderly, Geriatric clinic of Shri B.M.Patil Medical College and volunteers enrolled through advertisement. Screening for subjects was done from 15th October 2012 to 15th December 2012. Those subjects with PP > 60mmHg were selected for the study after thorough examination as per our inclusion and exclusion criteria.

7. RANDOMIZATION

Subjects who were selected from screening at Visit 1, 2 & 3 were allocated a subject number. The allocated volunteer randomization numbers were used to identify the volunteer during the entire study. They were then allocated for either yoga group (n=30) for yoga intervention or control group (n=30) for walking intervention using the random number table following baseline recordings.
8. INTERVENTION

8.1. Yoga Intervention

The participants allocated for yoga group were assigned for yoga practice for 6 days in a week for one hour daily in the morning from 06:00 hrs to 07:00 hrs for twelve weeks under the supervision of authorized yoga instructor. The yoga training included Loosening practices, Asanas (maintaining postures), Pranayama (breathing exercises) and Cyclic Meditation: yoga based guided relaxation technique (Pailoor S & Telles S et al., 2009). The integrated yoga module for elderly participants is shown in Table 9. Yoga intervention was given from 15th January to 17th April 2013 at Shri P.G.Halkatti Hall, BLDE University Campus.

8.2. Walking intervention

The protocol for the control group consisted of flexibility or loosening practices for 15-20 minutes followed by brisk-walk for 35-40 minutes and rest for 5 minutes for 6 days in a week for one hour daily in the morning from 06:00 hrs to 07:00 hrs for twelve weeks under the supervision of authorized instructor (Table 10). Walking intervention was given from 15th January to 17th April 2013 at BLDE University Campus.
### Table 9 Integrated yoga module for elderly subjects with hypertension

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Practice</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Opening Prayer</td>
<td>1 min</td>
</tr>
<tr>
<td>2.</td>
<td>Sukshma Vyayama (Loosening Practices)</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Loosening of Fingers</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Loosening of Wrist</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Shoulder rotation</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Ankle stretch/rotation</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Drill walking</td>
<td>5 min</td>
</tr>
<tr>
<td>3.</td>
<td>Breathing Practices</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Hands in and out breathing</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Ankle stretch breathing</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Straight leg raising breathing</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Lumbar stretch breathing</td>
<td>5 min</td>
</tr>
<tr>
<td>4.</td>
<td>Asana (Maintaining Postures)</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Utkatasana</td>
<td>15 min</td>
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<tr>
<td></td>
<td>Padhastasana</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Ardhachakrasana</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Shashankasana</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Ardha Ustrasana</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Bhujangasana</td>
<td>15 min</td>
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<tr>
<td></td>
<td>Ardha Salabrasana</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>Trikonasana</td>
<td>15 min</td>
</tr>
<tr>
<td>5.</td>
<td>Pranayama</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Anuloma Viloma Pranayama</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Brahmari Pranayama</td>
<td>5 min</td>
</tr>
<tr>
<td>6.</td>
<td>Cyclic Meditation [CM]</td>
<td>23 min</td>
</tr>
<tr>
<td>7.</td>
<td>Devotional Session – Chanting / Bhajans</td>
<td>5 min</td>
</tr>
<tr>
<td>8.</td>
<td>Closing prayer</td>
<td>1 min</td>
</tr>
</tbody>
</table>
**Table 10** Practices for control group participants

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Practice</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Loosening practices</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neck flexion/extension stretch</td>
<td>15-20 min</td>
</tr>
<tr>
<td></td>
<td>Neck lateral flexion stretch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shoulder stretch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>shoulder rotation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>wrist stretch/rotation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arm/trunk stretch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hip stretch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Side bend</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward bend</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lumbar extension stretch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lumbar flexion stretch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adductor stretch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ankle rotations</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hamstring stretch</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calf stretch</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Brisk-Walk</td>
<td>35-40 min</td>
</tr>
<tr>
<td>3</td>
<td>Rest</td>
<td>05 min</td>
</tr>
</tbody>
</table>
9. MEASUREMENTS AT EACH VISIT

**Visit 1: Screening-1\textsuperscript{st} Day**
- Medical history, including history of past use of medications, demographics (date of birth & sex) and personal history of alcohol & tobacco consumption.
- Measurement of brachial blood pressure (mmHg)

**Visit 2: Screening-2\textsuperscript{nd} Day**
- Measurement of brachial blood pressure (mmHg).

**Visit 3: Screening-3\textsuperscript{rd} Day**
- Measurement of brachial blood pressure (mmHg).
- General physical examination
- Estimation of blood glucose and lipid profile

**Visit 4: Baseline investigation**
- Anthropometric measurements such as height, weight & body mass index (BMI).
- Physiological parameters such as BP & heart rate.
- Measurement of arterial stiffness
  - Brachial-ankle pulse wave velocity (m/s)
  - Carotid-Femoral pulse wave velocity (m/s)
  - Aortic augmentation index
  - Arterial stiffness index at Brachial and Tibial arteries.
- Evaluation of cardiac autonomic activity
  - Heart rate variability analysis
- Estimation of serum nitric oxide concentration
- Estimation of serum malondialdehyde
- Estimation of reduced glutathione, serum vitamin C, serum super oxide dismutase.

**Visit 5: Post-intervention investigation**
- Anthropometric measurements such as height, weight & body mass index (BMI)
- Physiological parameters such as BP & heart rate.
Measurement of arterial stiffness
  ✓ Brachial-ankle pulse wave velocity (m/s)
  ✓ Carotid-Femoral pulse wave velocity (m/s)
  ✓ Aortic augmentation index
  ✓ Arterial stiffness index at Brachial and Tibial arteries.

Evaluation of autonomic activity
  ✓ Heart rate variability analysis

Estimation of serum nitric oxide concentration

Estimation of serum malondialdehyde.

Estimation of Erythrocyte reduced glutathione, serum vitamin C, serum super oxide dismutase
10. DETAILS OF MEASUREMENTS

All the recordings were made in the morning between 8.00hrs to 10.00hrs after supine rest for 10 minutes.

i. Anthropometric measurements

a. Height

Height was measured using a device (BIOCON™) mounted on the wall and is expressed in centimeters (cms).

b. Weight

Weight was measured using a weighing machine and is expressed in Kilograms (Kg).

c. Body Mass Index (BMI)

Body Mass Index was estimated from weight in Kilograms (Kg) divided by height in meters squared (m²) and was expressed as Kg/m².

ii. Physiological parameters

a. Heart rate (bpm)

Heart rate was determined using a digital physiograph (Physiopac, Medicaid Systems Ltd, India). It was calculated by using R-R Interval of electrocardiogram (ECG) and was expressed as beat per minute (bpm). ECG was recorded for five minutes using limb leads.

b. Measurement of blood pressure

- Systolic & Diastolic blood pressure (mmHg): As BP is more variable in older people, so we have taken average of nine BP readings (Supiano MA., 2009). Brachial BP was measured thrice with an interval of one minute on every visit for three consecutive days in a sitting posture using mercury sphygmomanometer (Pickering TG et al., 2005).

- Pulse pressure (mmHg): It is the pulsatile component of the blood pressure. It was estimated as the difference between systolic and diastolic blood pressure and expressed in mmHg.
o Mean arterial pressure (mmHg): It is an average arterial pressure in an individual during single cardiac cycle. It was estimated by adding 1/3rd of PP (mmHg) to the DBP (mmHg).

iii. **Assessment of arterial stiffness:**
Vascular function was assessed by oscillometric method using a non-invasive automatic device (Periscope, Genesis Medical Systems, India). Periscope is a validated 8-channel real time PC-based simultaneous acquisition (200 samples per second) and analysis system (Naidu MU et al., 2005). According to Nyquist’s criterion the minimum sampling rate should be twice the maximum input frequency which is sufficient to avoid aliasing and preserve all the input signal information (Faulkner EA et al., 1969). The significant frequency content of the pressure as well as ECG waveform was not more than 40 Hz; hence, a sampling rate of 200 samples per second was optimum. This device uses four BP cuffs and two-channel ECG leads to record arterial pressure waveforms and ECG (Lead I & II) simultaneously.

![Blood pressure cuffs and ECG electrodes placement for vascular analysis using Periscope.](image-url)
Figure 9b Vascular analysis using Periscope

Figure 10 Sheet 1: Results of vascular analysis given by Periscope at a glance
The recordings were made in supine position. BP cuffs were wrapped on both upper arm brachial artery and tibial artery above ankles. ECG electrodes were placed on the ventral surface of both wrists and medial side of the ankles (Figure 9a & b). The BP cuffs were connected to oscillometric pressor sensor and plethysmographic sensor located on the hardware of the system (Periscope) to determine pressure waveforms and volume pulse waveform. The data obtained in 10 seconds was stored in the computer for further analysis and to detect various arterial stiffness parameters. Periscope supports a sophisticated digital-signal algorithm to calculate all the results. As the device is fully automated and does not require any operator for handling any probe to record the waveforms, so it is devoid of any operator bias. Periscope is fully automatic, so once the test is started, the recording completes itself by displaying the results directly (Figure 10a & b).

Figure 10b Sheet 2: Results of vascular analysis given by Periscope at a glance
a. **Estimation of pulse wave velocity**

- Brachial-ankle PWV (baPWV), a measure of arterial stiffness (central artery & peripheral semi-muscular arteries) was estimated using arterial pressure waveforms (Brachial and Tibial artery) and ECG recordings (Lead I & II). The pulse transit time (PTT) in seconds elapsed between brachium and respective ankle was calculated as the time difference between the R-wave of ECG and foot of respective pulse wave. The distance between the brachium and ankle was calculated automatically according to the height of the subject. The PWV was calculated by dividing the distance by PTT (Figure 11).

\[
\text{brachial – ankle PWV} = \frac{L_{ba}}{PTT_{ba}}
\]

Where \( L_{ba} \) = Distance between respective brachium and ankle

\( PTT_{ba} \) = Pulse Transit Time (PTT) between brachium and respective ankle was calculated as the time difference between the feet of respective pulse wave originated from R-wave (QRS complex) of ECG.

![Figure 11 Pulse wave form and ECG and calculation of pulse transit time](image-url)
The carotid-femoral PWV (c-f PWV), a measure of central arterial (aortic) stiffness was calculated by the composite baPWV found out by averaging left and right baPWV. Periscope estimates the c-f PWV on the basis of equation (0.8333*Avg.baPWV-233.33) derived by regression analysis between baPWV and c-f PWV from the studies conducted elsewhere (Yamashina A et al., 2002).

\[
\text{Carotid – femoral PWV} = 0.8333 \times \text{Avg.baPWV} - 233.33
\]

b. Estimation of augmentation index
Periscope determines aortic pressure by Oscillometric PWV method. It estimates aortic pressure on the basis of regression equation derived by multivariate statistical analysis of invasive aortic pressure values (found by a fluid-filled catheter method) with respect to the brachial pressure and c-f PWV values obtained non-invasively by Periscope (Naidu MUR et al., 2012).

**Measurement of aortic pressure by Oscillometric PWV method:**
Aortic root pressure gradient is composed of two major components:
I. Systolic Pressure gradient – The rapid rise of pressure at the aortic root is contributed by the left ventricular pressure during systole. As soon as the left ventricle is emptied into the aorta, the aortic pressure falls rapidly. This gradient does not contribute to the aortic root pressure during diastole.

![Wave reflection diagram](image)

**Figure 12 Wave reflection**
II. Diastolic Pressure gradient – The pressure wave generated in the aorta during systole is propagated along the arterial tree which is resisted by the systemic vascular resistance from the branches at various points. From this various points of impedance mismatch at different arterial branches, the waves reflect back as a single wave (wave reflection) to the aorta during diastole and contribute for diastolic pressure gradient (Figure 12).

Thus, the aortic root pressure is mainly dependent on two components: Left ventricular systolic pressure and wave reflection pressure.

The timing of arrival of wave reflection at the aortic root is dependent on the arterial stiffness. The wave reflection arrives earlier during systole in the stiffened arteries due to increase in PWV and contributes to augmentation of aortic systolic pressure. Thus, the resultant aortic root pressure increases in proportion with the arterial stiffness.

Periscope uses brachial BP and c-f PWV to determine the aortic root pressure. It is based on the mathematical analysis of invasive aortic pressure values (Fluid-filled catheter method) with respect to the brachial BP and PWV found non-invasively. Aortic root pressure values are directly proportional to a combination of both the brachial pressure value and c-f PWV. A significant correlation was found in these parameter values when multivariate regression analysis was carried out. Equation relating aortic pressure value, brachial pressure and c-f PWV with respective coefficients was derived from this and added in the Periscope to determine equivalent aortic pressure.

The rise in the systolic pressure is called an augmentation pressure. The augmentation index (AIx) is the ratio of augmentation pressure to the aortic PP and is expressed in percentage. This oscillometric PWV method used for estimation of AIx by periscope has been validated (Naidu MUR et al., 2012). As it was reported that AIx is influenced by heart rate, an index normalized for a heart rate of 75 bpm (AIx@75) was used in this study (Wilkinson IB et al., 2000).
c. Calculation of arterial stiffness index

Arterial stiffness index (ASI), an another measure of local and peripheral arterial stiffness was estimated at brachial artery (bASI) and tibial artery (aASI) by quantifying the oscillometric envelopes derived from the oscillations in the respective artery (Naidu MUR et al., 2012).

\[
ASI = (\text{Systolic side Value of cuff pressure at 80\% of maximal oscillation amplitude of cuff}) - (\text{Diastolic side Value of cuff pressure at 80\% of maximal oscillation amplitude of cuff}).
\]

Figure 13 Oscillometric Envelope

**Oscillometric envelope**

An oscillometric envelope is a graph constructed by mapping the change in arterial pulse amplitude in response to changing cuff pressure (Acton A., 2013) (Figure 13). It is a graphical depiction of compressibility of the artery. It is derived from the oscillations in the artery during the deflation of BP cuffs while recording BP by
oscillometric method. The shape of the oscillometric envelope is bell-shaped in normal artery where as it is flattened in stiffened artery. It becomes harder to collapse the stiffened arteries by applying external pressure; hence the oscillometric envelope becomes flatter as the stiffness increases. The ASI value gives a clear indication of this flattening process (Figure 13). The ASI values increases with an increase in arterial stiffness.

iii. Assessment of heart rate variability

Heart rate variability (HRV) is an established tool for evaluation of autonomic activity (sympathetic and parasympathetic tone). HRV is the physiological variation in time interval between heart beats. It is measured by the variation in the beat-to-beat interval.

Procedure

ECG was recorded in the standard limb lead II configuration for five minutes using a four channel digital polygraph (Medicaid systems Pvt Ltd, Chandigarh, India). The subjects were asked to breathe normally during the ECG recording. ECG recordings were exported from the digital polygraph for HRV analysis. The recorded data were visually inspected off-line and only noise free data were included for analysis. No ectopic beats were found on offline scrutiny. HRV analysis software version 2.0 developed by the Biomedical Signal Analysis group, University of Kuopio, Finland was used for HRV analysis (Tarvainen MP et al., 2014). HRV analysis was done by Frequency domain method. A non-parametric Fast Furrier Transform (FFT) technique was used to obtain the Power spectral density of the RR Series (Figure 14).

Total power in the frequency range (0-0.40Hz) was divided into very low frequency (VLF: 0.0-0.04), low frequency (LF: 0.04-0.15Hz) and high frequency (HF: 0.15-0.40Hz).

- LF is a measure of both sympathetic and parasympathetic activity, but mainly reflects sympathetic activity.
HF measure reflects parasympathetic activity. The LF and HF components were expressed in normalized units (nu).

LF/HF ratio was calculated to assess overall balance between the sympathetic and the parasympathetic systems (sympathovagal balance).

HRV analysis was done as per the guidelines of a Task force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (Task force of the ESC and the NAS of Pacing and Electrophysiology, 1996).

Figure 14 Fast Furrier Transform (FFT) spectrum

iv. Evaluation of endothelial function

a. Estimation of serum nitric oxide concentration

Total serum nitric oxide concentration (NOx) was measured as an index of endothelial function. Serum NOx was estimated by improved Griess method using vanadium chloride as a reducing agent for reduction of nitrate to nitrite (QuantiChrom™ Nitric Oxide Assay Kit: D2NO-100, BioAssay Systems, USA).
The subjects were advised to abstain from foods such as cured meat, fish, cheese, herbal or black tea, beer, wine and malted beverages on the previous day to avoid dietary effect on NOx (Choi JW et al., 2001). To avoid change in the serum NO levels secondary to physical activity, subjects were given rest for at least 10 minutes before collection of blood sample.

**Principle**
Since NO is unstable and oxidized to nitrite and nitrate, it is common practice to quantitate total NO$_2$/NO$_3$ as a measure for NO level. Nitrate was reduced to nitrite by vanadium chloride (VCl$_3$) after deproteinization of serum sample by somogyi reagent (NaOH & ZnSO$_4$). The nitrite produced was determined by diazotization of sulfanilamide and coupling to naphthylethylene diamine.

**Reagents**
1. ZnSO$_4$ Solution (75mMol/L)
2. NaOH solution (55mMol/L)
3. Vanadium chloride III
4. Griess reagent
   a. Sulfanilamide
   b. N-Naphthylethylene diamine
5. NaNO$_2$ standard (1.0 mM/L)

**Procedure**
1. Deproteination
   150 µl of sample was mixed with 8 µl ZnSO$_4$ in 1.5 ml eppendorf tube. 8 µl of NaOH was added following vortex for one minute. The mixture was vortexed again and centrifuged for 10 min at 14,000 rpm. Clear supernatant obtained was transferred to a clean tube.
2. Standards
   1.0 ml of working standard (100 µM/L) was prepared by mixing 0.1 mL of 1.0 mM/L NaNO$_2$ standard with 0.9 mL of distilled water.
Following calibrators were prepared from the working standard.

<table>
<thead>
<tr>
<th>No.</th>
<th>Working standard</th>
<th>Distilled water</th>
<th>Nitrite (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500 µL</td>
<td>----</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>300 µL</td>
<td>200 µL</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>150 µL</td>
<td>350 µL</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>---</td>
<td>500 µL</td>
<td>0 (blank)</td>
</tr>
</tbody>
</table>

3. Reaction

i. Working reagent (WR) for all samples and standards was prepared by mixing per reaction tube

   a. 400 µL - Sulfanilamide
   b. 400 µL - N-Naphthylethylene diamine
   c. 200 µL - Vanadium chloride III

ii. 400 µL of deproteinated sample and calibrators were added in a separate labeled eppendorf tubes.

iii. Then 800 µL of working reagent was added to each tubes.

iv. Incubated for 10 min at 60ºC.

**Standard graph**
Measurement
Optical density (OD) was read at 540 nm (UV-1700, UV-visible spectrophotometer, Scimadzu).

Calculation
i. Standard graph was plotted using OD against standard concentrations.
ii. Slope was determined using linear regression fitting.
iii. The NO concentration of sample was calculated as

\[
Serum \ NO \ (\mu M) = \frac{OD_{sample} - OD_{blank}}{Slope}
\]

b. Augmentation index (Alx)
Procedure of measurement of augmentation index is described in section 10.iii.b.

v. Evaluation of oxidative stress and antioxidant status
The blood sample was collected in the morning with overnight fasting for estimation of biochemical parameters.

a. Estimation of Serum malondialdehyde (MDA)
Serum malondialdehyde (MDA), a marker of oxidative stress was estimated by Kei Satoh method (Satoh K., 1978).

Principle
Auto-oxidation of unsaturated fatty acids involves the formation of semi-stable peroxides, which then undergo a series of reactions to form malondialdehyde. Malondialdehyde reacts with Thiobarbituric acid to form pink colored chromogen. The resulting chromogen was extracted with 4.0 ml of n-butyl alcohol and the absorbance of which was measured at 530 nm.

Reagents
1. Trichloroacetic acid (TCA) reagent: 20g/dl TCA in 100 ml distilled water to prepare 20% TCA.
2. Sodium sulphate solution (2M): 28.4 gm of anhydrous sodium sulfate was mixed in 90 ml of distilled water by heating and stirring. Then distilled water was added to make final volume of 100 ml.

3. Thiobarbituric acid (TBA) reagent: 670 mg of TBA in 100ml of 2M sodium sulphate solution.

4. Sulphuric acid (0.05M)

5. N-butyl alcohol

**Standards**

Following calibrators were prepared from the working standard (10nmol/ml).

<table>
<thead>
<tr>
<th>No.</th>
<th>Working standard</th>
<th>Distilled water</th>
<th>MDA (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.0 ml</td>
<td>----</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>2.5 ml</td>
<td>0.5 ml</td>
<td>8.3</td>
</tr>
<tr>
<td>3</td>
<td>2.0 ml</td>
<td>1.0 ml</td>
<td>6.7</td>
</tr>
<tr>
<td>4</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>1.0 ml</td>
<td>2.0 ml</td>
<td>3.3</td>
</tr>
<tr>
<td>6</td>
<td>0.5 ml</td>
<td>2.5 ml</td>
<td>1.7</td>
</tr>
<tr>
<td>7</td>
<td>0 ml</td>
<td>3.0 ml</td>
<td>0</td>
</tr>
</tbody>
</table>

**Standard Graph**
Procedure
1. 300 µl of serum and 1.5 mL of TCA was taken in a test tube and kept for 10 min at room temperature.
2. Centrifugation at 3500 rpm for 10 min was done.
3. The supernatant was decanted and the precipitate obtained was washed with 0.05M Sulphuric acid.
4. 1.5 mL of 0.05M Sulphuric acid and 3 mL of TBA reagent were added to the precipitate.
5. The test tube containing the mixture was kept in a boiling water bath for 30 min.
6. Then the tube was cooled in cold water followed by addition of 2.4 mL of n-butyl alcohol with vigorous shaking to extract the chromogen.
7. Separation of organic phase was facilitated by centrifugation at 3000 rpm for 10 min.
8. The absorbance (OD) was read at the 530 nm wavelength using spectrophotometer.

Calculation

Concentration of serum MDA (nmol/ml)

\[
\text{Concentration of serum MDA (nmol/ml)} = \frac{\text{OD of Test}}{\text{Nano-molar Extinction Coefficient}} \times \frac{\text{Total volume of solution in cuvette}}{\text{Sample volume}}
\]

\[
= \frac{\text{OD of Test}}{1.56 \times 10^5} \times \frac{X}{109} \times \frac{X^{2.4}}{0.3}
\]

\[
= \text{OD of the Test x 51.28 nmol/ml.}
\]

b. Estimation of Reduced glutathione (GSH)

Blood reduced glutathione (GSH) was estimated by Earnest Beutler method (Beutler E et al. 1963).

Principle

Non-protein sulphhydryl groups of red blood cells (RBC) are present in the form of reduced glutathione (GSH). 5, 5’–dithiobis-2-nitrobenzonic acid (DTNB) is a
disulphide compound which is readily reduced by sulphydryl compounds, forming a highly colored yellow compound. Optical density was measured at 412 nm and it is directly proportional to GSH concentration.

Reagent

1. Precipitating solution: 1.67gm of glacial metaphosphoric acid, 0.2gm of disodium or dipotassium ethylene diamine tetra acetic acid (EDTA) and 30 gm of sodium chloride was dissolved in 100ml of distilled water.
2. Phosphate solution: 0.3M Na$_2$HPO$_4$ (di-sodium hydrogen phosphate) was prepared by dissolving 4.68gm in 100 mL distilled water.
3. 1% Sodium citrate: 1gm of sodium citrate was dissolved in 100ml distilled water.
4. DTNB reagent: 40mg 5, 5’dithiobis– (2-nitrobenzonic acid) was dissolved in 100ml of 1% sodium citrate.
5. Reduced glutathione standard (0.5 mg/ml): Take 5 mg of reduced glutathione and dissolved in 10 ml of distilled water.

Procedure

Three test tubes were taken and labeled as blank, standard and test. The procedure of the assay was as given below.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>--</td>
<td>--</td>
<td>0.2 mL</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>0.4 mL</td>
<td>--</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2 mL</td>
<td>1.6Ml</td>
<td>1.8 mL</td>
</tr>
</tbody>
</table>

Mixed well

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitating Solution</td>
<td>3.0 mL</td>
<td>3.0 mL</td>
<td>3.0 mL</td>
</tr>
</tbody>
</table>
Kept for five minutes, centrifuged and 1 ml supernatant was added in a separate labeled test tubes

<table>
<thead>
<tr>
<th></th>
<th>4.0 mL</th>
<th>4.0 mL</th>
<th>4.0 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate solution</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DTNB Reagent</td>
<td>0.5 mL</td>
<td>0.5 mL</td>
<td>0.5 mL</td>
</tr>
</tbody>
</table>

Mixed and absorbance was read at 412 nm against the blank within 5 minutes

**Calculation**

Concentration of Erythrocyte reduced glutathione

\[
\text{Concentration} = \frac{OD_{\text{test}}}{OD_{\text{Std}}} \times \frac{\text{Conc of Std}}{\text{Volume of test}} \times 100
\]

\[
= \frac{OD_{\text{test}}}{OD_{\text{Std}}} \times 0.04 \times 100
\]

\[
= \frac{OD_{\text{Test}}}{OD_{\text{Std}}} \times 50
\]

\[
= \ldots \ldots \ldots \ldots \text{mg/dl}
\]

c. **Estimation of superoxide dismutase (SOD)**

Superoxide dismutase (SOD) activity was measured by Marklund and Marklund method (Marklund S & Marklund G., 1998).

**Principle**

Superoxide anion is involved in auto-oxidation of pyrogallol at alkaline pH (8.5). The superoxide dismutase inhibits auto-oxidation of pyrogallol which can be determined as an increase in absorbance at 420 nm.

**Reagents**

1. Tris buffer (0.05M): 50 mM of Tris buffer and 1 mM of EDTA was mixed with distilled water and HCL was added to adjust the pH at 8.5. A final volume of 100 ml solution at pH 8.5 was prepared.
2. Pyrogallol (20mM): 25 mg pyrogallol was dissolved in 10 mL distilled water.

Procedure
1. Control: 2.9 ml of Tris buffer was taken in a cuvette to which 0.1 ml of Pyrogallol was added. Then absorbance (OD) was read at 420 nm after 1 min 30 sec and 3 min 30 sec.
2. Test: 2.8 ml of Tris buffer and 0.1 ml of serum was taken in a cuvette to which 0.1 ml of Pyrogallol was added. Then absorbance (OD) was read at 420 nm after 1 min 30 sec and 3 min 30 sec.
3. Difference in absorbance (ΔA/min) was calculated as

\[
\Delta A/\text{min} = \frac{\text{OD at 3 min 30 sec } - \text{OD at 1 min 30 sec}}{2}
\]

Calculation
Serum SOD activity = \(\frac{\Delta A/\text{min of control} - \Delta A/\text{min of Test}}{\Delta A/\text{min of control} \times 50} \times 100 \times \frac{1}{\text{volume of sample}}\)

\[
= \frac{C-T}{C \times 50} \times 100 \times \frac{1}{0.1}
\]

\[
= \frac{C-T}{C \times 50} \times 1000
\]

\[
= \text{---------- U/ml.}
\]

One unit of SOD is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

d. Estimation of serum vitamin C
Serum vitamin C was estimated by 2, 4-dinitrophenylhydrazine method (Roe JH et al., 1943; Brewster MA., 1996)

Principle
Ascorbic acid was oxidized by copper to form dehydroascorbic acid and diketogluconic acid. These products were treated with 2,4-dinitrophenyl
hydrazine (DNPH) to form the derivative bis-2,4-dinitrophenyl hydrazone. This compound in strong sulfuric acid undergoes rearrangement to form a colored product which was measured at 520 nm. The reaction was run in the presence of thiourea to provide a mildly reducing medium which helps to prevent interference from non-ascorbic acid chromogen.

Reagents
1. 10 % Trichloroacetic acid: 10 gm of Trichloroacetic acid (TCA) was dissolved in distilled water to prepare a final volume of 100 ml.
2. DTC reagent: 3.0 gm of 2, 4-dinitrophenyl hydrazine (DNPH), 0.4 gm Thiourea and 0.05 gm copper sulphate were added to 9N sulfuric acid. The final volume of 100 ml was prepared.
3. 65 % sulfuric acid: 65 ml of sulfuric acid was dissolved in 35 mL distilled water.
4. Stock standard: 100 mg ascorbic acid was dissolved in 100 mL of 5 % TCA.
5. Working standard (10µg/mL): 1mL of stock standard was dissolved in 100 mL of 5 % TCA.

Procedure
1. Deproteination
   500 µl of sample was mixed with 500 µl of 10% TCA in an eppendorf tube. Vortexed and then centrifuged. The clear supernatant protein free filtrate was used.
2. 500 µl of sample and standards were taken in a test tube separately to which 100 µl DTC reagent was added.
3. Incubated at 37°C for 3 hours.
4. 750 µl of 65% sulfuric acid was added to all the test tubes.
5. Vortexed and kept for 30 minutes at room temperature.
6. Absorbance was read at 520 nm.
Calculation

Concentration of Serum Vitamin C

\[
\frac{\text{OD of test}}{\text{OD of Std}} \times \frac{\text{Conc of Std}}{\text{Volume of test}} \times 100
\]

\[
= \frac{\text{OD of test}}{\text{OD of Std}} \times 0.005 \times 100
\]

\[
= \frac{\text{OD of Test}}{\text{OD of Std}} \times 2
\]

\[
= \ldots \ldots \ldots \text{mg/dl}
\]

Standard Graph

![Absorbance vs. Ascorbic acid (mg/dL)]

vi. Estimation of blood glucose

Fasting blood glucose was estimated by Trinder's method (Trinder P., 1969).

(Erba diagnostics Mannheim)

Principle

Glucose in sample was oxidized to yield gluconic acid and hydrogen peroxide in the presence of Glucose oxidase. The enzyme peroxidase catalyses the oxidative coupling
of 4-aminoantipyrine with phenol to yield a colored quinoneimine complex, the absorbance was proportional to the concentration of glucose in sample.

**Reagent**

1. **Enzyme reagent**

<table>
<thead>
<tr>
<th>Active ingredients</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose oxidase</td>
<td>≥ 2000 U/L</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>≥ 2000 U/L</td>
</tr>
<tr>
<td>Phenol</td>
<td>10 mmol/L</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>200 mmol/L</td>
</tr>
</tbody>
</table>

2. **Glucose standard:** 100mg/dl

**Procedure**

1. Three test tubes were taken and labeled as blank, standard and test. The procedure of the assay was as follows.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>--</td>
<td>--</td>
<td>10 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>10 µl</td>
<td>--</td>
</tr>
<tr>
<td>Enzyme reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

2. Mixed well and incubated at 37°C for 5 minutes.
3. Absorbance of test and standard was read against blank at 505/670 nm.

**Calculation**

Glucose (mg/dl)

\[
\text{Glucose (mg/dl)} = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{Concentration of standard (100mg/dl)}
\]
**Precision of the assay**

1. Inter-assay co-efficient of variability (CV): 2.34%

<table>
<thead>
<tr>
<th></th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (mg/dl)</td>
<td>68</td>
<td>185</td>
</tr>
<tr>
<td>SD</td>
<td>1.9</td>
<td>3.5</td>
</tr>
<tr>
<td>CV %</td>
<td>2.79</td>
<td>1.89</td>
</tr>
</tbody>
</table>

2. Intra-assay co-efficient of variability (CV): 2.47%

<table>
<thead>
<tr>
<th></th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (mg/dl)</td>
<td>72</td>
<td>165</td>
</tr>
<tr>
<td>SD</td>
<td>1.26</td>
<td>5.24</td>
</tr>
<tr>
<td>CV %</td>
<td>1.75</td>
<td>3.18</td>
</tr>
</tbody>
</table>

**vii. Estimation of lipid profile**

The blood sample was collected in the morning with overnight fasting for estimation of lipid profile (Erba diagnostics Mannheim).

**a. Estimation of Serum triglyceride**

Serum triglyceride was estimated by glycerol phosphatase-oxidase (GPO-PAP) method (Bucolo G & David H., 1973; Fossati P & Prencipe L., 1982; McGowan MW et al., 1983).

**Principle**

Triglycerides were enzymatically hydrolyzed by lipase to glycerol and free fatty acids. The glycerol was subsequently measured by a coupled enzymatic reaction system. The glycerol released was phosphorylated to glycerol-3-phosphate by glycerol kinase. The glycerol-3-phosphate was oxidized by glycerol phosphate
oxidase to produce dihydroxyacetone phosphate and hydrogen peroxide. Peroxidase catalyzed the reaction of hydrogen peroxide with 4-aminoantipyrine and 3, 5-Dichloro-2-hydroxybenzene sulfonate. The absorbance of chromogen formed was measured at 505 nm. The intensity of the chromogen (Quinoneimine) formed was proportional to the triglycerides concentration in the sample.

**Reagents**

1. Triglyceride reagent: ATP (2.5 mmol/L), Mg$^{2+}$ (2.5 mmol/L), 4-aminoantipyrine (0.8 mmol/L), 3, 5-Dichloro-2-hydroxybenzene sulfonate (1 mmol/L), Peroxidase (>2000U/L), Glycerol Kinase (>550 U/L), Glycerol phosphate oxidase (>8000U/L), Lipoprotein Lipase (>3500 U/L), Buffer (53mmol/L, pH 7.0 ± 0.1 at 20$^0$C).

2. Triglyceride standard (200mg/100ml).

**Procedure**

1. Three test tubes were taken and labeled as blank, standard and test. The procedure of the assay was as follows.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>--</td>
<td>--</td>
<td>10 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>10 µl</td>
<td>--</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Working Reagent</td>
<td>1000 µl</td>
<td>1000 µl</td>
<td>1000 µl</td>
</tr>
</tbody>
</table>

2. Mixed well and incubated at 37$^0$C for 10 minutes.

3. Absorbance of test and standard was read against blank at 505nm.

**Calculation**

Triglycerides (mg/dl)

\[
= \frac{\text{OD of test}}{\text{OD of standard}} \times \text{Concentration of standard (200mg/dl)}
\]
Precision of the assay

a. Inter-assay co-efficient of variability (CV): 4.15%

<table>
<thead>
<tr>
<th></th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (mg/dl)</td>
<td>81</td>
<td>140</td>
</tr>
<tr>
<td>SD</td>
<td>3.2</td>
<td>6.1</td>
</tr>
<tr>
<td>CV %</td>
<td>3.95</td>
<td>4.35</td>
</tr>
</tbody>
</table>

b. Intra-assay co-efficient of variability (CV): 4.15%

<table>
<thead>
<tr>
<th></th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (mg/dl)</td>
<td>82.1</td>
<td>139.5</td>
</tr>
<tr>
<td>SD</td>
<td>3.4</td>
<td>5.8</td>
</tr>
<tr>
<td>CV %</td>
<td>4.14</td>
<td>4.16</td>
</tr>
</tbody>
</table>

b. Estimation of Serum cholesterol

Cholesterol was estimated by cholesterol oxidase-peroxidase (CHOD-PAP) enzymatic method (Allian CC et al., 1974; Roeschlau P et al., 1974)

Principle

Cholesterol esters were hydrolyzed by Cholesterol esterase to cholesterol and free fatty acids. Free cholesterol was oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. This hydrogen peroxide combined with 4- aminoantipyrine to form a chromophore (quinoneimine dye) which was measured at 505 nm.

Reagents

1. Reagent
   - Good’s buffer (50mmol/L)
   - Phenol (5 mmol/L)
Chapter III  Study Plan & Procedure

- 4-aminoantipyrine (0.3 mmol/L)
- Cholesterol esterase (≥200 U/L)
- Cholesterol oxidase (≥ 50 U/L)
- Peroxidase (≥ 3 kU/L)

2. Standard
   - Cholesterol (200mg/100ml)

**Procedure**

1. Three test tubes were taken and labeled as blank, standard and test. The procedure of the assay was as follows.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>--</td>
<td>--</td>
<td>10 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>10 µl</td>
<td>--</td>
</tr>
<tr>
<td>Reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10 µl</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

2. Mixed well and incubated at 37°C for 10 minutes.
3. Absorbance of test and standard was read against blank at 505nm.

**Calculation**

\[
\text{Cholesterol (mg/dl)} = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{Concentration of standard (200mg/dl)}
\]

**Precision of the assay**

a. Inter-assay co-efficient of variability (CV): 2.38%

<table>
<thead>
<tr>
<th></th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (mg/dl)</td>
<td>122.2</td>
<td>216.02</td>
</tr>
<tr>
<td>SD</td>
<td>3.1</td>
<td>4.82</td>
</tr>
<tr>
<td>CV %</td>
<td>2.53</td>
<td>2.23</td>
</tr>
</tbody>
</table>
b. Intra-assay co-efficient of variability (CV): 2.44%

<table>
<thead>
<tr>
<th></th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (mg/dl)</td>
<td>116.25</td>
<td>196.83</td>
</tr>
<tr>
<td>SD</td>
<td>2.61</td>
<td>4.69</td>
</tr>
<tr>
<td>CV %</td>
<td>2.5</td>
<td>2.38</td>
</tr>
</tbody>
</table>

c. **Estimation of HDL cholesterol**

High density lipoprotein (HDL) cholesterol was estimated by phosphotungstic acid (PTA) method (Burstein M et al., 1970).

**Principle**

Phosphotungstic acid precipitates low and very low density lipoproteins (LDL & VLDL) in the presence of divalent cations such as magnesium. The high density lipoprotein (HDL) cholesterol which remains unaffected in the supernatant was estimated using cholesterol reagent.

**Reagents**

1. Precipitating reagent: Phosphotungstic acid (0.77 mmol/l) & Magnesium chloride (17.46 mmol/l)
2. Cholesterol working reagent
   - Good’s buffer (50mmol/L)
   - Phenol (5 mmol/L)
   - 4-aminoantipyrine (0.3 mmol/L)
   - Cholesterol esterase (≥200 U/L)
   - Cholesterol oxidase (≥ 50 U/L)
   - Peroxidase (≥ 3 kU/L)
3. HDL cholesterol standard (50mg/dl)
Procedure

1. Precipitation: 500 µl of precipitating reagent was added to 250 µl serum and standard. Mixed well and kept for 10 minutes at room temperature to allow reaction, and centrifuged at 4000 rpm for 10 minutes. The clear supernatant was used for further reaction.

2. Three test tubes were taken and labeled as blank, standard and test. The procedure of the assay was as follows.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>--</td>
<td>--</td>
<td>50 µl</td>
</tr>
<tr>
<td>Standard</td>
<td>--</td>
<td>50 µl</td>
<td>--</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 µl</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cholesterol working reagent</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

3. Mixed well and incubated at 37°C for 10 minutes.

4. Absorbance of test and standard was read against blank at 500nm.

Calculations

HDL Cholesterol (mg/dl)

\[
\text{HDL Cholesterol (mg/dl)} = \frac{\text{OD of test}}{\text{OD of standard}} \times \text{Concentration of standard (50mg/dl)}
\]

Precision of the assay

a. Inter-assay co-efficient of variability (CV): 5.76%

<table>
<thead>
<tr>
<th></th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (mg/dl)</td>
<td>36.8</td>
<td>62.08</td>
</tr>
<tr>
<td>SD</td>
<td>1.86</td>
<td>4.02</td>
</tr>
<tr>
<td>CV %</td>
<td>5.05</td>
<td>6.47</td>
</tr>
</tbody>
</table>
b. Intra-assay co-efficient of variability (CV): 5.3%

<table>
<thead>
<tr>
<th></th>
<th>Level 1</th>
<th>Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean (mg/dl)</td>
<td>41.2</td>
<td>68.2</td>
</tr>
<tr>
<td>SD</td>
<td>2.01</td>
<td>3.9</td>
</tr>
<tr>
<td>CV %</td>
<td>4.88</td>
<td>5.71</td>
</tr>
</tbody>
</table>
11. STATISTICAL ANALYSIS

- The obtained data was expressed in mean and standard deviation.
- Level of significance: Statistical significance was established at p< 0.05.
- An Unpaired ‘t’ test was used to find the difference between pre-intervention or baseline values of study and control groups.
- Paired ‘t’ test (normal distribution data) and Wilcoxon signed rank test (non-normal distribution data) were applied to determine the significant difference between pre-intervention and post-intervention values within the group.
- Analysis of Covariance (ANCOVA) was applied to find the differences in the post-intervention values between study and control groups while controlling the pre-test values. It evaluates whether there is any difference in post-intervention or dependent variable (DV) between groups by reducing the within group error variance (while controlling for the effects of other continuous variables that are not of primary interest, known as covariates). ANCOVA is strongly recommended for randomized controlled studies to find the effect of treatment (Van Breukelen GJ et al., 2006). In ANCOVA, the pre-intervention or pre-test values are used as a covariate and the post-intervention values of study and control group are used as dependent variables. The purpose of using the pre-intervention values as a covariate in randomized controlled study design is to reduce the error variance and to correct the baseline imbalances (eliminate systematic bias). However, the random assignment of subject to groups guards against baseline imbalances (systematic bias), so the purpose of the ANCOVA in randomized studies is mainly to reduce error variance (Dimitrov DM & Rumrill PD Jr., 2003). ANCOVA informs whether there is an overall statistically significant difference in post-intervention values between the different interventions once their means had been adjusted for pre-intervention or baseline values.
- Data were analyzed by using SPSS software version 20.
12. REFERENCES


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