CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Instruments used

- Mercury sphygmomanometer (Diamond BP MR120, Bombay, India)
- TANITA TBF-410 Pro Body Composition Analyzer (Tanita Corporation of America, Inc. USA)
- Weighing machine (ESSAE, EEROKA Limited, Bangalore, India)
- Stadiometer (Standard Steel, Ambala, Haryana, India)
- Fully automated biochemistry analyzer (ERBA-EM 200, Erba Lachema, Czech Republic).

3.1.2 Chemicals used

- Acetic acid (Loba Chemie Pvt. Ltd., Colaba, Mumbai)
- Ferric chloride (Loba Chemie Pvt. Ltd., Colaba, Mumbai)
- Ferrous sulphate (Loba Chemie Pvt. Ltd., Colaba, Mumbai)
- Sodium acetate (S. D. Fine Chem Ltd., Boisar)
- Sodium hydroxide (Sisco research laboratories Pvt. Ltd., Maharashtra)
- MDA, 95% (Loba Chemie Pvt. Ltd., Colaba, Mumbai)
- Thiobarbituric acid (Loba Chemie Pvt. Ltd., Colaba, Mumbai)
- Trichloroacetic acid (TCA) (Loba Chemie Pvt. Ltd., Colaba, Mumbai)
- 2,4,6-tripyridyl-S-triazine (TPTZ) (Loba Chemie Pvt. Ltd., Colaba, Mumbai)

3.1.3 Source of data

Subjects who had enrolled for yoga camps conducted at different places of Dakshina Kannada, Karnataka, between May 2014 and June 2015, were included in this study. After routine investigations and considering the inclusion and exclusion criteria, study participants
(n=200) were recruited. Routine investigations included fasting blood glucose, serum creatinine and thyroid profile.

3.1.4 Inclusion criteria

- Subjects were aged 25 -35 yrs of both genders.
- Subjects who had body mass index (BMI) ≥23 to ≤40.
- Subjects willing to participate in the study

3.1.5 Exclusion criteria

- Overweight or obese subjects with diabetes or thyroid abnormalities.
- Subjects under medication for any other illness
- Pregnant or lactating women or women on oral contraceptives
- Subjects who were unable to perform yoga.

3.1.6 Sample size

The sample size was calculated based on the results obtained from a pilot study. The pilot study included 32 participants, of which 14 were overweight (23≤BMI ≤24.9) and 18 were obese (25≤BMI ≤40).

3.1.6.1 Pilot study

A pilot study was conducted with 18 obese adults aged 31.4 ± 3.0 years and BMI 32.1 ± 2.9 kg/m² and 14 overweight adults aged 29.1 ± 2.1 years and BMI 24.0 ± 0.5 kg/m² to check the feasibility of the study protocol. The subjects underwent 10 days of residential yoga training which was designed for the study. The assessments for anthropometric variables, body composition and serum lipid profile were made before (pre test) and after 10 days (post test) of yoga intervention. Significant changes were observed in the bodyweight, BMI, triglycerides (TAG), total cholesterol and LDL cholesterol (LDL-C) following intervention.

Yoga training resulted in significant decrease in body mass index (BMI), in the subjects of the pilot study, which is considered for calculating the effect size. The mean change in the BMI of overweight group was 1.59 ± 0.24 kg/m² and mean change in the BMI of obese group was 1.76 ± 0.29 kg/m². Sample size was calculated using G*Power software, Version 3.0.10
(181) where alpha was 0.05 and power was 0.95. The effect size was 0.638 and sample size was 130.

However, a total of 200 samples were collected in the main study as suggested by the review committee considering the possibility of dropouts. By considering the increased health risk associated with obese individuals, more number of subjects were included in the obesity group (n=124) compared to the overweight group (n=76).

![Sample size calculation with G*Power software](Fig. 3.1)

3.1.7 Study design
The recruited subjects were divided into two groups based on BMI, i.e., overweight group and obese group (7).

(i) Overweight group: includes subjects with $23 \leq \text{BMI} \leq 24.9$ kg/m$^2$.

(ii) Obese group: includes subjects with $25.0 \leq \text{BMI} \leq 40$ kg/m$^2$.

Subjects of both the groups underwent three months of yoga intervention (minimum 75 practice hours in 3 months). The study participants were assessed for the study parameters at day-1 (pre test) and at the end of 3 months (post test1). Even though the study was planned for three months, as per suggestions from the review committee, data was collected at the end of the next 3 months, to find the sustained effect of yoga in the study subjects. After 3 months, those subjects who had continued with the yoga intervention for the next three months with a minimum 75 practice hours over 3 months, were considered as regular yoga practice group or continued intervention group (CIG) and those who did not do a minimum of 75 hours of yoga practice sessions in the further 3 months were considered as irregular yoga practice group or discontinued intervention group (DIG). Assessments of the study parameters were made at the end of six months from both CIG and DIG subjects (post test 2).

3.1.8 Study plan
Abbreviations: Continued intervention group (CIG): The subjects of this group practiced yoga intervention for 3 months with a minimum 75 yoga practice sessions and further followed up yoga practice for next 3 consecutive months with a minimum 75 yoga practice sessions.
Discontinued intervention group (DIG): The subjects of this group did yoga intervention for three months (of minimum 75 hours of practice sessions) and further did not do minimum of 75 hours of yoga practice sessions in the next three consecutive months.

3.1.9 Assessments

The subjects of the present study were assessed for the study parameters at 3 time points, viz., at day-1 (pre test), at the end of 3 months (post test1) and at the end of six months (post test 2).

3.1.10 Study variables
In the present study physiological variables, viz., anthropometric measurements, body composition, biochemical variables, viz., serum lipid profile, total antioxidant status and malondialdehyde level were studied

3.1.10.1 Physiological variables

- Anthropometric variables
  - Body weight, height, BMI, hip circumference and waist circumference
- Body composition
  - Fat mass, total body water and muscle mass
- Blood pressure

3.1.10.2 Biochemical variables

- Serum lipid profile
  - Serum triglycerides (TAG)
  - Total cholesterol (TC)
  - LDL cholesterol (LDL-C)
  - HDL cholesterol (HDL-C)
- Oxidative stress markers
  - Serum total antioxidant status
  - Serum malondialdehyde
3.2 Methods

3.2.1 Instrumentation

All the biochemical investigations were carried out in a fully automated biochemistry analyzer (ERBA-EM 200). All the methods employed for the study were standardized and the quality control was done.

3.2.2 Collection of sample for the assessment of biochemical variables

Venous blood sample (5.0 mL) was drawn from all the participants after overnight fasting on the first day of the study, which was labelled as ‘pre test’. The serum was separated from the plain tube after clot formation and either analysed immediately or kept at -20°C until further investigation. At the end of three months, 2nd sample was collected for the assessment of study variables, which was labelled as ‘post test1’ and at the end of six months 3rd sample was collected, which was labelled as ‘post test 2’.

3.2.3 Estimation of serum lipid profile

The estimation of serum lipid profile included serum total cholesterol, LDL-C, HDL-C and serum TAG. These parameters were estimated by using ERBA Lipid Profile Reagents from Transasia bio-medicals.

3.2.3.1 Estimation of serum TAG

Concentration of serum TAG was estimated by enzymatic single reagent method. Serum TAG are hydrolysed to glycerol and fatty acids by lipase. In presence of glycerol kinase, ATP phosphorylates glycerol to glycerol-3-phosphate and ADP. Glycerol-3-phosphate was further oxidized by glycerol phosphate oxidase (GPO) to produce dihydroxy acetone phosphate and H₂O₂. In presence of peroxidase (POD), H₂O₂ couples with 4-aminoantipyrine and 4-chlorophenol to produce red quinoneimine dye which has an absorbance maxima at 546 nm. The intensity of colour produced is directly proportional to the concentration of TAG in the sample.

Reference range: 40-160 mg/dL
3.2.3.2 Estimation of serum total cholesterol

Cholesterol esters in the serum were hydrolysed to cholesterol and fatty acid by cholesterol esterase, which is further oxidized by cholesterol oxidase (CHOD) to cholester–3-one and H₂O₂. In presence of peroxidase (POD), H₂O₂ couples with 4-aminoantipyrine and 4-chlorophenol to produce red quinoneimine dye which has an absorbance maxima at 546 nm. Absorbance of quinonemine measured at 505 nm is proportional to cholesterol concentration in the serum specimen.

Reference range: 150-200 mg/dL.

3.2.3.3 Estimation of HDL cholesterol (HDL-C)

HDL-C estimated by modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol methyl-ether coupled classical precipitation method. LDL, VLDL and chylomicrons of serum reacts with PVS and polyethylene-glycol methyl-ether, forms inaccessible complex, leaving behind the HDL in solution. Now the HDL-C in serum is estimated by a series of enzymatic reactions, which are initiated, by the oxidation of cholesterol to cholestenone by cholesterol oxidase, accompanied by the formation of H₂O₂. In a second reaction catalysed by peroxidase, 4-aminoantipyrine and phenol react with H₂O₂ to form red coloured quinoneimine. Absorbance at 600 nm, is directly proportional to HDL-C concentration.

Reference range: Females: 42-88 mg/dL; Males: 36-80 mg/dL

3.2.3.4 Estimation of LDL cholesterol (LDL-C)

LDL-C estimated by modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol methyl-ether (PEGME) coupled classical precipitation method by using optimized quantities of PVS and PEGME. LDL, VLDL and chylomicrons of serum reacts with PVS and polyethylene-glycol methyl-ether, forms inaccessible complex, leaving behind the HDL. HDL reacts with enzymes cholesterol oxidase (CHOD) and cholesterol esterase (CHER). Addition of specific detergent reagent (MES buffer + EDTA + TODB) releases LDL from the inaccessible complex. This LDL reacts with CHOD and CHER accompanied by the formation of H₂O₂. In a second reaction catalysed by peroxidase, 4-aminoantipyrine and phenol react with H₂O₂ to form red coloured quinoneimine. Absorbance at 505 nm is directly proportional to LDL-C concentration.
**Reference range:** <100 mg/dL

### 3.2.4 Estimation of stress markers

#### 3.2.4.1 Estimation of serum malondialdehyde (MDA)

Estimation of MDA level in the serum was done by thiobarbituric acid method (182). MDA formed by the breakdown of poly unsaturated fatty acids (PUFA) serves as a convenient index to determine the extent of lipid peroxidation, thereby oxidative stress. The lipoperoxides of serum are heated in an acid medium to liberate protein bound malondialdehyde (MDA). This MDA reacts with two molecules of thiobarbituric acid (TBA) and produces pink coloured adduct, which is read at 535 nm.

**Reference range:** 0.6-1.4 mmol/L

#### 3.2.4.2 Estimation of serum total antioxidant status (TAS)

Estimation of total antioxidant status of serum was done by using ferric reducing ability of plasma (FRAP) method (183). The antioxidants present in the serum reduces ferric-tripyridyltriazine (Fe$^{3+}$-TPTZ) complex to blue coloured ferrous tripyridyltriazine (Fe$^{2+}$-TPTZ). This blue coloured complex has absorption maximum at 593 nm which is read using spectrophotometer.

**Reference range:** 630 - 1634 µmol/L

### 3.2.5 Assessment of physiological variables

The physiological variables measured in the study include weight, height, BMI, waist circumference, hip circumference and waist to hip ratio.

#### 3.2.5.1 Weight

The weight of every subject was measured in light clothing without shoes after emptying bladder. The digital scale (ESSAE, EEROKA LTD, India) was used to measure the weight, was reset before every weighing procedure, and weight was documented in kgs.

#### 3.2.5.2 Height
For the measurement of height, subjects were asked to stand with bare feet kept together, and head at the level of a Frankfort plane (an imaginary line from lower border of the eye orbit to the auditory meatus). Height was measured using a wall mounted stadiometer and documented in centimetres.

3.2.5.3 Body mass index (BMI)

BMI was calculated as follows (39)

\[ BMI = \frac{\text{Weight (kg)}}{[\text{Height (m)}]^2} \]

3.2.5.4 Waist circumference and hip circumference

Waist circumference was measured with a horizontal tape midway between the lower rib margin and iliac crest, at the end of gentle expiration. Hip circumference was measured around the widest portion of the buttocks, with the measuring tape parallel to the floor. For both measurements, the individuals were made to stand with their feet close together, arms at the sides, and body weight evenly distributed, and in little clothing. The subjects were relaxed, and the measurements were taken at the end of a normal expiration. Each measurement was repeated twice for an average; if the measurements were within 1 cm from one another, the average was calculated. If the difference between the two measurements exceeded 1 cm, a third measurement was taken (184).

3.2.5.5 Body composition

Body composition was measured by using Tanita TBF-410 Pro body composition analyser. A standard method for recording was used, with the participant standing on the analyzer. All relevant parameters such as height, weight, age, sex, and ethnicity were entered in the recording unit. The duration for recording was approximately 5 minutes. The analyser records fat percentage, fat mass, total body water and lean body mass.

3.2.5.6 Blood pressure

Blood pressure was recorded before and after the yoga sessions by using a standard mercury sphygmomanometer, auscultating over the right brachial artery. The first clear tapping sound was noted as systolic pressure and the reading at which korotkoff sounds appeared muffled was noted as diastolic pressure (185).
3.3 Institutional ethical clearance

Ethical clearance for the study was obtained from the Institutional Ethics Committee. (Appendix 1) Participants who were willing to sign the informed consent form were included in the study.

3.4 Intervention

Yoga practice which is used as intervention in the present study constituted a varied set of practices comprising physical postures (asanas), breathing practices (pranayamas), cleansing practices (kriyas), relaxation (yoga nidra) and meditation (Table 3.3).

Selected subjects had residential yoga training program of 10 days, every day for 60 minutes. The same yoga training was continued for minimum of 3 months at home with a minimum of 75 sessions in three months. Assessments were also taken from those subjects who continued and discontinued the intervention up to 6 months as a follow-up period. Throughout the 6 months of study period participants were regularly monitored through phone and every fortnight a yoga therapist visited the participants. After 10 days of residential yoga training, the study participants continued their yoga sessions at their home and attendance sheet was provided to each participant, to mark the duration and sessions of yoga practiced.

Along with yoga intervention, participants were asked to take a low calorie diet that is planned individually taking into account the patient’s obese or overweight status in order to create a deficit of energy from 500 to 1000 kcal/day. The nutritive value of foods consumed by the study participants was calculated by applying the database available at http://www.medindia.net/calories-in-indian-food/
Table 3.1 *Yoga* intervention module followed in the study

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Yoga Practice</th>
<th>Rounds</th>
<th>Duration (Min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><strong>Sukhasana</strong> (Easy posture)+ Prarthana mantra (Universal Prayer)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Standing series of <em>asanas</em></td>
<td></td>
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<tr>
<td></td>
<td><strong>Suryanamaskar</strong></td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td><strong>Ardhachakrasana</strong></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>Trikonasana (Triangular pose)</strong></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Supine series of <em>Asanas</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Uttitapadasana</strong></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><strong>Vipareetakarani</strong></td>
<td>1</td>
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</tr>
<tr>
<td></td>
<td><strong>Naukasana</strong></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Pavanamuktasana</strong></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Prone series of <em>Asanas</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Bhujangasana</strong></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><strong>Shalabasana</strong></td>
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<tr>
<td></td>
<td><strong>Dhanurasana</strong></td>
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<td></td>
</tr>
<tr>
<td></td>
<td><strong>Navasana</strong></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Instant relaxation Technique</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>6.</td>
<td>Pranayama Series</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Bhastrika (bellows breathing)</strong></td>
<td>36-50 strokes</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><strong>Kapalabhati (High frequency yoga breathing)</strong></td>
<td>250-300 strokes</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td><strong>Anulom-vilom (Alternate nostril breathing)</strong></td>
<td>20-25 rounds</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td><strong>Right nostril breathing</strong></td>
<td>20-25 rounds</td>
<td>3</td>
</tr>
<tr>
<td>7.</td>
<td>Deep Relaxation technique</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>8.</td>
<td><strong>Total Duration</strong></td>
<td></td>
<td><strong>60 min</strong></td>
</tr>
</tbody>
</table>
3.5 Statistical analysis

Data was analyzed based on both descriptive and inferential statistical methods.

3.5.1 Descriptive method

Collected data of all the variables is quantitative in nature. So, summarized by mean, SD and analysed at 95% confidence interval

3.5.2 Inferential method

To compare all the variables between different time points ANOVA for repeated measures was used, further Post hoc analysis was performed by Bonferroni test. Comparison of the effect between the groups and the genders was assessed by Mann-Whitney U test. The level of significance in the present study was 0.05. Statistical analysis of the data was done using IBM SPSS Statistics (Version 19.0.0) software package.