CHAPTER - III

METHODS AND PROCEDURE

To achieve the objectives of the study the research worker has to plan the entire process of the work in term of research design suited to the study. Therefore the design of the present study systematically under the following headings:

1. Selection of Subjects
2. Variables Studied
3. Tools used for the collection of data
4. Collection of data
5. Statistical techniques used

1. Selection of Subjects:

In the present study purposive sampling method has been used for the collection of data. The research worker selected a total of 850 subjects (450 males and 400 females as shown in Table No. 3.1) to measure the digital finger ratio 2D:4D out of the total of 850; 300 were non sportspersons who have never participated in any competitive sport/game and remaining 550 were elite sports persons both male and female who had participated and got first, second and third position at least at national/ inter-university levels in the discipline of Boxing, Wrestling, Judo, Volleyball, Basketball, Football, Handball, Shooting and Archery.
Table No. - 3.1

Categorization of Subjects for Digital Finger Ratio (2D:4D)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of Game</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Boxing</td>
<td>42</td>
<td>38</td>
<td>80</td>
</tr>
<tr>
<td>2.</td>
<td>Wrestling</td>
<td>37</td>
<td>36</td>
<td>73</td>
</tr>
<tr>
<td>3.</td>
<td>Judo</td>
<td>28</td>
<td>21</td>
<td>49</td>
</tr>
<tr>
<td>4.</td>
<td>Volley Ball</td>
<td>36</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>5.</td>
<td>Basket Ball</td>
<td>30</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>6.</td>
<td>Football</td>
<td>25</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>7.</td>
<td>Handball</td>
<td>32</td>
<td>26</td>
<td>58</td>
</tr>
<tr>
<td>8.</td>
<td>Shooting</td>
<td>35</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>9.</td>
<td>Archery</td>
<td>35</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>10.</td>
<td>Non-Sports Persons</td>
<td>150</td>
<td>150</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>450</strong></td>
<td><strong>400</strong></td>
<td><strong>850</strong></td>
</tr>
</tbody>
</table>

The age of all the subjects selected for the present study above was 17 years. Similarly the 240 subjects (120 males and 120 females as shown in Table No. 3.2) were selected for the testing of Salivary Testosterone Hormone (STH) out of these 240; 216 were elite sportspersons 12 from each category of game (both males and females) remaining 24 subjects were selected from non sportspersons (12 males & 12 females)
Table No. - 3.2

Categorization of Subjects for Digital Finger Ratio (2D:4D) Salivary Testosterone Hormone (STH)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Category</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Boxing</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>2.</td>
<td>Wrestling</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>3.</td>
<td>Judo</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>4.</td>
<td>Volley Ball</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>5.</td>
<td>Basket Ball</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>6.</td>
<td>Football</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>7.</td>
<td>Handball</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>8.</td>
<td>Shooting</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>9.</td>
<td>Archery</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>10.</td>
<td>Non-Sports Men</td>
<td>12</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>120</strong></td>
<td><strong>120</strong></td>
<td><strong>240</strong></td>
<td></td>
</tr>
</tbody>
</table>

2. Variables Studied:

In the present study following two variables were studied:

a) First variable was digital finger ratio (Ratio between the length of Index finger and Ring finger) i.e. 2D:4D.

b) Second variable was Salivary Testosterone Hormone (Level of testosterone hormone present in the subjects Saliva). The most common method used to evaluate your steroid hormone levels is a blood test. However, the blood hormone levels represent your body’s total hormone content. 95% are bound to certain types of proteins, which carry them through your bloodstream. The
other 5% represents your free or bio available hormones. These are available to move easily throughout the body and are utilized by the brain, uterus, skin, prostate and breasts can utilize them. Saliva testing is proving to be the most reliable medium for measuring hormone levels. Hormone levels in saliva accurately represent the amount of hormone delivered to receptors in the body, unlike serum which represents hormone levels that may or may not be delivered to receptors of the body. Clinically, it is far more relevant to test the amount of hormones delivered to the tissue receptors as this is a reflection of the active hormone levels of the body.

For the present study due to following reasons saliva was selected to measure the level of testosterone instead of blood:

- Saliva is more stable than blood. It can be stored for a few days without it affecting the test results.
- Our hormone saliva test kit, test the “free” or bio available hormones, which are moving throughout your body.
- Saliva tells what the amounts of hormones are in your body that are able to be used by your cells. While blood hormone tests only tell about the circulating hormones regardless of their availability to your cells.
- Saliva is a far more accurate and relevant test than blood tests in measuring bio available steroid hormones such as estrogen, progesterone, testosterone, DHEA-S and cortisol.
- Saliva is an easy way to show the relationship between partner hormones like estrogen and progesterone, and DHEA-S and cortisol. These partner hormones need to be in proper balance.
- It is more convenient to collect than blood as the sportsmen or women are not ready to give blood samples.

3. Tool Used for the Collection of Data

Following tools were used for the collection of data for different variables:

(i) **Digital Vernier Caliper**: Digital Finger Ratio (2d:4D) was measured with digital vernier Caliper.
(ii) **Salivary Testosterone Hormones:** All the samples collected from the subjects were sent to the Thyrocare Technologies Limited, D-37/1 TTC MIDC, Navi Mumbai-400703 for the analyses of level of testosterone present in saliva.

4. **Collection of Data**

For the collection of data for sports category the investigator has visited the following coaching camps and tournaments:-

1. All-India inter-university Air-Pistol and Air-Rifle Shooting Championship (Men and Women) 2010-2011 held at Jiwaji University Gwalior.
2. All-India inter-university Archery Championship (Men and women) 2010-2011 held at Kurukshetra University, Kurukshetra.
3. North Zone and All-India inter-university Volleyball championship (Men) 2010-2011 held at Kurukshetra University, Kurukshetra.
4. North Zone and All-India inter-university Basketball championship (women) 2011-2012 held at Kurukshetra University, Kurukshetra.
5. All India Inter-University Boxing Championship (Men and Women) 2012-13 held at Bilaspur University, Bilaspur.
6. All-India inter-university Wrestling Championship (Men and Women) 2011-12 held at Ch. Devi Lal University, Sirsa.
7. Coaching camp of Kurukshetra University, Kurukshetra and Maharshi Dayanand University, Rohtak in the discipline of Wrestling, Boxing and Judo teams for both men and women.

8. Coaching camp of Kurukshetra University, Kurukshetra and Maharshi Dayanand University, Rohtak in the discipline of Basketball, Volleyball, Football and Handball for both men and women teams.


10. National coaching camps held at Sports Authority of India regional Center, Joshi Chauhan, Sonepat (Haryana).

11. National coaching camps held at Nehru Stadium, National Stadium and IG Indoor stadium, New Delhi.

12. National coaching camp shooting held at Dr. Karni Singh Shooting Range, Tughlakabad, Delhi.

13. For the non sports men and women categories data was collected from the students who were pursuing their master’s degree from Kurukshetra University and have never ever participated in any competitive sports/game.

**Procedure for Measurement of Digital Finger Ratio**

Digital fingers of all the subjects of the study were measured with the help of vernier caliper. Length of index finger and ring finger was measured with the help of digital vernier caliper in millimeter from the basal (lowest) finger crease to the fingertip along the medial line bisecting the finger. All the measurements were taken twice. Ratios were calculated by dividing the length of the second digit (index finger) by the length of the fourth digit (ring finger) for only right hands as many authors (Brown et al., 2002b; Manning, 2002a; Manning et al., 2000; McFadden and Bracht, 2003; McFadden and Shubel, 2002; Peters et al., 2002; Hönekopp J and Watson S. 2010) have found stronger sexual dimorphism in digit ratio on the right hand than the left. But in the present study digit ratio was calculated by dividing the length of the second digit (2D) by the length of the fourth digit (4D) for the left and right hand separately and then calculating the mean of these two ratios.
Collection of Saliva

In the present study the second variable which was to be tested was level of Testosterone Hormone, which was tested with the help of saliva instead of serum. About 2-3 ml of saliva was collected early in the morning in a clean and sterilized glass tube without force or inducement before eating, drinking or brushing the teeth. The subjects were told only they can rinse their mouth with water before saliva collection. All samples were sent to Centralised Processing Laboratory (CPL) of Thyrocare Technologies Limited (ISO 9001:2008 & Accredited to College of American Pathologist) at Navi Mumbai for further analyses in the frozen form.

Collecting of Saliva samples from the women:

Samples of saliva were collected on day 19, 20, 21 of menstruation cycle of the females subjects, counting the first day or period as day 1, as these days should reflect the luteal phase, which is the ideal time to measure both progesterone (which should be at its peak) and estradiol in order to calculate the most accurate ratio between the two.

Principle of Testing Salivary Testosterone Hormones

The principle of the following enzyme immunoassay test follows a two-step competitive binding scenario. Competition occurs between an unlabeled antigen
(present in standards, control and samples) and a biotin-labelled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. In the second step, the streptavidin-horseradish peroxidase conjugate binds to any bound biotinylated testosterone. The washing and decanting procedures remove unbound materials. After the last washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution of testosterone in the sample. A set of standards is used to plot a standard curve from which the amount of testosterone in samples and controls can be directly read. The absorbance is measured on a microtiter plate reader. The intensity of the color formed is inversely proportional to the concentration.

**Procedural Precautions**

1. Control materials was included in every run at a high and low level for assessing the reliability of results.

2. Deionized or distilled water was used when the use of water was specified for dilution or reconstitution.

3. In order to reduce exposure to potentially harmful substances, gloves was worn when handling kit reagents and human specimens.

4. All kit reagents and specimens were brought to room temperature and mixed gently but thoroughly before use. Repeated freezing and thawing of reagents and specimens was avoided.

5. The control was included in every run and fall within established confidence limits.

6. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage were indicated when assay values for the control did not reflect established ranges.

7. When reading the micro plate, the presence of bubbles in the microwells affected the optical densities (ODs). Carefully bubbles were removed before performing the reading step.

8. The substrate solution (TMB) is sensitive to light and should remain colourless, if properly stored. Instability or contamination was indicated by the development of a blue color, in which case it was not used.
9. When dispensing the substrate and stopping solution, pipettes was not used in which these liquids would have come into contact with any metal parts.

10. To prevent contamination of reagents, a new disposable pipette tip was used for dispensing each reagent, sample, standard and control.

11. Various lot numbers of kit components were not mixed within a test and any component beyond the expiration date printed on the label was not used.

12. Kit reagents were regarded as hazardous waste and disposed off according to national regulations.

Limitations

1. All the reagents within the kit were calibrated for the direct determination of testosterone in human saliva. The kit was not calibrated for the determination of testosterone in serum, plasma or other specimens of human or animal origin.

2. Any samples or control sera containing azide or thimerosal were not compatible with this kit, as they may lead to false results.

3. Only calibrator A might have been used to dilute any high saliva samples. The use of any other reagent may lead to false results.

Specimen Pre-treatment

1. Specimen samples were centrifuged. The supernatants were transferred into clean tubes.

2. The tubes containing the supernatant were placed in a waterbath and heated at 60-70°C for 1 hour.

3. Allow heated samples to reach room temperature before assaying.

Reagents And Equipment Needed But Not Provided

1. Precision pipettes to dispense 50, 100, 150 and 300 µl

2. Disposable pipette tips

3. Distilled or deionized water

4. Plate shaker

5. Benchtop centrifuge
6. Microwell plate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater* (see assay procedure step 13).

7. Waterbath

Reagents Provided

1. 
   **Rabbit Anti-Testosterone Antibody Coated Microwell Plate-Break Apart Wells:**
   
   Ready To Use.
   
   Contents: One 96 well (12x8) polyclonal antibody-coated microwell plate in a resealable pouch with desiccant. Storage: Refrigerate at 2-8oC
   
   Stability: 12 months or as indicated on label.

2. 
   **Testosterone-Biotin Conjugate (Biotin Conjugate) Concentrate:**
   
   Requires Preparation.
   
   Contents: Testosterone-Biotin conjugate in a protein-based buffer with a non-mercury preservative.
   
   Volume: 300 µl/vial
   
   Storage: Refrigerate at 2-8oC
   
   Stability: 12 months or as indicated on label.
   
   Preparation: Dilute 1:50 in biotin conjugate assay buffer before use. Discard any unused solution.

3. 
   **Streptavidin-Horse Radish Peroxidase (HRP Conjugate) Conjugate Concentrate:**
   
   Requires Preparation.
   
   Contents: Streptavidin-HRP conjugate in a protein-based buffer with a non-mercury preservative.
   
   Volume: 400 µl/vial Storage: Refrigerate at 2-8oC
   
   Stability: 12 months or as indicated on label.
   
   Preparation: Dilute 1:50 in HRP conjugate assay buffer before use. Discard any unused solution.
4. **Testosterone Saliva Calibrators** - Ready To Use.

    Contents: Six vials containing testosterone in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of testosterone. Storage: Refrigerate at 2-8°C. Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

    Listed below are approximate concentrations, please refer to vial labels for exact concentrations

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>Concentration</th>
<th>Volume/Vial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator A</td>
<td>0pg/ml</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>Calibrator B</td>
<td>2pg/ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Calibrator C</td>
<td>10pg/ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Calibrator D</td>
<td>50pg/ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Calibrator E</td>
<td>200pg/ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Calibrator F</td>
<td>800pg/ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

5. **Control** - Ready To Use.

    Contents: One vial containing testosterone in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of testosterone. Refer to vial label for expected value and acceptable range.

    Volume: 1.0 ml/vial   Storage: Refrigerate at 2-8°C
Stability: 12 months in unopened vial or as indicated on label. Once opened, the control should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

6. **Biotin Conjugate Assay Buffer - Ready To Use.**

Contents: One vial containing a protein-based buffer with a non-mercury preservative.
Volume: 13 ml/vial.
Storage: Refrigerate at 2-8oC
Stability: 12 months or as indicated on label.

7. **HRP Conjugate Assay Buffer - Ready To Use.**

Contents: One vial containing a protein-based buffer with a non-mercury preservative.
Volume: 20 ml/vial  Storage: Refrigerate at 2-8oC
Stability: 12 months or as indicated on label.

8. **Wash Buffer Concentrate - Requires Preparation.**

Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative. Volume: 50 ml/bottle   Storage: Refrigerate at 2-8oC
Stability: 12 months or as indicated on label.

Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

9. **TMB Substrate - Ready To Use.**

Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer. Volume : 16 ml/bottle, Refrigerate at 2-8oC
Stability: 12 months or as indicated on label.

10. **Stopping Solution - Ready To Use. Contents: One vial containing**

Storage: Refrigerate at 2-8oC
Stability: 12 months or as indicated on label.
Assay Procedure

Specimen Pre-treatment

Centrifugation and heating at $60^\circ-70^\circ$C for one hour. All regents must reach room temperature before use. Calibrators, controls and specimen samples were assayed in duplicate. Once the procedure had started, all steps were completed without interruption.

1. Working solutions of the testosterone-biotin conjugate, streptavidin-HRP conjugate was prepared and washed buffer.

2. The required number of microwell strips were removed. The bag was resealed and any unused strips were returned to the refrigerator.

3. Pipette 100 µl of each calibrator, control and specimen sample into correspondingly labeled wells in duplicate.

4. Pipette 100 µl of the testosterone-biotin conjugate working solution into each well (Using a multichannel pipette was recommended).

5. Solution was incubated on a plate shaker (approximately 200rpm) for 1 hour at room temperature.

6. Wells were washed 5 times with 300 µl of diluted wash buffer per well and the plate was tapped firmly against absorbent paper to ensure that it was dry. (The use of a washer was recommended).

7. 150 µl of the streptavidin-HRP conjugate working solution was pipette into each well (using a multichannel pipette was recommended).

8. Incubated on a plate shaker (approximately 200rpm) for 30 minutes at room temperature.

9. The wells were washed again in the same manner as step 6.

10. Pipette 150 µl of TMB substrate into each well at timed intervals.

11. Incubated on a plate shaker for 10-15 minutes at room temperature (or until calibrator A attains dark blue color for desired OD).

12. Pipette 50 µl of stopping solution into each well at the same timed intervals as in step 10.
13. Read the plate on a microwell plate reader at 450nm within 20 minutes after addition of the stopping solution.

14. If the OD exceeds the upper limit of detection or if a 450nm filter is unavailable, a 405 or 415nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of controls and samples.

Calculations

1. Calculated the mean optical density of each calibrator duplicate.

2. Drawn a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.

3. Calculated the mean optical density of each unknown duplicate.

4. Read the values of the unknowns directly off the calibrator curve.

5. If a sample reads more than 800 pg/ml then diluted it with calibrator A at a dilution of not more than 1:8. The result obtained were multiplied by the dilution factor.

Expected Normal Values

As for all assays each laboratory should collect data and establish their own range of expected normal values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Range (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>38-120</td>
</tr>
<tr>
<td>Females</td>
<td>5-32</td>
</tr>
</tbody>
</table>

5. Statistical Techniques Used

The statistical tools are used to convert the quantitative data into qualitative responses, So that it may be easy to make the calculation and this process is known as quantification of data. The selection of statistical tools depends upon the nature of the data and number of variables included in the investigation. In order to analysis the data in the present study the following statistical techniques were used manually as
a) **Mean**

Arithmetic mean was calculated by adding up all the Observations and dividing the sum by the number of individuals.

\[
\overline{X} = \frac{\sum X}{N}
\]

Where  
\( N \) = Total Number of subjects  
\( \sum X \) = Sum of all individual values.

b) **Standard Deviation**

It measures the absolute dispersion of variability. It is calculated by following method.

\[
S.D. = \sqrt{\frac{\sum x^2 - \left(\frac{\sum x}{n}\right)^2}{n-1}}
\]

Where  
\( \sum x^2 \) = Sum of squares of the individual values.  
\( (\sum x)^2 \) = The square of the sum of the individual values.

c) **Standard Error of Difference (S.E.D.)**

\[
S.E.D. = \sqrt{\frac{(SD_1)^2}{N_1} + \frac{(SD_2)^2}{N_2}}
\]

Where  
\( S.D_1 \) = Standard Deviation of First Group.  
\( S.D_2 \) = Standard Deviation of Second Group.  
\( N_1 \) = Number of Sources in First Group.  
\( N_2 \) = Number of Sources in Second Group.

d) **t-Test**

This test was applied to determine whether the observation difference between two sample means \( X_1 \) and \( X_2 \) were indicative of real difference or it is due to the sampling error. The t-ratio was calculated with the following formula of t-test:
\[ t = \frac{M_2 - M_1}{\text{SED}} \]

Where \( M_1 \) = Mean of First Group.
\( M_2 \) = Mean of Second Group.

**e) Karl Pearson’s Co-efficient of Correlation**

\[ r = \frac{\sum XY}{\sqrt{\sum X^2 \sum Y^2}} \]

Where \( \sum X \) = Deviation of x-series from the mean of x.
\( \sum y \) = Deviation of y-series from the mean of y.

**f) ANOVA**

The purpose of analysis of variance is to test differences in means (for groups or variables) for statistical significance. This is accomplished by analyzing the variance, that is, by partitioning the total variance into the component that is due to true random error (i.e., within-group SS) and the components that are due to differences between means.