CHAPTER III

METHODOLOGY
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In this chapter, selection of subjects, selection of variables, selection of tests, reliability of instruments, competency of tester, reliability of data, pilot study to construct practice schedule of asanas, orientation of subjects, schedule for practice of asanas, collection of data, administration of tests, experimental design and statistical procedure used are explained.

SELECTION OF SUBJECTS

Twenty-four trained intercollegiate Basketball players were selected at random from the colleges affiliated to Madurai Kamaraj University, Madurai. From the selected players, twelve players were randomly assigned as subjects for control group and the other twelve players for experimental group. The experimental group underwent twelve weeks practice of selected asanas whereas the control group did not undergo any type of training. The age, height and weight of the selected subjects ranged from eighteen to twenty five years, 1.63 to 1.71 meters and 52 to 63 kilograms respectively. Their means were 19 years and four months, 1.68 meters and 58 kilograms respectively.
A qualified physician assured that the subjects did not have any malfunction in their endocrine system. A copy of the fitness certificate of the subjects is given in the appendix-IX. Subjects of the study volunteered to participate with a written consent and they were free to withdraw their consent in case they felt discomfort during the period of training. A form for written consent is shown in the appendix - X. However, there were no dropouts in the study. All the subjects had similar academic work and regular activities in accordance with the requirements of the college curriculum. The subjects were not involved in any other activity except the intramural competitions that could not be avoided.

RATIONALE USED IN THE SELECTION OF SUBJECTS

To give due credence to the purpose of the present investigation, six main players from each of the four teams were selected. From the selected players, twelve players were randomly assigned as subjects for control group and the other twelve players for experimental group. The six players chosen for this study were mainly involved in play for more time than other players in that particular team. It was felt that these subjects were sufficient, taking into consideration of the number and nature of variables, nature and duration of training, testing procedures, time factor and practical difficulties of the investigator to collect the data and processing them. Above all, such subjects were adequate to draw meaningful conclusions and generalisations.
SELECTION OF VARIABLES

Most of the available data refer to blood levels of various hormones rather than rates of secretion. An increase of blood concentration during exercise may indicate an increased rate of hormone secretion by an endocrine gland. Plasma cortisol was increased due to heavy, prolonged or exhausting exercises (Shephard, 1982).

Resting plasma cortisol concentrations are not greatly affected by aging that increases heart rate, systemic blood pressure and associated catecholamine secretion with stressful exercise or cold exposure. They are greater in the elderly than in the younger person (Shephard, 1982).

Cortisol is the main glucocorticoid, comprising more than 30% of the total plasma 17-hydroxycorticoids. 15-30 \( \mu g \) of cortisol is secreted per day by the adrenal cortex in an adult and 60-70% of this is secreted between midnight and morning 8 o’clock. Because of the diurnal variation, normal plasma cortisol values in the same person may be 25 \( \mu g \) per dl at 7 to 8 A.M and 6 to 10\( \mu g \) per dl after 4.P.M. (Rapael, 1976).

If there is a stress imposed on the person, the ACTH (Adrenocorticotropic Hormone) secretion is elevated and so the cortisol secretion is elevated, no matter what is the hour of the day. Further, inspite of the high plasma cortisol level, the ACTH continues to remain high in stress (Chaudhuri, 1991).

It is amazing that almost any type of stress, whether it be physical or neurogenic, will cause an immediate and marked increase in ACTH secretion,
followed within minutes by greatly increased adrenocortical secretion of cortisol (Guyton, 1971).

Athletes who sweat heavily during long periods of exercise need to be concerned with loss of electrolytes, especially sodium (Bruce, 1986).

Epithelial cells of the renal tubules, sweat glands and glands of the alimentary system have enzymatically-controlled mechanisms for transporting electrolytes across cell membranes. The electrolyte 'pumps' respond to mineralocorticoids by concerning sodium and chloride and by wasting bodily potassium. A good example of such a system is found in the distal tubule of the mammalian nephron. The processes are accelerated by mineralocorticoids. Absence of mineralocorticoid activity may result in luteal wastage of sodium and retention of potassium. Sufficient mineralocorticoid helps the body to achieve electrolyte homeostasis (Williams, 1981).

Asanas, as these exercises are called, may be best translated, as postures. It is interesting to note that, as against western exercise, a posture, once the position is assumed, is held for a long time. Apart from the general development that is claimed to follow from these postures, some of them are said to have specific therapeutic value (Belnam, 1938).

The yoga theory about the endocrine glands and their profound effect on body, mind and soul, was not accepted as scientific until endocrinology was 'born' in the west, which was as late as May 1899. Even then it was a long time before it
was discovered that the ductless glands were responsible for the nutrition of the body and its growth, for our sex life, for blood circulation and other important functions including the working of the mind (Indradevi, 1990).

Hence, the investigator selected plasma cortisol, plasma sodium and plasma potassium as dependent variables for this study.

Elevation of adrenal cortex hormones is considered helpful in doing a movement explosively and effectively to give better performance. The investigator selected a group of asanas to examine their influence on plasma cortisol, plasma sodium and plasma potassium.

**SELECTION OF TESTS**

**Radio Immuno Assay (RIA)**

Radio immuno assay is an elegant technique in analytical biochemistry and plays a significant role in the diagnosis of disease. If the substance to be analysed is in very low quantities, of the order of micrograms, nanograms, conventional methods like the gravimetric and colorimetric methods fail. Only the property of radio activity comes to the rescue as even nanogram amounts of radio-labeled substance give appreciable counts detectable by electronic counters. These counts can be conveniently related to the concentration of the substance assayed. As such, the method finds an extensive application in the assay of many substances which are present in trace amounts in blood (Ramakrishnan and Swamy, 1995).
Controls

Most laboratories now depend on commercially prepared kit reagents to perform the various clinical ligand assay available. The advantages of using commercially prepared controls are several. The commercial manufacturer has facilities to ensure consistent, accurate concentrations, chemical stability and a long-lasting supply of the preparation that are almost certain to exceed the analogous facilities of even the best user laboratory (Rose et al. 1986).

Photometry

Photometry is the technique used to study the chemical nature or more frequently the concentration of substances by employing the property of absorption of light of a definite wavelength by molecules or particles. This analytical procedure also requires small quantities of substances for simple and rapid estimation that offers a high order of accuracy. Substances can not be separated by photometry but can only be estimated.

In this method, a definite colour is imparted to the non-luminous flame by the burning of say sodium ions, potassium ions, etc. The intensity of colour is proportional to the concentration of these ions. The different ions in serum will give the emission spectrum due to their burning. By using suitable filters, the light due to any one ion is allowed to fall on the photocell and measurements are taken in the galvanometer. Flame photometer is a versatile instrument used for the estimation of serum Na⁺ and K⁺ in the routine of a clinical biochemical laboratory.
Hence, the investigator selected RIA Controls for testing plasma cortisol and Flame Photometer for testing plasma Sodium and Potassium.

**RELIABILITY OF INSTRUMENTS**

All the instruments were in good condition and workable, purchased in reputed companies. The calibrations were tested and found to be accurate enough to serve the purpose of the study.

**COMPETENCY OF TESTER**

The operation of semi auto analyser and flame photometer was done under the supervision of an experienced microbiologist. The investigator learned the procedure and methods to prepare the solutions and operate the instrument for testing plasma cortisol, sodium and potassium. The competency of tester was evaluated together with the reliability of the tests. The plasma cortisol, sodium and potassium levels of ten players were estimated twice under identical conditions for determining the competency of the tester. The scores thus obtained were analysed by using intraclass correlation.
Table 1

INTRACLASS RELIABILITY COEFFICIENTS OF SELECTED DEPENDENT VARIABLES

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coefficient of Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Cortisol</td>
<td>0.98*</td>
</tr>
<tr>
<td>Plasma Sodium</td>
<td>0.85*</td>
</tr>
<tr>
<td>Plasma Potassium</td>
<td>0.92*</td>
</tr>
</tbody>
</table>

* Significant at 0.01 level of significance

The table value required for significance at 0.01 level of confidence is 0.76.

RELIABILITY OF DATA

The reliability of data was ensured by establishing the reliability of instruments, competency of tester, reliability of the test and subject reliability.

PILOT STUDY TO CONSTRUCT PRACTICE SCHEDULE OF ASANAS

To construct the practice schedule of asanas, ten trained basketball players were selected at random from the selected subjects and they underwent practice of asanas under the keen observation of the experts and the investigator. The asanas
selected for this study under the practice schedule have been recommended to influence the adrenal cortex hormones (Yogeshwar, 1982). Based on the response and interest of the subjects during the pilot study, training schedule was constructed. However, the individual differences were considered. The following basic principles of asanas were followed while constructing the practice schedule.

**Principles of asanas**

1. Practice of asanas without the backing of yama and *niyama* is mere acrobatics.
2. Before starting to practice asana, the bladder should be emptied and the bowels evacuated.
3. Taking a bath or a shower bath before and after practicing asanas refreshes the body and mind.
4. The best time to practice is either early in the morning or late in the evening.
5. Do not practice asanas after being out in the hot sun for several hours.
6. Asanas should be done in a clean airy place, free from insects and noise.
7. Do not do them on the bare floor or on an uneven place, but on a folded blanket laid on a level floor.
8. No undue strain should be felt in the facial muscle, ears and eyes or in breathing during the practice.
9. In the beginning, keep the eyes open. You can keep your eyes closed only when you are perfect in a particular asana.

10. During the practice of asanas, it is the body, alone which should be active while the brain should remain passive, watchful and alert.

11. In all the asanas, the breathing should be done through the nostrils only and not through the mouth.

12. After completing the practice of asanas always lie down in Savasana for at least 10 to 15 minutes, as this will remove fatigue (Iyengar, 1982).

**ORIENTATION OF THE SUBJECTS**

The investigator clearly explained the selected variables in the study and the purpose of training schedule to the subjects. Before the commencement of the training programmes, a week was spent to teach the asanas postures for the experimental group. Four ‘one hour’ sessions were spent on alternate days to have thorough knowledge of practicing asanas.

Though the control group did not undergo any training, they were also given a thorough knowledge about the test items followed in this study.

**SCHEDULE FOR THE PRACTICE OF ASANAS**

The bodily postures help to strengthen the body and stabilise the mind. That posture in which a man can remain longest without effort is for him the best. The very word ‘asana’ means “easy, comfortable”, and so the postures should
have their full effect. To gain its effects, it is necessary to remain in one posture motionless for a specific period. The asanas in the practice schedule and the duration of practice, as prescribed by Kuvalayananda (1925), have been shown in Table 2 given below.

Table 2
SCHEDULE OF ASANAS

<table>
<thead>
<tr>
<th>Name of the Asana</th>
<th>Duration of asana</th>
</tr>
</thead>
<tbody>
<tr>
<td>Savasana</td>
<td>15 minutes to 20 minutes</td>
</tr>
<tr>
<td>Sarvangasana</td>
<td>½ minute to 6 minutes</td>
</tr>
<tr>
<td>Matsyasana</td>
<td>¼ minute to 3 minutes</td>
</tr>
<tr>
<td>Halasana</td>
<td>1 minute to 4 minutes</td>
</tr>
<tr>
<td>Bhujangasana</td>
<td>10 seconds to 1 minute</td>
</tr>
<tr>
<td>Dhanurasana</td>
<td>10 seconds to 1 minute</td>
</tr>
<tr>
<td>Shalabasana</td>
<td>10 seconds to 1 minute</td>
</tr>
<tr>
<td>Savasana</td>
<td>15 minutes to 20 minutes</td>
</tr>
</tbody>
</table>

The procedure of practicing each asana, prescribed in the schedule, is given hereunder. Figure 1 to 7, showing the asana postures are copied from the book entitled “Lights on Yoga” written by Iyenkar.
\textit{Savasana (Corpse Pose)}

'Sava' means a corpse. In this asana the subject has to imitate a corpse. Once life has departed, the body remains still and no movements are possible.

![Savasana Image]

\textbf{Figure 1 Savasana}

\textbf{Procedure}

The subject lies flat on the back full length like a corpse. He keeps the hands a little away from the thighs, with the palms up. Then he closes the eyes and keeps the heels together and the toes apart. He concentrates on deep and fine exhalations, in which the nostrils do not feel the warmth of breath. He relaxes completely and breathes out slowly. He stays in the pose from 15 to 20 minutes.
Sarvangasana (The Pan – Physical Pose)

‘Sarvanga’ (‘Sarva’- all, whole, entire, complete; ‘anga’ - limb or body) means the entire body or all the limbs. In this pose, the whole body benefits from the exercise, hence the name.

Procedure

The subject first lies supine on his seat with all his muscles completely relaxed and his mind thoroughly concentrated. Then he slowly raises his legs through the hipjoint till they make a right angle with the ground, all the while maintaining stiff knees, upto now he does not bring into action his arms and elbows which play only a passive part. But, here, he raises his whole body with
weight on his arms, resumes the position as shown in figure -2. At this point, the player must see that his chest presses against his chin. Further, in order to complete the chin-lock, he bends his forearm through the elbows and with his hands press his trunk against the chin, until it well sets in the jugular notch. In this practice, the posterior part of the neck lies close along the ground; the trunk and the legs are in a straight line and the mind is free on the thyroid.

*Matsyasana (The Fish Pose)*

‘Matsya’ means a fish. This posture is dedicated to *Matsya*, the Fish Incarnation of Vishnu, the source and maintainer of the universe and of all things.

![Matsyasana](image)

**Figure 3 Matsyasana**

**Procedure**

The subject first takes his seat with his legs fully stretched out. He then bends one of his legs, preferably the right in the knee joint and folding it upon itself, sets the same in the opposite hip joint, so as to allow the foot lie stretching at the root of the thigh with its sole upturned. The other leg is similarly folded and
set in the opposite hipjoint. Both the heals he adjusts in the adjacent portion of the abdomen. After this, the player lies supine on his seat. Then he is resting his weight on the elbows. He raises his trunk and head and throwing the latter backward with an arched spine, makes a bridge on his seat. Subsequently he makes hooks of his forefingers and with these lays hold of the opposite toes, which are now available on their wrong side.

Halasana (The Plough Pose)

‘Hala’ means a plough, the shape of which this posture resembles, hence the name.

Figure 4 Halasana

Procedure

It is a part of Sarvangasana and a continuation thereof. The subject releases the chin lock, lowers the trunk slightly, move the arms and legs over the head and rests the toes on the floor. He tightens the knees by pulling up the hamstring muscles at the back of the thighs and the trunk.
Bhujangasana (The Cobra Pose)

'Bhujanga' means a serpent. The pose resembles a serpent about to strike.

Figure 5 Bhujangasana

Procedure

The subject first lies prone on his seat with his muscles thoroughly relaxed while getting ready for the cobra pose. The player touches the ground with his forehead and keeps his hands, one on each side of the chest, bending them in the elbows. The soles are made to look upward. The player raises his head and bends the neck backward as far as possible. The chin is completely thrown out. During this attempt, the player lifts the body up from the trunk until the pubis is in contact with the floor and stays in this position with the weight on the legs and palms. Then, he contracts the anus and the buttocks.
Dhanurasana (The Bow Pose)

‘Dhanu’ means a bow. The hands here are used like a bow-string to pull the head, trunk and legs up and the posture resembles a bent bow.

![Dhanurasana](image)

**Figure 6 Dhanurasana**

**Procedure**

The subject lies full length on floor on the stomach, face downward. He exhales and bends the knees. Then he stretches the arms back and holds the left ankle with the left hand and the right ankle with the right hand. Now, he exhales completely, pulls the legs up by raising the knees above the floor, and simultaneously lifts the chest off the floor. The arms and hands act like a bowstring to tighten the body like a bent bow. The player lifts up the head and pulls it as far back as possible.
Shalabhasana (The Locust Pose)

‘Shalabha’ means a locust. The pose resembles that of a locust resting on the ground, hence the name.

Figure 7 Shalabhasana

Procedure

The subject lies full length on the floor on the stomach, face downwards. He stretches the arms back. He exhales, lifts the head, chest and legs off the floor simultaneously as high as possible. The hands should not be placed and the ribs should not rest on the floor. Only the abdominal front portion of the body rests on the floor and bears the weight of the body. Then, he contracts the buttocks and stretches the thighs, knees and ankles. He does not bear the weight of the body on the hands but stretches them back to exercise the upper portion of the back muscles. He stays in the position as long as he can with normal breathing. In the beginning, it is difficult to lift the chest and the legs off the floor, but this becomes easier as the abdominal muscles grow stronger.
The time for holding each asana in the final stage is slowly increased. Thus the concept of *asanjaya*, where one practices the asana with greater ease and maintains it comfortably and stably for a longer duration, could be achieved. In such type of effortless, easy and comfortable maintenance of the final stage, various muscles and joints are stretched smoothly without any resistance. This is known as passive stretching where the muscles and tendons are not stretched beyond their natural limits. When the big muscles of the extremities are undergoing a passive stretch, asanas work on the trunk areas and the smooth muscles of the visceral organs. The mild pressure in the internal organs results in stimulation of the autonomic nervous system as the walls of these organs undergo a mild stretching and relaxation, alternatively.

In most of the asanas, the abdominal area is influenced. During the maintenance phase of the asanas, the abdominal cavity undergoes pressure changes that are reflected on the visceral organs like stomach, lungs, colon, kidney, urinary bladder etc. If breathing is allowed to be continued while maintaining the asanas, there is an alternate positive and negative pressure on the viscera. These pressures and consequent stretching of the walls of the visceral organs stimulate visceroreceptors, which are sensitive to stretching. The alternate positive and negative pressure changes brought about in the abdominal and the pelvic region promote and preserve the health of the endocrine glands (Gore, 1991).
Hence, the investigator selected the above-mentioned group of asanas in which abdominal pressure is produced (Yogeshwar, 1982). These pressures may control the hyper or hypo secretion of human hormones in general and adrenal cortex activities in particular.

**COLLECTION OF THE DATA**

Blood samples were collected from experimental group and control group one week before competition during rest. In order to find out the influence of asanas on plasma cortisol, in the morning and evening separately, blood samples were drawn from the subjects at 8 a.m. and 4 p.m. respectively. Then the blood samples were also collected just five minutes prior to competition and immediately after competition before and after the experimentation. The blood samples were collected from the non-dominanted forearm vein of the subjects by a qualified lab-technician. All blood samples were collected from the subjects prior to which they refrained from exercise, alcoholic drinks and any other strenuous physical activity, which may elevate the secretion of adrenal cortex hormones for twenty-four hours. The blood samples from each subject were drawn using separate disposable, highly sterilised syringe to avoid possible HIV (AIDS) infection. A qualified lab-technician was assigned to collect blood samples to gain faith from the subjects.
The subjects from the experimental group were treated with the practice of asanas for twelve weeks both in morning and in evening, as mentioned in Table 2. The asanas were practiced between 6 a.m. and 7 a.m. and between 5.30 p.m. and 6.30 p.m. for five days in a week. The practice was given under the watchful eyes of the investigator. Attendance was calculated for the experimental group by dividing the total number of training sessions by the number of sessions present. It was 93% for the experimental group. The control group did not undergo any type of training.

**ADMINISTRATION OF TESTS**

Blood samples were allowed to clot for 1 hour at room temperature and were then centrifuged in order to obtain the plasma. Plasma was taken from each sample and they were stored at -20°C within 2 hours after collection.

**DETERMINATION OF PLASMA CORTISOL**

*(EIA DSL-10-2000 Active tm)*

Temperatures of all kit reagent specimens were brought to room temperature. Each kit contains reagents such as gars-coated microtitration strips, Cortisol antiserum (blue), cortisol standards, cortisol controls, cortisol enzyme conjugate concentrate, conjugate diluent, TMB chromogen solution, wash concentrate and stopping solution. The reagents and samples were thoroughly mixed by gentle inversion before use. The stopping solution and TMB chromogen solution were carefully added into the wells in the same order. A clean disposable
pipette tips were used for each reagent, standards, control or specimen. The reagents were carefully kept against excessive heat and sunlight.

PREPARATION OF REAGENTS

Wash solution was prepared by diluting wash concentrate 10-fold with deionised water. The enzyme conjugate concentrate was diluted at a ratio of 1 part enzyme conjugate concentrate into 50 parts conjugate diluent. The enzyme conjugate solutions were prepared just before use.

ASSAY PROCEDURE

All specimens and reagents were allowed to come to room temperature. The liquid reagents were thoroughly mixed by gentle inversion before use. Duplicates were assayed in standard, control and unknown. 25 μl of each standard, control and unknown were pipetted into the appropriate wells. 100 μl of the enzyme conjugate solution was added to each well using a semi-automatic dispenser. The well holder was gently tapped for 5-10 seconds. 100 μl of the cortisol Antiserum was added to each well using a semi-automatic dispenser. The wells were incubated at room temperature on a shaker at 500-700 rpm for 45 minutes. Each well was washed five times with the wash solution using an automatic microplate washer. The plate was blotted on absorbent material. 100 μl of the TMB chromogen solution was added to each well and incubated the wells at room temperature for 10-15 minutes on a shaker at 500-200 rpm. 100μl of the
Stopping solution were added to each well and then the plate was shaken by hand for 5-10 seconds. The absorbance of the solution was read within thirty minutes for which a microplate reader was set to 450nm.

**CALCULATION OF RESULTS**

The mean absorbance readings for each standard, control and unknown were calculated. The mean absorbance readings for each of the standards were plotted along the y-axis versus the cortisol concentrations in µg/dl along the x-axis. A best fitting curve was drawn through the mean of the duplicate points. The cortisol concentrations of the controls and unknown were determined from the standard curve by matching their mean absorbance reading with the corresponding cortisol concentrations (Diagnostics Systems Laboratories, 1998). The collected data were recorded in µg/dl.

**SCORING:** The cortisol concentration was recorded in µg/dl.

**DETERMINATION OF PLASMA SODIUM AND POTASSIUM BY EVANS ELECTROSELENIUM (E.E.L) FLAME PHOTOMETER**

In this instrument, the solution under test was passed under carefully controlled conditions as a very fine spray in the air supply to a burner. In the flame the solution evaporated and the salt dissociated for giving neutral atoms. Some of these, though only a very small proportions, moved into a higher energy state. It was the light emitted when these excited atoms fall back to the ground state, which
was used in flame photometry of this type-emission flame photometry. Light of characteristic wavelengths was emitted and passed through a suitable filter and the amount of current thus produced was measured. This varied with the concentration of sodium, for example, in the solution that was being tested. Using solutions of known sodium content, a calibration curve was constructed and this was used for reading the sodium content of the fluids examined. Several gases have been used for the flame. These include acetylene, propane, butane and coal gas. Both the gas pressure and air pressure were carefully regulated so as to maintain a constant steady flame, which was blue in colour and had no yellow streaks. Whilst ordinary coal gas was more variable in composition than the others it was satisfactorily used. The temperature of these flames differed considerably, decreasing as we go from acetylene to coal gas. This is important in regard to the amount of light emitted and the extent to which one element can increase the excitation of another.

Figure 8 Flame Photometer
E.E.L Model
Air from a compressed air cylinder filtered with suitable reducing values or from an air compressor, was introduced into the all-metal atomizer through a control valve (2) at a pressure indicated on a gauge (3) mounted at the front of the instrument. This stream of air draws liquid from the sample being tested in beaker (4) up the inlet tube (5) and atomizes it to a fine spray. The atomizer clips into a plug (6) at one end of the spray chamber (7) in which the larger droplets fall from the air stream and flow to waste through the drainage tube (8). Gas was introduced into the spray chamber through the inlet tube (9) which is connected by synthetic rubber tubing to the automatic gas pressure stabilizer (10) and to a control valve (11). The gas-air mixture burns in a broad, flat flame and the hot gases pass up a
well-ventilated chimney (12). Gauze in the burner tube prevents serious firing back of the flame. The light emitted by the flame is collected by a reflector (13) and focussed by lens (14) through the interchangeable optical filters (15) on to an EEL barrier layer selenium photocell (16). This is connected in series with a calibrated potentiometer (7) and a galvanometer unit (18) (Varley, 1997).

**Resting Sodium:** The normal plasma sodium is 135 – 145 mEq. per liter (Cromwell, 1996).

**Resting Potassium:** The normal concentration of extra cellular potassium is recorded between 3.5 and 5.0 mEq./liter (Cromwell, 1996).
EXPERIMENTAL DESIGN AND STATISTICAL PROCEDURE

The experimental design used in this study was $2 \times 2 \times 3$ factorial design. The first factor indicates 'groups' of experimental and control. The second factor denotes 'treatments' namely practice of asanas to experimental group and no such practice to control group. The third factor indicates 'competition' during rest, five minutes prior to competition and immediately after competition.

This study comes under $2 \times 2 \times 3$ factorial design. The data pertaining to the variables in the study was examined by Factorial Analysis of Variance with repeated measures on the last two factors (ANOVA). If the main effects were found significant, Scheffe's test was applied as a post-hoc test. If the interaction were found significant, simple effect test was applied. If the F-ratio for simple effect was found significant, Scheffe's test was used as post-hoc test (Broota, 1992).