CHAPTER III

METHODOLOGY

In this chapter selection of the subjects, selection of variables, experimental design, pilot study, criterion measures, reliability of data, reliability of instruments, tester's reliability, subject reliability, exercise selected and nutritional supplementation of iron, test administration, collection of data and the statistical techniques used have been explained.

3.1 SELECTION OF SUBJECTS

The purpose of the study was to find out the effect of specific nutritional supplementation, desupplementation and resupplementataion on anemic profile status among college women. To achieve the purpose of the study, the investigator conducted a sample survey to assess the symptoms of the anemic. Based on the survey, the investigator selected 15 anemic women students as subjects. To confirm the anemic status selected college women were tested for their hemoglobin levels and the college women who were having less than 12 gm/dl hemoglobin were considered as the anemic for this study, as the normal adult women required is 12 – 16 gm/dl hemoglobin.

3.2 SELECTION OF VARIABLES

The investigator reviewed a number of research journals, magazines and books on anemic profile status and selected the following variables for the study:

Dependent Variables

1. Iron
2. Total Iron Binding Capacity
3. Ferritin
4. Folic Acid
5. B12
6. Hemoglobin
7. Red Blood Cell

Independent variable

1. 8 weeks Nutritional Supplementation
2. 8 Weeks Nutritional Desupplementation
3. 8 Weeks Nutritional Resupplementation

3.3 EXPERIMENTAL DESIGN

Random group design was followed in this study.

The selected anemic college women (N=15) were provided with specific nutritional supplementation for eight weeks. After the completion of 8 weeks nutritional supplementation, they were stopped the nutritional supplementation for 8 weeks and this phase was considered as desupplementation phase. After the completion of desupplementation period of 8 weeks the subjects were started providing with nutritional supplementation and this phase of 8 weeks was considered as resupplementation phase. Prior to the experimental treatments, all the subjects were measured of their anemic profile status and determined their (1) Iron (2) Total Iron Binding Capacity (3) Ferritin (4) Folic Acid (5) B12 (6) Hemoglobin and (7) Red Blood Cell, the data obtained were considered as initial scores of the anemic profile status. Data were obtained at the end of nutritional supplementation phase (completion of 8 weeks of nutritional supplementation), end of desupplementation (completion of 16 weeks), and end of resupplementation phase (completion of 24 weeks). Thus, anemic profile of the subjects was determined four times as detailed in Table I on all the seven variables selected. The obtained data was subjected to statistical analysis using Repeated measures ANOVA. In all cases 0.05 level was fixed to test the significance of the results.

Table I

Showing Anemic Profile Status Tested under different Phases of Experiment

S.No.	Scores	Phase	Tested at
1	Initial Scores (IS)	Initial	Prior to Experiment
2	Supplementation (SS)	Supplementation	8th week of experiment
3	Desupplementation (DS)	Desupplementation	16th week of experiment
4	Resupplementation (RS)	Resupplementation	24th week of experiment

3.3 PILOT STUDY

The pilot study was conducted ensure the suitability, frequencies and duration of testing and the effects of nutrition. Further it helps to know the subjects' capacity to know the satisfactory effects and know the difficulty of conducting experimental programme and to set a clear understanding about the duration of time which is required for conducting the test. It also helped the investigator to know the administration of different nutritional supplementations to the subjects.

3.4 CRITERION MEASURES

The following criterion measures were adopted to measure the test.

1 The number of serum iron cells were determined by suitable dilution and enumerated over a definite area in a laboratory condition and measured in micrograms per deciliter (µg/dL)

2 Total Iron Binding Capacity (TIBC) is done Ferrozine Method.

3 Ferritin was measured by Turbidimetery-Latex method

4 Folic Acid is measured by Folic Acid Red Blood Cell Test (Quantaphase II Folate/vitamin B12)

5 B12 was measured by Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12"

6 Hemoglobin was estimated by the acid hematin method using Sahle's haemocytometer in a Laboratory

7 The number of red blood cells (RBC) were determined by suitable dilution and enumerated over a definite area in a laboratory condition.

3.5 RELIABILITY OF DATA

The reliability of data was ensured by establishing the instrument reliability, tester's competency and subject reliability.

3.6 RELIABILITY OF INSTRUMENTS

The research scholar collected the blood samples from the subjects through qualified lab technicians and the tests were conducted in Chennai Clinical Laboratory. The lab is a reputed one and the instruments used by them are standard ones. Hence, the reliability of the instruments were accepted for the purpose of this study.

3.7 TESTER'S RELIABILITY

To determine the reliability of test measurements involved in this study, blood samples who were not the subjects of this study were collected twice and the measures on criterion variables were determined and found to be reliable. The test and retest scores were subjected statistical treatment and the obtained values proved that there was significant.

Tester's competency and reliability of tests were established by Test, retest, process. As very high correlation was obtained, the tester competency in taking measurement and test reliability were accepted.

The co-efficient of reliabilities were significant at the 0.01 level for tests under investigation.

3.8 SUBJECT RELIABILITY

The intra class correlations obtained through test and retest method on the iron deficient college women also proved the subject reliability as the subjects for this study was randomly selected from the same population.

Before the commencement of experiment, the reliability of the data was established through test and retest method. The correlation of coefficient obtained for the testes variables were given in Table II.

Table II

Intra Class Correlation Coefficient of Test – Retest Scores

S.No	Variables	Coefficient of Correlation
1	Serum Iron	0.93*
2	Total Iron Binding Capacity	0.92*
3	Ferritin	0.91*
4	Folic Acid	0.89*
5	Vitamin B12	0.88*
6	Hemoglobin	0.91*
7	Red Blood Cells	0.92*

* Significant at 0.01 level

3.9 NUTRITIONAL SUPPLEMENTATION

The experimental group was given eight weeks Nutritional Supplementation for iron deficiency among college women selected. The subjects were provided with lotus stem with one whole egg during nutritional supplementation period. The nutritional supplementation was provided to the subjects under the supervision of dieticians. The description of the nutritional supplementation is described below.

Lotus Stem

Lotus stem is a great source of iron. Deep frying will not reduce the iron content but will greatly increase the fat content of your diet. One could be able to purchase the lotus stem from any local vendor. The low cal version of cooking Lotus stem is described below.

Ingredients:

Lotus stem -100 gm Salt - to taste Yellow chilli powder -1 tsp Mashed potatoes - 40 gm Chopped green chilli - 1 tsp Chopped ginger - 1 tsp Grated cottage cheese - 40 gm Garam masala powder - 1/2 tsp Grated cheese - 20 gm

Method:

Clean and boil the lotus stem. Grate the boiled lotus stem and mix it with the rest of the ingredients. Skewer the mixture and cook them in the tandoor till they are done.

LOTUS STEM - dry - Nutritive Value of Common Foods

Energry(Ecals)	**234**
Moisture(gm)	**9**
Protein(gm)	**4**
Fat(gm)	**1**
Mineral(gm)	**8**
Fibre(gm)	**25**
Carbohydrates(gm)	**51**
Calcium(mg)	**405**
Phosphorous(mg)	**128**
Iron(mg)	**60**

Hard Boiled Egg

Put the eggs in a single layer in a saucepan, covered by at least an inch or two of cold water. Starting with cold water and gently bringing the eggs to a boil will help keep them from cracking.. Adding a half teaspoon of salt is

thought to help both with the preventing of cracking and making the eggs easier to peel. Put the burner on high and bring the eggs to a boil. As soon as the water starts to boil, remove the pan from the heat for a few seconds. After a minute, remove the pan from the cover, and let sit for 12 minutes. Strain out the water from the pan, fill the pan with cold water, strain again, fill again, until the eggs cool down a bit. Once cooled, strain the water from the eggs.

Nutritional Value Per 100 g Chicken Egg whole, Hard Boiled

Energy	647 kJ (155 kcal)
Carbohydrates	1.12 g
Fat	10.6 g
Protein	12.6 g
Tryptophan	0.153 g
Threonine	0.604 g
Isoleucine	0.686 g
Leucine	1.075 g
Lysine	0.904 g
Methionine	0.392 g
Cystine	0.292 g
Phenylalanine	0.668 g
Tyrosine	0.513 g
Valine	0.767 g
Arginine	0.755 g
Histidine	0.298 g

Alanine	0.700 g
Aspartic acid	1.264 g
Glutamic acid	1.644 g
Glycine	0.423 g
Proline	0.501 g
Serine	0.936 g
Water	75 g
Vitamin A equiv.	149 µg (19%)
Thiamine (vit. B_1)	0.066 mg (6%)
Riboflavin (vit. B_2)	0.5 mg (42%)
Pantothenic acid (B_5)	1.4 mg (28%)
Folate (vit. B_9)	44 µg (11%)
Vitamin B_{12}	1.11 µg (46%)
Choline	294 mg (60%)
Vitamin D	87 IU (15%)
Vitamin E	1.03 mg (7%)
Calcium	50 mg (5%)
Iron	1.2 mg (9%)
Magnesium	10 mg (3%)
Phosphorus	172 mg (25%)
Potassium	126 mg (3%)
Zinc	1.0 mg (11%)
Cholesterol	424 mg

3.10 DESUPPLIMENTATION PHASE

In order to find out the effect of desupplementation of specific nutrition, the nutritional food cooked lotus stem and one hard boiled chicken egg was stopped for 8 weeks. After the completion of desupplementation phase, blood samples were collected from all the subjects to collect data on selected variables.

3.11 RESUPPLEMENTATION PHASE

In order to find out the effect of resupplementation of specific nutrition, the nutritional food cooked lotus stem and one hard boiled chicken egg was given for 8 weeks under the supervision of dietician. After the completion of resupplementation phase, blood samples were collected from all the subjects to collect data on selected variables.

3.12 TEST ADMINISTRATION

Blood Collection

The subject was asked to sit on an arm chair comfortably. An examination of the superficial vein of the left fore arm was made to select the site for venous puncture. The skin was cleared with spirit and allowed to dry. A tourniquet was tied around the upper arm. The subject was asked to flex and

extend the wrist joint to make the veins more prominent. Thumb of the left hand was placed on the lower part of the cleared area and gentle traction was given to fix the vein. A 3 ml sterilized syringe with needle was used to puncture the vein and blood flowed in the syringe. Five millimeter of blood was collected from each subject and stored in a stoppered container with antioogulant. The collected blood samples were subjected to the following estimations.

3.12.1 Hemoglobin

Hemoglobin was estimated by the acid hematin method using Sahle's haemocytometer

Principle

The hemoglobin was converted into acid haematin by reacting with dilute hydrochloric acid. The resulting brownish mixture was matched with a standard in a colorimeter.

Procedure

Upto two marks of the square tube, the 0.1N hydrochloric acid was taken. To this 20 micro liter of blood was added and then mixture was allowed to stand until acid hematin was developed. Distilled water was added drop by

drop till the colour matched with standard colour of the haemometer. Once the colour matched, the readings were recorded directly.

Result

The results were expressed in gram percentage.

3.12.2. ENUMERATION OF RED BLOOD CELLS

The number of red blood cells (RBC) were determined by suitable dilution and enumerated over a definite area.

Procedure

Blood was drawn upto 0.5 mark of red blood cells pipette. The blood on the sides of the pipette was wiped off. Hayem's fluid was drawn into red blood cells pipette upto 101 mark carefully avoiding air bubbles. The contents were mixed gently taking care to avoid haemolysis. First few drops were discarded and then a small drop of mixture was placed at the edge of the cover slip, placed on the haemocytometer, which was focussed under the microscope. It was allowed undisturbed for five minutes so that the cells could settle on the haemocytometer. The number of cells in five small squares were counted.

Calculation

Number of cells in 5 small squares = X

Number of cells in a square millimeter area (25 small squares) = X x 5

Depth (height between the cover slip and counting chamber, = 0.1mm

Dilution factor = 200

Number of cells in 1 cu.mm = X x 5 x 10 x 200

= X x 10000

The results were expressed in millions / cu.mm of blood.

3.12.3. ENUMERATION OF SERUM IRON : FERROZINE METHOD

Purpose

To measure the iron in Serum

Principle

The major advantages of ferrozine are the high molar absorptivity of the ferrous ferrozine complex (28,000), its water solubility, and stability over the pH range of 4-9. It also can be manufactured economically and isolated in a state of high purity.In an acidic medium transferrin bound iron dissociates into

free ferrous and ferric ions. Ascorbic acid is then used to reduce the ferric iron to the ferrous state. Ferrozine reacts with ferrous iron to form a magenta complex which absorbs at 560 nanometer. The absorbance is directly proportional to the amount of iron in the serum.

REAGENTS

Acid Dissociating Reagent (R1): a solution of HCl containing at least 26.2 mmol/L thiourea.

Acid Dissociating Diluent (1a): a solution of HCl and a surfactant.

Iron Color Reagent (R2): a solution of HCl containing 26.2 mmol/L thiourea, 10 mmol/L Ferrozine®, and a surfactant.

Ascorbic Acid (1b): Cat. No. 102-25: 1 vial containing 1.5 g ascorbic acid

Cat. No. 102-15: 1 vial containing 0.75 g ascorbic acid

Procedure

Combine the Acid Dissociating Reagent (R1) with the Acid Dissociating Diluent (1a). Dissolve the Ascorbic Acid (1b) in this solution. Allow sufficient time for the Ascorbic Acid to completely dissolve before beginning the serum iron assay. The R1 working reagent is stable for one week at 2-8°C.

The R2 Color Reagent is ready for use. The reagents included are stable until the expiry date stated on the labels at 18-26EC. The Color Reagent (R2) should be protected from light.

Scoring

The number of serum iron cells were determined by suitable dilution and enumerated over a definite area in a laboratory condition and measured in micrograms per deciliter (µg/dL)

3.12.4 TOTAL IRON BINDING CAPACITY (TIBC)

Purpose

To measure the Total Iron Binding Capacity.(TIBC)

Principle

The major advantages of ferrozine are the high molar absorptivity of the ferrous ferrozine complex (28,000), its water solubility, and stability over the pH range of 4-9. It also can be manufactured economically and isolated in a state of high purity. In an acidic medium transferrin bound iron dissociates into free ferrous and ferric ions. Ascorbic acid is then used to reduce the ferric iron to the ferrous state. Ferrozine reacts with ferrous iron to form a magenta

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REAGENTS

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Iron Color Reagent (R2): a solution of HCl containing 26.2 mmol/L thiourea, 10 mmol/L Ferrozine®, and a surfactant.

Ascorbic Acid (1b): Cat. No. 102-25: 1 vial containing 1.5 g ascorbic acid

Cat. No. 102-15: 1 vial containing 0.75 g ascorbic acid

Procedure

Pipette into a clean dry test tube serum 0.5 ml and TIBC Saturating Reagent (Iron Buffer Reagent) 1.0 ml. Mix well and allow to stand at room temperature for 10 min and add TIBC precipitating reagent iron colour reagent approx 50 mg. Mix well and allow to stand at room temperature for 10 min. Centrifuge at 2500 – 3000 rpm in the supernatant as above mentioned in the Iron Assay.

TIBC in (µg/dl) = Abs T-(AbsSD+AbsB) / (Abs S - Abs B) X 300

Scoring

The number of TIBC were determined by suitable dilution and enumerated over a definite area in a laboratory condition and measured in micrograms per deciliter (µg/dL)

3.12.5 FERRITIN

Immuno-turbidimetric determination of ferritin in serum or plasma - the kit contains specific anti-ferritin antibodies - ratio sample/reagent 1/ reagent 2: 1/20/10 - wavelength 570 nm - calibrator (ref 11525a) and control (ref 11210; ref 11212; ref 11211a) are available separately - measuring range from 4 to 1000 ng/ml - n° of tests is referred to automatic analyzer on hitachi 911-912. note: reagent 1 and reagent 2 are filled in a universal trapezoidal vial.

Scoring

The test measures the amount of iron in the body. Iron is important for red blood cell production and scores recorded in ng/ml (nanograms/milliliters)

3.12.6 FOLIC ACID AND VITAMIN B12

Purpose

To measure vitamin Folate - also known as Folic Acid and Vitamin B12.

Procedure

Both vitamins are measured by using the Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radioassay kit (1). The assay is performed by combining serum or a whole blood hemolysate sample with 125 I-folate and 57 Co-vitamin B12 in a solution containing dithiothreitol (DTT) and cyanide. The mixture is boiled to inactivate endogenous folate-binding proteins and to convert the various forms of vitamin B12 to cyanocobalamin. The reduced folate and its analogs are stabilized by DTT during the heating. The mixture is cooled and then combined with immobilized affinity-purified porcine intrinsic factor and folate-binding proteins. The addition of these substances adjusts and buffers the pH of the reaction mixture to 9.2. The reaction mixture is then incubated for 1 hour at room temperature.

During incubation, the endogenous and labeled folate and B12 compete for the limited number of binding sites on the basis of their relative concentrations. The reaction mixtures are then centrifuged and decanted.

Labeled and unlabeled folate and vitamin B12, binding to immobilized binding proteins, are concentrated in the bottom of the tube in the form of a pellet. The unbund folate and B12 in the supernatant are discarded, and the radioactivity associated with the pellet is counted. Standard curves are prepared by using the pre-calibrated folate/B12 standards in a human serum albumin base. The concentration of the folate and vitamin B12 in the patient serum or folate in a patient's whole blood is calculated from the standard curve.

In the erythrocyte folate procedure, the sample is first diluted 1:11 with a solution of 1 g/dL ascorbic acid in water and either incubated for 90 min prior to assay or frozen immediately for later assay. The 90-min incubation or the freeze-thaw is necessary for hemolysis of the red blood cells; either allows the endogenous folate conjugates to hydrolyze the conjugated pterylpolyglutamates prior to assay. The sample is further diluted 1:2 with a protein diluent (human serum albumin), resulting in a matrix similar to that of the standards and serum samples.

Scoring

The scores of folic acid and vitamin B12 were recorded in ng/dl.

3.13 STATISITICAL PROCEDURE

Based on the blood samples collected from all the subjects prior to experimental treatment (initial scores), after 8 weeks on completion of nutritional supplementation, which was considered as the effect of nutritional supplementation on the subjects. Data were collected on selected variables after 16 weeks after completion of desupplementation phase which was considered as scores of desupplementation. After the completion of resupplementation phase, that is, 24 weeks, the subjects were again tested for their scores to determine the resupplementaion phase. The differences among means of initial, nutritional supllementation, desupplementation and resupplementation scores were subjected to statistical treatment using repeated analysis of variance (Repeated ANOVA).

When the F ratio was found to be significant, Scheffe's post hoc test was used to find out the paired mean significant difference. (Thirumalaisamy, 1998).