

MATERIALS AND METHODS

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This chapter has been discussed under the following heads :

- A. General objectives of the study.
- B. Specific objectives of the study.
- C. Sample selection.
- D. Experimental design
- E. Dosage level and duration of dosing of the hematinic compound.
- F. Parameters and techniques used for data collection.
- G. Statistical analysis.

A. To restate, the general objectives of the present study were :

- I. To study the impact of prophylactic iron supplementation at 60 mg elemental Fe for 60 days at a stretch, twice in a school year on iron status.
- II. To study the impact of this level and duration of prophylactic supplementation of Fe twice in a school year, on :
 - (i) selected areas of Cognitive Function, namely, concentration, memory, discrimination, perception and visual motor coordination in underprivileged school girls, 8-15 yrs of age;
 - (ii) selected parameters of Physical Work Capacity, namely, pulse rate and blood lactic acid, in underprivileged school girls, 8-15 yrs of age;

(iii) growth status.

B. The specific objectives of the present study were :

I. To study the iron status of school girls (8-15 yrs) in terms of :

1. Percent prevalence of anemia.
2. Impact of Fe supplementation on Hb status.
3. Impact of Fe supplementation on parameters of iron status, namely, serum iron, total iron binding capacity and percent saturation of transferrin.

II. 1. To assess the relationship between Hb status and Cognitive Function, in specific areas of concentration, discrimination, memory, perception and visual motor coordination, in underprivileged school girls, 8-15 yrs of age.

2. To study the impact of prophylactic supplementation with 60 mg elemental iron for 60 days at a stretch, twice in a school year, on selected areas of Cognitive Function stated above.

3. To evaluate the effect of withdrawing the Fe supplementation for four months, on Hb and Cognitive Function status.

4. To analyse the effect of Fe supplementation on Cognitive Function by the variable of age.

5. To analyse the effect of Fe supplementation on Cognitive Function by the variable of Hb status.

- III.
1. To investigate the relationship between Hb status, iron status and Physical Work Capacity (PWC), using specific parameters, namely, pulse rate and blood lactic acid, both pre and post exercise, in underprivileged school girls, 8-15 yrs of age.
 2. To study the impact of prophylactic supplementation with 60 mg elemental Fe for 60 days at a stretch, twice in a school year, on selected parameters of PWC stated above.
 3. To evaluate the effect of withdrawing the Fe supplementation for 4 months, on the Hb status and PWC parameters.
 4. To analyse the effect of Fe supplementation on PWC by the age variable.
 5. To analyse the effect of Fe supplementation on PWC by the Hb status as the variable.
 6. To study the relationship between iron status and the selected parameters of PWC.
- IV.
1. To study the impact of supplementation with 60 mg elemental Fe for 60 days at a stretch, twice in a school year on specific measures of growth, namely, height-for-age and weight-for-age.
 2. To analyse the effect of Fe supplementation on growth parameters by the variable of Hb status.

C. Sample selection

(a) Sampling universe

There are 163 primary schools in Baroda, under the Baroda Municipal Corporation. These schools cater to the underprivileged population and are free of cost. They provide scholarships to those children who belong to the scheduled caste/scheduled tribes. There are some schools exclusively for boys, exclusively for girls while others are coeducational.

(b) Sampling frame

Out of 163 schools, 52 schools are exclusively for boys, 45 schools cater exclusively to girls and the rest are coeducational. The 45 exclusively girls schools constituted the basic frame for sampling.

(c) Sample selection

Four schools, exclusively for girls were purposively selected for the study. The schools were in close vicinity to each other. Two of the schools selected were morning shifts, functioning from 7.30 a.m. to 12.30 p.m. and two were afternoon shifts, functioning from 12.45 pm. to 5.45 p.m.

Permission to conduct the study in these schools was obtained from the administrative authorities of the Primary School Board and the Principals of the respective schools.

The study sample constituted of the pre-adolescent and adolescent school girls. The criteria used for selection of the subjects were :

1. Those girls whose birth dates were recorded and were between the age of 8-15 years.

Table 2. Comparison between initial and final study sample.

Parameters	Initial screening of 207 subjects	Final study population of 166 subjects	't' value
Age (in yrs mths)	9.86 \pm 0.1035	9.76 \pm 0.1015	0.69
Hb (g/dl)	10.49 \pm 0.088	10.42 \pm 0.090	0.59

Note : All values are mean \pm SE.

The final age-wise breakup of the sample was as under :

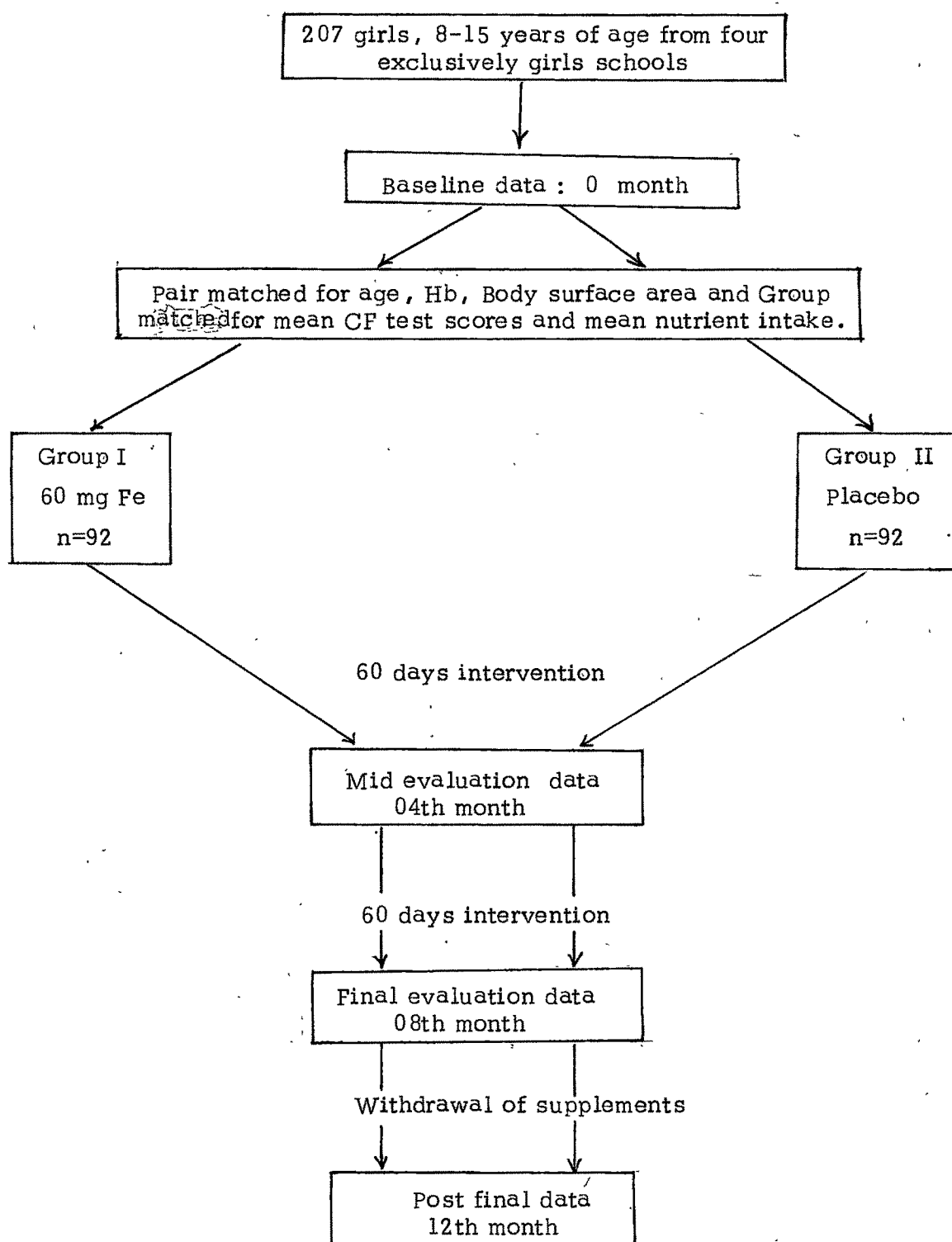
<u>Age group</u>	<u>Initial sample</u>	<u>Final study population.</u>
8 yrs 0 mths to 8 yrs 11 mths	44	42
9 yrs 0 mths to 9 yrs 11 mths	42	40
10 yrs 0 mths to 10 yrs 11 mths	40	38
11 yrs 0 mths to 11 yrs 11 mths	38	28
12 yrs 0 mths to 12 yrs 11 mths	18	06
13 yrs 0 mths to 13 yrs 11 mths	25	12
	<u>207</u>	<u>166</u>

2. Those girls who belonged to the low socio-economic group with a family income not greater than Rs.500/- per month.
3. Parents of the selected subjects should give written consent for participation in the study and also for blood withdrawal during the course of the study.

Based on the above criteria 207 subjects were enrolled for the study. However, only 184 subjects constituted the baseline sample as 17 subjects could not be pair matched (See Experimental Design) and 2 subjects were not cooperative in giving the preliminary data and showed unwillingness to participate in the study and 4 subjects had attained menarche and were dropped from the study. However, by the end of the year long study, 166 subjects formed the total study population due to attrition in sample over time. This was both due to subjects dropping out from the study on their own and also due to 5 subjects who had attained menarche over the year-long study and who therefore had to be deselected.

As indicated in Table 2, independent 't' test established that the preliminary data obtained on the 166 subjects was not significantly different from that on 207 subjects. This ensured that the final study population of 166 girls was representative of the initially selected population.

Fig. 4

EXPERIMENTAL PLAN

D. Experimental Design

Hemoglobin (Hb) and red cell morphology (RCM) were performed on all the 184 subjects. They were stratified by age, using one year age intervals. Within each age category the subjects were pair matched for Hb levels and baseline cognitive function test scores to fulfill objective II; and for Hb levels and body surface area to fulfill Objective III. The body surface area was calculated by the formula of Banerjee and Sen (1955).

One subject from each pair was then randomly assigned to either of the groups, namely, the experimental group and the placebo group. Each group consisted of 92 subjects.

The experimental plan is summarised in Figure 4.

The study was conducted over one calendar year. Iron supplementation (experimental group) at a dose level of 60 mg elemental Fe, in the form of ferrous sulfate (FeSO_4) and sugar tablets (placebo group) were given twice, at the commencement of each school term for 60 days at a stretch (i.e. over one school year).

Data were collected every four months i.e. baseline (0 month), at the commencement of the study; mid-evaluation data (04th month), at the end of the first school term; final-evaluation data (08th month), at the end of the second school term; and post-final data (12th month), following a withdrawal of supplements.

The parameters studied at each evaluation were :

Parameter	Baseline	Mid	Final	Post final
Height (Ht)	✓	—	✓	—
Weight (Wt)	✓	—	✓	—
Red Cell Morphology (RCM)	✓	—	—	—
Hemoglobin (Hb)	✓	✓	✓	✓
Serum Iron (SI)	—	—	✓	—
Total Iron Binding Capacity (TIBC)	—	—	✓	—
Percent Transferrin Saturation (TS)	—	—	✓	—
Cognitive Function (CF) Tests	✓	✓	✓	✓
Physical Work Capacity (PWC) Tests	✓	✓	✓	✓
Dietary Survey	✓	—	—	—

E. Dosage of hematinic compound

The subjects were pair matched and divided into two groups (as described in the experimental design). One group was assigned as the experimental or Fe treated group, receiving 60 mg elemental Fe as FeSO_4 and the other was assigned as the control or placebo group, receiving a sugar tablet. The ferrous sulfate tablets were obtained from Rup Pharma, Navsari, Gujarat and the sugar tablets for the placebo group were specially manufactured for the study by Alembic Limited, Baroda.

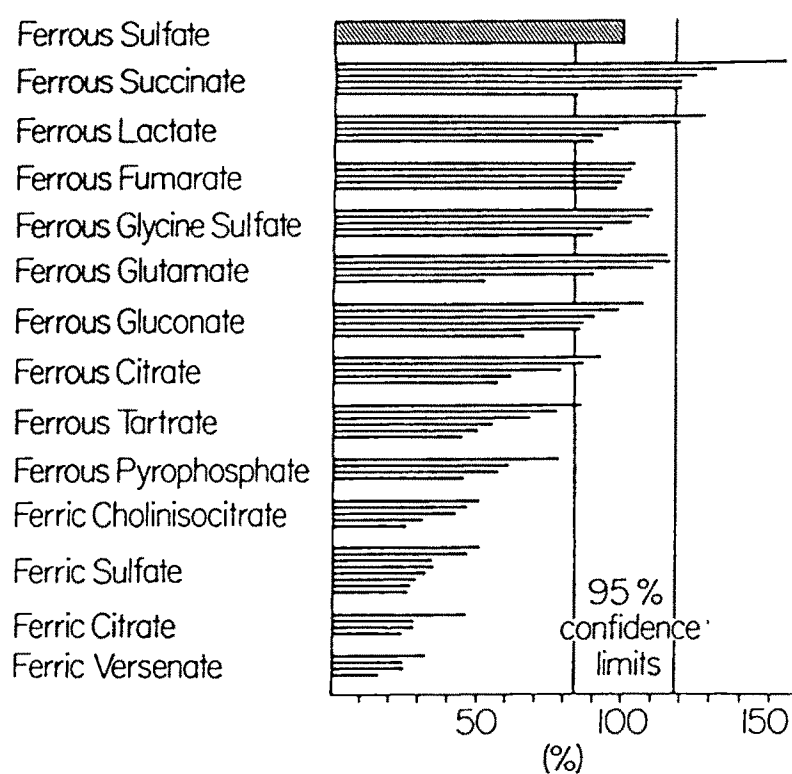


FIG 5

A comparison of the absorption rates with different iron salts. In these studies a daily dose of 30 mg iron as ferrous sulfate was alternated with another iron salt in similar dosage labelled with a second isotope over a period of 10 days. Taken from: Brise and Hallberg, 1962.

(a) Hematinic compound selection

The type of iron used for supplementation must be one which is readily assimilated and does not cause any undesirable effects in the human body, it should be stable under locally prevailing storage conditions. Its absorbability should be shown to be sufficiently good to justify its use.

Although, numerous pharmaceutical products are available, the advantages claimed for each have little foundation. In fact, certain general statements can be made. Preparations of ferrous salts in a dosage of 30 mg are absorbed about three times as well as are preparations of ferric salts, and the discrepancy is even greater with larger amounts (INACG, 1981). Varying the particular salt of ferrous iron has little effect; sulfate, lactate, fumarate, glycine sulfate, glutamate and gluconate are absorbed to about the same degree. The one exception may be ferrous succinate, which has been reported to be about 30% better absorbed than ferrous sulfate (Brise and Hallberg, 1962) (Fig. 5.1).

However, ferrous sulfate is by far, the cheapest source of ferrous iron available and hence is the compound of choice for oral administration for almost all cases of iron deficiency (INACG, 1977).

Thus, the compound used for hematinic supplementation in the present study was FeSO_4 .

(b) Basis for Non-inclusion of folic acid in the hematinic compound

Bradfield (1968) in a study on 156 school children (7-13 years) studied the effect of iron treatment with or without vitamin B-complex, which included folic acid, for a period of 6 weeks. No additional benefit of the B-complex vitamins (including folic acid) along with iron was observed indicating that folic acid deficiency was not prevalent in this population.

Margolis et al (1981) in an attempt to examine the major cause of anemia, supplemented 344 Eskimo children, 6-17 years of age with 3 mg elemental Fe/Kg body wt/day for 6 months. Results indicated an improvement in Hb status and other parameters (serum ferritin, percent saturation of transferrin and free erythrocyte protoporphyrin) of nutrition, indicating anemia of iron deficiency origin.

Saraya et al (1971) observed an increase in serum folate levels along with an improved iron status on iron supplementation. The authors postulated that in iron deficiency, folic acid is trapped in the liver and cannot enter the circulatory pool. Hence, serum folate levels may be low in iron deficiency but is not necessarily indicative of folate deficiency.

Tarvady (1982) could not demonstrate any beneficial effect of folate supplementation on Hb status greater than that in the placebo group. Indicating folic acid deficiency

was not the cause of anemia in the preschoolers studied. Gopaldas and Kale (1985), also demonstrated an absence of folate deficiency in 8-15 year old underprivileged school boys, in Baroda, based on the predominantly microcytic hypochromic red cell picture.

Further, Bothwell et al (1979) suggest that mixed preparations should be avoided since they create uncertainty as to the specificity of any response obtained, quite apart from the unnecessary additional expense. Also if there are other deficiencies it is important to identify them rather than to obscure their presence with multiple therapy.

Based on the literature available and on baseline data of the present study on RCM, which indicated a predominant microcytic hypochromic or anisocytic red cell picture, the subjects were intervened with iron supplements (as FeSO_4) alone. Details of RCM data are given in the chapter on Results and Discussion.

Studies conducted on the hematological response of iron therapy have used various levels of ferrous (Fe^{2+}) iron in school children.

These dosages ranged from :

Dose	Fe Form	Period	Investigator
(a) 15 mg elemental Fe/day + Vitamin supplements.	FeSO ₄	10 weeks	Bradfield et al, 1968.
(b) 20 mg elemental Fe/day + Folic acid	FeSO ₄	60 days each at the commencement of each school term.	Gopaldas et al, 1983
(c) 40 mg elemental Fe/kg body weight/day.	FeSO ₄	60 days	Gopaldas and Kale, 1985
(d) 3 mg elemental Fe/kg body weight/day	FeSO ₄	6 months	Margolis et al, 1981
(e) 82.5 mg elemental Fe/day + Folic acid + Vitamin B ₁₂	Fe ²⁺ Fumarate	10 weeks	Singla et al, 1982.
(f) 200 mg Fe/day + Multi-vitamins	FeSO ₄	40 days	Khanduja and Agarwal, 1970

A clear distinction needs to be made between the prevention and treatment of Fe deficiency anemia. The former calls for a modest dose level for a prolonged period of time, while the latter calls for large dose of iron in a short interval of time.

Gopaldas et al (1983) however, indicated the non-efficacy of 20 mg elemental Fe as FeSO₄ for 60 consecutive days, twice in a school year, in improving the Hb status of school girls, 10-15 years of age indicating higher Fe needs for older girls.

Gopaldas et al (1985a and 1985b) demonstrated the hematological and functional benefits of 40 mg elemental Fe (FeSO_4) dose, administered for a period of 60 days at a stretch, on boys 8-15 years of age.

In order to prevent side effects and bring about a slow hematological response for mildly anemic cases, INACG (1981) suggested, a single dose of 36 to 74 mg iron should be administered. WHO (1975) agreed to use a dose of 3 mg elemental Fe as Fe^{2+} ascorbate/kg body weight per day. The mean weight of the subjects of the present study was 21 kg (ranging from 14.0 kg to 36.0 kg) at baseline. In view of the WHO recommendation a dose of 60 mg elemental Fe as FeSO_4 was calculated for the study subjects.

(c) Iron content of the tablets

The experimental group received FeSO_4 tablets, calculated to yield 60 mg elemental Fe. These tablets were analysed chemically for iron content by the method of Wong (Oser, 1976).

Procedure

A random sample of the iron tablet was taken and powdered. A known weight of the powdered sample was digested with a mixture of concentrated HNO_3 and concentrated H_2SO_4 (5 : 1) in a long necked Kjeldahl's flask till a clear extract was obtained. The volume was made upto 100 ml with water.

To a 50 ml volumetric flask, 2.5 ml aliquot of the digested solution of the tablet was added and made to mark. Two ml of iron-free concentrated H_2SO_4 were then added and mixed by whirling for 2 minutes. Then 2 ml of saturated potassium persulfate solution were added and it was diluted to 25 ml with water.

Standards were prepared in the same way by taking 2.5 ml of standard iron solution containing 0.1 mg of ferric iron per ml and treating it as described above. A blank was prepared using water instead of standard iron solution.

Ten ml of all solutions i.e. blank, standards and unknown were measured into separate test tubes. To each was then added 0.5 ml of saturated potassium persulfate solution followed by 2 ml of 3 N potassium thiocyanate solution. It was then mixed by inversion and read within the next 30 minutes, setting the instrument to zero with the blank at 540 nm.

Iron content was obtained by the following formula :

$$\begin{aligned} \text{mg iron in} \\ \text{one tablet} &= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{conc. of std.} \\ &\times \frac{5}{2.5} \times 100 \times \frac{\text{Mean wt. of tablet}}{\text{Wt. of sample}} \end{aligned}$$

Details of the preparation of reagents is given in Appendix 1.

(d) Administration of the supplements

The administration of the supplements was done in the schools everyday by the investigator. A register was maintained and receipt of

the supplement by the subject was marked. Subjects who were consistently absent were dropped from the study.

(e) Intervention period

Although, the response in Hb should be virtually complete after 2 months, therapy should be ideally maintained for a longer period to allow for accumulation of storage iron (INACG, 1981): Thus, a supplementation period of 60 days (2 months) each, twice (i.e. a total of 4 months) in the school year was selected.

(f) Parameters and techniques for data collection

For each subject enrolled for the study, written consent from the parents was obtained for participation and blood withdrawal in the study. A proforma in simple Gujarati (Appendix 2) was used for this purpose. The ages of the subjects were rechecked and the family income obtained was ascertained through this proforma.

The sample size for each parameter tested was as follows :

Parameter			Sample size
1	Height-for-age	...	166
2.	Weight-for-age	...	166
3.	RCM	...	166
4.	Hb	...	166
5.	SI	...	102
6.	TIBC	...	102
7.	TS	102
8.	CF tests	...	130
9.	PWC tests	...	166
10.	Dietary survey	...	122

Parameters to fulfil Objective I

A. Hematological status

a. Blood hemoglobin (Hb)

The preferred method is that in which hemoglobin is measured photometrically after conversion to cyanmethemoglobin or hemiglobincyanide (HiCN). The advantages of the cyanmethemoglobin technique are that it entails dilution with a single reagent, measures all forms of circulating hemoglobin (with the exception of sulphemoglobin) and employs standards with exceptionally long stability (Cook, 1984).

Principle

In solution the ferrous iron (Fe^{2+}) of the hemoglobins are oxidised to the ferric state (Fe^{3+}) by potassium ferric cyanide to form hemiglobin (methemoglobin). In turn, hemiglobin reacts with the cyanide ions provided by potassium cyanide to form cyanmethemoglobin. The time necessary for full colour development is shortened to 3 minutes if dihydrogen potassium phosphate is substituted for sodium bicarbonate in the Drabkin's reagent (Appendix 3). The addition of a nonionic detergent enhances erythrocytic lysis and minimizes turbidity resulting from lipoprotein precipitation.

The method was standardised and then applied to the subjects.

Method

The blood samples were collected in the schools. A finger other than the thumb or the little finger was first wiped with alcohol and then

dried with a clean filter paper. A bold prick was made using a sterile disposable blood lancet, so as to obtain free flowing blood. The first drop of blood was wiped off using filter paper. Then, 0.02 ml of blood was pipetted into a graduated hemoglobin pipette taking care to avoid the formation of air bubbles. The tip of the pipette was wiped free of blood with a filter paper and the blood was transferred into a test tube containing 5 ml of Drabkin's reagent. The contents were mixed by whirling the tube and allowed to stand for a minimum period of ten minutes. The reading was taken in a Klett Summerson Photoelectric Colorimeter at a wave-length of 540 nanometers. The instrument was set at zero using 5 ml Drabkin's solution as the reagent blank.

A series of standards were run using standard Hb solutions, obtained from CSIR centre for biochemicals, New Delhi. A calibration curve was prepared using this standard. A factor of 0.08 g Hb was established from the standardisation and each Klett reading was multiplied by this value to obtain the Hb concentration in g/dl. (Details of standardisation are given in Appendix 4.)

Modification of the method

Filter paper technique (NIN, 1974)

To facilitate working in the field, where large number of samples had to be transported, the modified filter paper technique was used. The 0.02 ml of blood sample drawn out from the finger tip,

was blown out, in concentric circles onto a Whatman Grade I filter paper. This was allowed to dry. Each piece of filter paper was labelled and stored between butter paper, in a box. In the laboratory, the blood stained portion of the filter paper was cut and immersed into a test tube containing 5 ml of Drabkin's reagent for elution. To enable complete elution it was allowed to stand for 30 to 45 minutes. The reading was taken in a Klett Summerson Photoelectric Colorimeter at 540 nm after shaking the tubes well. The Klett readings were multiplied by the factor 0.08 to obtain Hb (g/dl) values. To the Hb values, a correction factor of 4.4% was applied as suggested by NIN (1974) where it was observed that the filter paper method underestimates the actual Hb content (as measured by the direct method by 4.4%).

The major limitations of the filter paper technique are :

1. Sometimes the hemoglobin may not be completely eluted at the time of reading, making an underestimate of the value.
2. The blood blown out onto the filter paper may be absorbed on the filter paper differently, depending on the viscosity of the blood. Thin blood spreads much more than thick blood. The peripheral absorption may not be visible to the naked eye and may be cut out before elution.

b. Red Cell Morphology (RCM) examination

A thin blood film was used to study the morphology of erythrocytes (Dacie and Lewis, 1977).

Only in uncomplicated iron deficiency anemia do hypochromia and microcytosis develop in tandem. The hypochromic anemias are characterised by red cells that are deficient in hemoglobin. Hypochromic red cells are easily recognised by their washed out appearance and their broadened areas of central pallor. Among the secondary changes in configuration affecting severely hypochromic red cells are conspicuous variations in size (anisocytosis) and in shape (poikilocytosis) (Kapff and Jandl, 1981).

Procedure

Clean, dry, grease-free glass slides were taken and wiped free from dust immediately before use. A small drop of blood was placed in the centre line of the slide 1 to 2 cm from one end. The spreading slide was placed at an angle of 45° to the slide and then moved back to make contact with the drop. The drop spread out quickly along the line of contact of the spreader with the slide. The film was spread by a rapid, smooth, forward movement of the spreader. The spreader slide had a smooth edge and was narrower in breadth than the slide on which the film was made.

A reference number was written in glass marking pencil on the slide itself. Smears were fixed immediately by immersing in water-free methanol, for a few seconds and then brought to the laboratory for staining (Leishman's stain) and microscopic examination.

Staining procedure

The fixed slides were first covered with 10 to 15 drops of the stain. After one minute they were flooded with double the volume of phosphate buffer (Appendix 5) of pH 6.8 to 7.0. The buffer was mixed with the stain by blowing and was allowed to stand for nine minutes. It was then washed in a stream of water, the back of the slides was wiped clean and set upright to dry.

Microscopic examination of the slides

Observations were made under the microscope first under low power (10x) then under high power (40x) lens. The observations made by the investigator were cross checked by a trained technician.

Using this technique the blood smears were examined and recorded only at baseline (0 month).

C. Serum iron, total iron binding capacity and percent transferrin saturation

Blood sample collection

With the help of a trained technician 7 ml of venous blood per subject was drawn out from 102 subjects willing to give blood. The blood samples were drawn between 11.00 a.m. and 2.00 p.m. The blood was put directly into clean test tubes from syringes and was allowed to stand for clot formation. The serum was separated from the clotted blood by centrifuging at 3000 rpm for 15 minutes and then drawing off the serum carefully with a pasteur pi pette.

The fact that a restricted supply of iron for erythropoiesis is responsible for anemia can readily be established. The most helpful indicators of iron supply are the plasma iron concentration and the transferrin saturation (Bainton and Finch, 1964). While erythrocyte protoporphyrin concentration is more stable an indicator of balance between marrow iron requirement and available Fe, it is also abnormal in lead poisoning and certain rare abnormalities of porphyrin synthesis. A useful estimate of the quantity of iron stored in the tissues, and potentially available for the synthesis of Hb, is provided by plasma ferritin concentration, the relationship is distorted by inflammation or liver disease (Lipschitz et al, 1974).

The latter two tests call for elaborate techniques requiring expensive equipment which were not available in our present laboratory. As per the suggestion of Dr.B.S.Narasinga Rao, Director, National Institute of Nutrition, Hyderabad, serum samples for subjects whose Hb levels were 12 g/dl or greater were stored in sealed containers for ferritin estimation in the freezer compartment of the refrigerator for subsequent analysis at NIN. However, these samples had to be discarded due to an insufficient freezing temperature in the freezer compartment of the refrigerator. Thus, only the tests for serum iron, total iron binding capacity and transferrin saturation (by calculation) could be performed.

A specific protein, transferrin, transports Fe through the plasma. In normal circumstances transferrin is only about one-third saturated with Fe (Bothwell et al, 1979).

It is generally believed that the most reliable indicator of iron supply to developing red cells is not the plasma iron concentration itself but rather the degree to which circulating transferrin is saturated with iron and there is good evidence that the iron supply is restricted when the saturation falls below 15% (Bainton and Finch, 1964). Because of this, plasma iron determination should be performed in conjunction with measurements of the iron binding capacity. The result usually provided by the clinical laboratory is the total iron binding capacity (TIBC) which represents the sum of endogenous iron bound to plasma and the additional iron which can be specifically bound. In many methods it is unsaturated iron binding capacity (UIBC), or concentration of iron that can be taken up by the unbound transferrin in native plasma, which is measured directly. This figure is then added to the plasma iron concentration to obtain TIBC (Cook, 1984).

Plasma/Serum iron concentration methods

The plasma/serum iron concentration method which are generally considered the most accurate are those involving deproteinization. They have in common four basic steps : plasma or serum is acidified to dissociate the iron-transferrin complex, the iron is reduced, plasma proteins are precipitated with trichloroacetic acid or heating and removed by centrifugation, and finally a sensitive iron chromogen is added to the supernatant to determine the ferrous iron concentration colorimetrically.

An alternative and simpler approach to the measurement of the plasma iron concentration is one in which an acid buffer and a reducing agent are first added in order to release iron from transferrin. A ferrous chromogen is then directly added without protein precipitation. The background absorbance of the plasma is obtained either in a second tube or in the same tube prior to adding the chromogen. However, there is a major disadvantage to this approach. The sensitivity is low because of the high background density of the plasma, especially in the presence of raised concentrations of Hb, bilirubin or lipids.

Iron binding capacity methods

The most popular methods for determining the iron binding capacity of plasma or serum are those in which iron in excess of the binding capacity of transferrin is added. The unbound iron is then physically removed from the sample prior to analysis. The usual approach is to add an insoluble material which complexes with unbound iron and that can be removed on centrifugation. Various compounds have been used for this purpose. Cook (1970) found that MgCO_3 was probably the most satisfactory of the adsorbents currently available and thus recommended its use.

Ramsay's dipyriddy method (1954) seemed to be best suited for these estimations and was thus employed.

Determination of Serum Iron (Ramsay's method)

Principle : Ferrous iron gives a pink colour with 2,2' dipyridyl .

A solution of dipyridyl in acetic acid is added to the serum followed by a reducing agent. Proteins are removed by heating in boiling water and then centrifuging.

For preparation of reagents see Appendix 6a.

Method

To 1 volume of serum, 1 volume of water, 0.5 volume each of the 0.2 M sulfite and 0.2% dipyridyl were added in a glass stoppered tube which could be centrifuged. This was heated in a boiling water bath for 5 minutes; cooled and then 1 ml of chloroform added, stoppered and vigorously shaken for 30 seconds. After removing the stopper, it was centrifuged for 5 minutes at 300 rpm. Reshaking and recentrifuging it, if the supernatant was not completely clear. Readings were taken at 520 nm in the spectronic 20, using a water blank instead of serum.

For standard, the working standard was put through in the same way.

Calculations

$$\text{mcg iron per 100 ml serum} = \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 300$$

By this method readings are linear with concentration to atleast 500 mcg/dl. To obtain the calibration curve, 5 ml of the stock solution was diluted to 100 ml with distilled water and tubes set up containing 0.4, 0.8, 1.2, 1.6 and 2.0 ml of this. Each was made to 2 ml with water and the colour was

developed as described in the method and read against the blank.

These corresponded to 100, 200, 300, 400 and 500 mcg/dl.

Determination of TIBC

As described earlier this method involves the addition to the serum an amount of iron more than sufficient to saturate the iron binding protein. Then, adsorbing the excess iron onto MgCO_3 and finally determining the total bound iron by the dipyrldyl method.

For preparation of reagents see Appendix 6b.

Method

Four ml of FeCl_3 solution was added to 2 ml serum. After standing for 5 minutes, 400 mg MgCO_3 (100 mg for each ml FeCl_3) were added shaking frequently and vigorously for 30-60 minutes. Then, 4 ml of supernatant fluid was pipetted out after centrifugation, for iron determination. To the supernatant fluid, 1 ml each of the 0.2 M sulfite and 0.2% dipyrldyl was added and the same procedure as for serum iron determination was followed.

Calculations

Since the volume of serum in 4 ml supernatant was 1.33 ml and same standard i.e. 3 mcg/ml was used (2 ml standard solution, 2 ml water and 1 ml each of dipyrldyl and sulfite solutions), then :

TIBC in mcg/100 ml serum

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \frac{100}{1.33} \times 6 \text{ (i.e. 450)}$$

Precaution

The most important precaution to be taken in this method is that the glassware used must be cleaned by placing them in boiling 5N HCl or leave overnight, and then washed with glass distilled water and only then used for the estimation of iron, because contamination iron plays havoc with the results if care is not taken.

Percent saturation or Transferrin saturation

Transferrin saturation (TS) is calculated by dividing the concentration of serum iron by the total iron binding capacity and multiplying by 100 to express the result as percent.

The measurement of TS is widely used as a confirmatory test in the diagnosis of iron deficiency (INACG, 1979).

Although, many automated techniques to measure SI, TIBC are now available and also provide more reproducible results, the initial investment for these equipment is high. Thus, the most reliable and reproducible manual techniques were employed for this study and all possible precautions taken.

Parameters to fulfill Objective II

(a) Blood hemoglobin (Hb)

as described for Objective I.

(b) Cognitive Function (CF) tests

Iron deficient children are reported to have narrowed attention span, poor discrimination, perception and impaired visual motor

coordination along with some behavioral problems.

Thus, in this study the tests of cognitive function selected were specific for certain areas, namely, concentration, discrimination, memory, perception and visual motor coordination, known to be affected in iron deficient anemic children.

The battery of tests selected were in consultation with Dr. S. Anandalakshmi, a noted Child Psychologist and currently Director, Lady Irwin College, New Delhi. The entire battery of tests, which also include two tests from the Gujarati adaptation of the Weschler's Intelligence Scale for Children (WISC) (Bhatt, 1973) was standardised on a similar population of school age children, before final use on the study population.

Testing Situation

The CF tests were carried out individually in a separate room in privacy. This prevented any consultation between and distraction to the subject. The total time involved for all tests for each subject was 25 minutes.

The tests suggested by Dr. Anandalakshmi were :

1. Clerical task, to test : attention, concentration and discrimination.
2. Visual memory test, to test : intellectual deterioration in which the ability to remember non-verbal material is evaluated.



The tests selected from the Gujarati adaption of WISC were :

1. Digit Span is a test of how large a quantity of a given kind of material can be reproduced perfectly after one presentation.
2. Mazes are a series of printed line mazes, steeply graded in difficulty. This test is intended as a measure of foresight, planning capacity (perception) and visual motor coordination, which are important in practical social sufficiency.

Both Visual memory and Digit Span tests were specifically chosen for immediate memory.

Details about the tests -

1. Clerical Task

In this test, each subject was given a printed text in Gujarati (Appendix 7) and was asked to encircle all matras ( and  ; vowels 'a' and 'o' of Gujarati) in the entire script as quickly as possible. The number of matras encircled in the test period of five minutes was recorded. The score was calculated as a ratio of the number of matras encircled to the total number of matras in the text. The ratio was then multiplied by 10 which was the maximum obtainable score.

2. Visual Memory Test

Ten items were displayed to the subject on a flat surface for a period of one minute. The items were then covered with a piece of cloth.

The subject was required to recall as many items as possible within the next one minute. The maximum score obtainable in this test was 10 and the items displayed were familiar everyday use items like pen, pencil, eraser, knife, spoon etc.

3. Digit Span

This is the simplest type of recall test with verbal materials. The subject was presented with series of digits gradually increasing in length. Each list was given only one presentation and at the end of each series the subject was to repeat the digits vocally. It was important that the series be repeated in correct order. Different series were presented for a forward recall and a backward recall. The series for forward recall started from 3 digits upto 9 digits and that for backward recall from 2 digits to 8 digits (Appendix 8).

The longest group of digits the subject could recall in the correct order, determined the memory span for digits. Maximum score obtainable in this test was 17, but for simplification in analysis it was proportionately reduced to ten.

4. Mazes

Maze is a test with a more or less complex pathway having blind alleys in which the subject can get lost, or atleast expend unnecessary time and energy.

A set of mazes A, B, C and 1, 2, 3, 4 and 5 were given to the subject (Appendix 9). Each subject was asked to start with Maze C* saying, "This is a maze, you are to start here", pointing to the cross in the centre, "and find your way out, here", pointing to the exit, "without going up any blind alley or crossing any lines. Do you understand?" The subject was then given a pencil and was asked to "go ahead". With the instructions not to lift the pencil until the maze is completed. Each maze had a time limit, hence a stop watch was started immediately, as the subject found her way out and completed Maze C with not more than one error and within the time limit, full credits were given for Maze A and Maze B and that earned for Maze C. She then continued to test Maze 1 and beyond with each successive maze in the same manner.

The test was discontinued on two consecutive failures i.e. score of zero. The time limit and maximum number of errors allowed for each maze was specified (Table 3).

* This was as per the procedure suggested for children, 8 years or older in age in the WISC manual (Bhatt, 1973).

Table 3. Time limit and maximum number of errors allowed for each maze.

Maze No.	Errors allowed	Time Limit (seconds)
Maze A	2	30
Maze B	2	30
Maze C	2	30
Maze 1	3	30
Maze 2	3	45
Maze 3	5	60
Maze 4	6	120
Maze 5	8	120

The subject was considered to have failed in a maze if she could not reach the exit within the time allowed or if her errors were more than the number allowed for that maze. Occasionally a subject would draw a line through one or more walls directly to the exit, this was complete failure.

Scoring

Mazes A, B and C

- 1 Within the time limit and no errors : 2 points.
- 2 Within the time limit and more : 1 point.
than allowed errors

Mazes 1 - 5

- 1 Within the time limit and no errors : 3 points.
- 2 Within the time limit and only one error : 2 points.
- 3 Within the time limit and 2 but no more : 1 point.
than maximum number of allowed errors

Maximum score possible was 21, but this was reduced to 10 for simplification in analysis. Appendix 10 gives the scoring sheet for Mazes and Visual memory.

Definition of errors for Mazes

1. In general, crossing an imaginary line into a major blind alley was an error. The particular blind alley entrances which are counted as places of error are marked with dotted lines on the mazes (Appendix 9). Any wandering around within a blind alley was part of the error. If the subject returns to the correct path and then re-enters the blind alley, it was a second error.

2. Crossing a line was counted as an error.

3. Lifting the pencil off the paper was counted as an error.

In order not to penalise the subject twice, the pencil lifting error did not count if it was done to correct another error. For example, if after crossing a line the subject lifted the pencil to return to the path, it was counted as only one error.

Table 4 . Comparison at baseline, Hb and Cognitive Function test scores between the two treatment groups.

Group	N	Hb (g/dl)	Cognitive Function test scores					Total Scores
			Clerical Task	Digit Span	Visual Memory	Mazes		
Iron treated	65	10.28 +0.14	4.00 +0.16	3.88 +0.16	7.89 +0.19	4.36 +0.22		20.11 +0.54
Placebo	65	10.39 +0.14	4.11 +0.12	4.04 +0.13	7.77 +0.15	4.71 +0.21		20.55 +0.46
't' values		0.5308 ^{NS}	0.4836 ^{NS}	0.8083 ^{NS}	0.4906 ^{NS}	0.3355 ^{NS}		0.6139 ^{NS}

All values are mean \pm SE

NS - Difference between the means of the two groups - non significant.



After the completion of all the four tests the subjects were given sweets or biscuits as a token of appreciation for their cooperation.

Total score : A total score of all the four ~~sub tests~~ was calculated. The maximum obtainable score was 40.

The CF tests were conducted on 83 pairs of subjects matched for initial age, Hb, individual and total CF test scores. However, by the end of the study data on only 65 pairs of subjects could be retained for analysis because the rest could not attempt the Clerical Task at baseline evaluation due to their inability to read the text; especially so for the younger children. Also, some of these subjects performed the test on subsequent testing as they had learned to read and this could not be attributed to the treatment. As such, it would have vitiated the results.

Thus, the baseline data (0 month) on CF tests was analysed for only 65 pairs of subjects using the independent 't' test to compare the mean scores for each test, between the two treatment groups. The results of the comparison (Table 4) revealed no significant difference between the two treatment groups for any of the CF tests. This ensured that both the groups were highly comparable and whatever differences emerged later during the course of the study, could be attributed to the intervention or treatment effect.

Parameters to fulfil Objective III

(a) Blood Hb as described for objective I.

Table 5. Matching criteria^a for the two treatment groups at baseline for Physical Work Capacity.

Groups	N	Age in years	Hemoglobin g/dl	Body surface area (cm ²)
Fe treated group	83	10.18±0.1627	10.443±0.131	9187.277±123.441
Placebo group	83	10.12±0.2185	10.410±0.125	9156.789±130.016
't' value		0.22 ^{NS}	0.18 ^{NS}	0.17 ^{NS}

^a All values are mean ± SE.

NS Difference between the means non-significant.

(b) Physical Work Capacity (PWC)

The PWC was measured in terms of changes in pulse rate and blood lactic acid on sub maximal exercise in response to hematinic supplementation. The test was carried out on subjects who had earlier been pair matched for their age, mean Hb and body surface area. A comparison for these criteria between the two groups was made at baseline. Results of the independent 't' test (Table 5) indicated no significant difference between the two treatment groups for any of the matching criteria.

This further ensured that the two treatment groups were highly comparable and whatever differences emerging later, during the course of the study could be attributed to the intervention or treatment effect.

In the recent past, numerous tests to measure work output have been devised. Of these the major tests most commonly used by well equipped laboratories are :

1. The treadmill test used by Gardener et al, 1977; Edgerton et al, 1981; Ohira et al, 1981; Flores et al, 1984.

This test involves progressively increasing work loads, by varying (increasing) speed, inclination and run duration systematically and also measuring oxygen consumption using a respirometer or a Douglas bag or other sophisticated equipment.

2. The bicycle ergometer which may be -
 mechanically braked as used by Vellar and Hermansen, 1971
 or magnetically braked as used by Satyanarayan et al, 1979,
 or electrically braked as used by Areskog et al, 1969;
 Ericsson, 1970; Davies et al, 1973.

However, these tests are not of practical utility in the field conditions, as they require special conditions like temperature and humidity controls using air conditioners etc. Also, only in an experimental laboratory condition can the oxygen uptake measures be made.

In the field conditions, the need for simple innovative exercise tests is called for. Simplest of these has been the measure of worker's productivity i.e. ability to perform his/her regular routine work (Basta et al, 1979; Rahamathullah, 1983). However, this may be valid only for adults and not for children. Attempts have been made to design simple tests which could be used for children. These tests are :

The Harvard Step test (Basta and Churchill, 1973), a test of running & jogging (Hunding et al, 1981), a test of running and jumping (Seshadri and Malhotra, 1982), a test of walking, climbing, running, skipping and sweeping (Vijayalakshmi and Selvasundaram, 1983) and a test of simple jumping (Gopaldas et al, 1985b).

Of these tests the Harvard Step Test (HST) can be standardised and can form an important tool in the measurement of PWC.

Application of the original HST in the Indian population has been found to be unsatisfactory because of their relatively short stature (Banerjee and Chatterjee, 1983). Also, the original HST was standardised on boys 15 to 40 years of age and the height of children less than 15 years would play an important role in choosing the final height of the bench.

A preliminary study conducted on girl subjects from a similar environment as that of the present study population revealed that the 12" high jumping (stepping) board did sustain interest in schoolers and was convenient too.

Hence, the modified HST was used in the present study as described.

Methodology

In an open space of about 6' by 6', the 12" jumping board was placed. A square at a distance of approximately 3 feet was drawn in white chalk all around the jumping board. This was done to ensure that every child would walk the same distance to climb the bench from each side, after the jump. Each subject was asked to step onto the bench, keeping knees straight and feet together. She was then told to jump off the bench. The subjects were instructed to go around the bench, clockwise and anti-clockwise alternately, after each jump. Rhythm was maintained by making the subjects get onto the bench every three seconds. The three seconds gap was maintained by using a stop watch and sounding a bell. Each subject was made to carry out the test for a fixed period of five minutes.

The measures to test PWC used were :

- 1 Pre and post exercise pulse rate.
- 2 Pre and post exercise blood lactic acid.

Pulse rate : The transport oxygen from the lungs to the tissues plays a critical role to meet the increased energy demands on exercise or at rest. The cardio-vascular adjustments in anemia include an increase in cardiac output, brought about by a higher heart rate or a larger stroke volume or both. Rushmer (1970) reported a linear relationship of coronary circulation with O_2 consumption during exercise. Thus, pulse rate, the simplest measure of heart rate was determined, both pre and post exercise.

Methodology

The pulse rate at the wrist was counted for the first 10 seconds. This time period was standardised during the preliminary study on similar but not the same children. It was observed that the pulse rate started to drop significantly after the first 15 seconds post exercise. Also, because, blood lactate measurements were to be performed immediately prior to and after exercise, much time could not be lost on this measure alone. This procedure enabled to obtain fairly consistent and reproducible results for both pulse rate and blood lactate estimations.

Blood lactic acid

Post exercise lactate concentrations have been directly related to the degree of anemia and indicate the relative contribution of anaerobic metabolism to the overall stress of the exercise task (Gardner et al, 1977). Finch et al (1979) demonstrated that the accumulation of lactate was a result of excessive production, as the clearance from the blood was shown to be unaffected in the anemic rats.

Methodology

Prior to the exercise, the finger tip of the subject was wiped clean of sweat and sterilised with alcohol, dried and a bold prick was made using a sterile blood lancet to obtain free flowing blood. The blood was drawn out using a 0.1 ml graduated micro-pipette and was transferred immediately into labelled test tubes containing 0.9 ml of 10% trichloroacetic acid samples were collected in duplicate. The samples were collected over ice since avoidance of doing so resulted in erratic duplicate results. The same procedure was repeated on the finger of the other hand after the subject had completed the 5 minute exercise. Special care was taken to remove all traces of perspiration from the finger to be pricked, as sweat is known to contain high levels of lactate. Hence, a stack of tubes, properly labelled, containing 0.9 ml of 10% TCA in an ice box was transported daily to the school during data collection. The lactate estimations were performed within 4 hours of sample collection.

The blood lactate was estimated using the method of Barker and Summerson, 1941 (Varley, 1976). Although, the method calls for the use of 0.2 ml blood for the estimation, the method was modified for use with only 0.1 ml blood. This was done because it was extremely difficult to obtain 0.2 ml blood each time from a subject by the finger prick technique. Thus, the volume of the reagents to be added were scaled down to half those in the original method.

Method for blood lactate estimation

Barker and Summerson, 1941 (Varley, 1976).

Principle

In this method, the protein-free filtrate is treated with copper sulfate (CuSO_4) and solid calcium hydroxide (Ca(OH)_2) to remove glucose. A portion of the filtrate from this process is treated with H_2SO_4 to convert lactic acid to acetaldehyde, the amount of which is measured by means of the purple color given by p-hydroxydiphenyl in the presence of copper.

Reagents used are described in Appendix 11.

Methodology

The protein from the blood was removed by using 10% TCA. To the filtrate (equivalent to 0.2 ml blood), 1 ml of 20% CuSO_4 solution was added. This volume was made up to 10 ml with distilled water. Further, one gram of powdered Ca(OH)_2 was

added and the tubes shaken vigorously. The mixture was allowed to stand at room temperature for atleast half an hour, shaking occassionally. It was then centrifuged at 3000 rpm for 5 minutes.

To 1 ml of the supernatant, 0.05 ml of 4% CuSO_4 was added followed by exactly 6 ml of conc. H_2SO_4 , mixing well while adding conc. H_2SO_4 . The test tubes were placed upright in a boiling water bath for 5 minutes. The test tubes were then removed, cooled below 20°C in ice cold water. Then 0.1 ml of p-hydroxydiphenylreagent was added and the test tubes were placed in a water bath, at 30°C for 1/2 hour, redispersing the precipitate reagent atleast once during this time.

The tubes were then placed in vigorously boiling water bath for exactly ninety seconds. After being cooled, it was read in the colorimeter at 560 nm wavelength. The instrument was adjusted using a reagent blank.

The method was standardized using lithium lactate solution. With each set of readings a tube containing 5 ml lithium lactate solution, containing 0.01 mg lactic acid/ml and another tube with water as blank were also run simultaneously.

Parameters to fulfill Objective IV

1 Blood Hb as described for Objective 1.

2 Growth parameters.

(a) Height-for-age (Jelliffe, 1966)

Height is a linear measurement made up of the sum of four components; legs, pelvis, spine and skull (Jelliffe, 1966).

Methodology

A measuring tape was fixed vertically on a smooth wall in the school, perpendicular to the ground, taking care to see that the floor area was smooth and not rough. The subject was asked to remove her shoes ; stand with the centre of her back touching the scale; feet together and parallel, and with heels, buttocks, shoulders and back of the head touching the wall. The head was held comfortably erect, arms hanging loosely by the side. A smooth thin ruler was held on top of the head in the centre, crushing the hair at right angle to the scale and height read off from the lower edge of the ruler to the nearest 0.5 cm (Jelliffe, 1966). Each reading was taken twice to ensure the correctness of the measurement.

(b) Weight-for-age (Jelliffe, 1966)

Weight is a measurement of body mass. It is important that the age of the subject is correctly known and presence of pathological weight (e.g. due to edema etc.) be ruled out.

Methodology

An adult platform weighing balance (also known as personal or bathroom weighing scale) was used, as it is portable and convenient to use in the field. The weighing scale was checked for accuracy against a Libra-Detecto Weight-cum-Height Meter and also periodically checked with a 10 kg standard weight after weighing every 10th child. The scale was adjusted to zero before each measurement. The subject was asked to stand on the platform of the scale without touching anything, looking forward and with minimum clothing on. The weight was recorded in a register to the nearest 0.25 kg.

Baseline dietary survey

Diet surveys provide relevant information on dietary adequacy/inadequacy and the causes of inadequacies in the population group under study.

Actual weighment of foods consumed by the family and by the individual concerned is a time consuming and unsuited technique for diet surveys in the community setting. Hence, the oral questionnaire or 24 hour recall method may be used which is simple and a practical field tool (Pasricha, 1959).

Therefore, it was decided to use the 24-hour dietary recall method in the present study to obtain information regarding the food and nutrient intake of the girl subjects in both the Experimental and Placebo groups.

Table 6 : Comparison of dietary intake in the iron treated versus placebo group of underprivileged school girls (8-15 yrs) at baseline evaluation.

Nutrients	Recommended Daily Allowances	Iron treated group(61)	Placebo group (61 a)	Independent 't' values
Fats (g)		22.87 \pm 8.42	22.39 \pm 6.98	0.0438
Proteins (g)	35-50	36.76 \pm 10.10	36.46 \pm 10.29	0.0208
Calories (kcal)	1800-2200	1333.25 \pm 297.91	1268.30 \pm 305.26	0.1522
Iron (mg)	20-35	23.50 \pm 8.66	24.25 \pm 8.23	0.0628
(Retinol)				
Vitamins A (mcg)	400-750	161.98 \pm 121.88	149.42 \pm 130.04	0.0704
β carotene (mcg)	1600-3000	276.62 \pm 435.74	317.38 \pm 806.36	0.0444
Ascorbic acid (mg)	30-50	20.51 \pm 8.64	22.33 \pm 8.06	0.0909

Note : All values are mean \pm SE.

Difference in the various nutrients for the two treatment groups was non significant.

Methodology

For each subject included in the diet survey, first a home visit was paid to explain to the mother the purpose of the survey and to request her to carefully observe what her child, who was a subject of the study, ate from morning to night on the next day. On the following day the family was visited and the mother of the subject was carefully questioned as regards the food items cooked for the family the previous day and the quantity of the cooked food eaten by the child subject. The mother quantified her observations of raw and cooked foods with the help of her domestic plates and utensils. The standard cup, glass, bowls and spoons helped the investigator to convert the indicated food quantities in grams or millilitres. All responses of the mother were recorded on the proforma developed by the National Institute of Nutrition, Hyderabad. The quantities of raw food items eaten by the child were calculated. The nutrient intake of the subject was calculated using the food composition tables (Gopalan et al, 1981) and recorded on the proforma.

The data presented in Table 6 reveals that the experimental and placebo groups were well matched with respect to calories, fats, proteins, iron and ascorbic acid intakes. However, the calorie, retinol and ascorbic acid intakes were far below the recommended daily allowances. Low intakes of ascorbic acid, a known enhancer

of iron absorption, could be one of the contributory factors to the widespread anemia in the study population. Further, although dietary intake of iron appeared to meet RDA, the bio-availability of the cereal-pulse diets consumed by the subjects could have been low (Shah et al, 1984).

G. Statistical analysis of the data

- 1 Mean and standard error were calculated for each parameter.
- 2 The paired 't' test was employed to study the impact of intervention on the subjects in each group, when comparing the data over time i.e. from baseline to mid to final to post final evaluations.
- 3 The independent 't' test was employed to establish whether or not there was any significant difference between the means of the two treatment groups for each parameter at baseline, mid, final and post-final evaluations.
- 4 Coefficient of correlation (r) was calculated whenever the relationship between two parameters was to be tested.

The paired 't' test and the correlation of coefficient analysis were done using the computer.

The levels of significance were $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ for the sake of clarity as there were several comparisons to be made.

Formulae used were as described by Snedecor and Cochran (1968).