CHAPTER - II

MATERIALS AND METHODS

MATERIALS AND METHODS

As mentioned in the preceding section, the present studies were concerned with certain aspects of human fetal and neonatal development in relation to gestational age, size for gestational age and plane of maternal nutrition.

The aspects studied were overall growth status and nutritional status with regard to iron and magnesium.

The studies carried out on the fetus included :

(a) stores of iron in the liver, spleen and kidney in relation to maternal status.

(b) magnesium content of selected tissues in relation to maternal magnesium status.

Both these aspects were studied in relation to gestational age and body weight of the fetus in relation to gestational age.

The studies carried out on the neonates were concerned with maternal and neonatal status with special reference to iron and magnesium in relation to gestational age and body weight.

Studies were also made on the effects of a food supplement, namely, Dhokla, a fermented food, on pregnancy weight gain at different stages and birth weight. In these studies, growth of the infants during the first six months of life was also monitored in the different groups.

Additional studies were carried out on body weight and somatic measurements of the newborn infants and fetuses. The growth pattern of selected fetal tissues was also studied.

The subjects used in the investigations for the first two aspects hailed from both low and high income groups in urban Baroda. These were women admitted to the local general hospitals or private nursing homes for medical termination of pregnancy or delivery. The women in the low income group who participated in the studies on magnesium and iron status and somatic measurements of the neonates hailed from the Jamunabai hospital, a general hospital where patients come mainly from urban Baroda. For fetal studies, the subjects hailed from Sayaji hospital, a general hospital which caters to both urban and rural poor sections of the society. The families in the low income group belonged to those of manual labourers and unskilled workers with an annual income per capita of Rs.1200-1500 or less whereas the corresponding figures

for the high income families, which were from the professional class was Rs.3000 or more. This difference in economic status was associated with differences in dietary intake and nutritional status. Differences in the dietary intake of pregnant women between the two groups have been documented by Rajalakshmi (1980). The typical diet consumed by pregnant women in the low and high income groups provide, respectively, about 1570 and 2020 Kcal, 38 and 49g of protein, 400-500 and 1290 mg of calcium and 375 and 477 µg of vitamin A from carotene and 15 and 165 µg of preformed vitamin A and 220-230 and 225-230 mg of magnesium and 20-27 and 22-30 mg of iron.

The subjects used for the supplementation studies hailed from an urban slum area, namely, Nava Yard situated within the Baroda corporation limit. Their incomes and food intakes were somewhat lower as compared to the LIG studied in the hospitals. The home diet of these women provided 1200-1400 Kcal, 35g of protein, 130 mg calcium, 500 µg of carotene and 20 mg of iron.

Fetal and neonatal studies

Fetuses less than 20-22 weeks of gestational ages were largely derived by medical termination of pregnancy. Those above 20-22 weeks were mostly derived from spontaneous abortions and still births. A scrutiny of the data for spontaneous abortions suggested that they fell into two distinct categories, namely, fetuses with extremely unsatisfactory or reasonably satisfactory development, suggesting a possible differences in the cause of abortion i.e. either poor development, and consequently rejection as a likely factor in the former case or sudden trauma as a likely factor in the latter case. Only those in the latter category were included in the study sample. The fetuses were kept on ice as soon as they were aborted, transported to the laboratory and kept in the frozen state till dissection and further analysis. For the assessment of gestational age, the mother was questioned about the last menstrual date with the help of the local calender, and using local events, if necessary. Gestational age was taken as menstrual age minus two weeks to allow for ovulation. The subjects were usually able to recall this quite precisely. The reliability of this approach was ascertained by comparing the results of prospective and retrospective investigations on selected samples.

blood
Maternal venous sample was collected within 8 hrs after abortion and within 48 hrs after delivery. Cord blood was used in the case of the neonates. Samples of mixed arterio-venous cord blood were collected from the placental end of the cord at the time of delivery.

The parameters investigated in the fetuses and their mothers are detailed below :-

(i) fetal weight
(ii) fetal somatic measurements
(iii) fetal weight and organ weights, namely, spleen, liver, kidney, heart, brain, adrenal and placenta in relation to gestational age
(iv) iron content of selected tissues, namely, spleen, liver and kidney in relation to maternal blood hemoglobin, serum iron and serum iron binding capacity.
(v) magnesium content of selected tissues, namely, spleen, liver, kidney, heart and muscle in relation to maternal serum magnesium levels.

The parameters studied in the neonates and their mothers are detailed below :-

(i) birth weight
(ii) birth weight in relation to gestational age

TABLE-13 : Number of subjects studied in low (LIG) and high (HIG) income groups.

Studies on :			
		Fetuses	Mothers
I. Iron status :			
	LIG	75	41
	HIG	35	18
		Newborn infants	Mothers
	LIG	75	75
	HIG	53	53
		Fetuses	Mothers
II. Magnesium status :			
	LIG	65	18
		Newborn infants	Mothers
	LIG	72	72
	HIG	34	34
III. Somatic measurements:		Fetuses	Newborns
	LIG	161	206
	HIG	52	107
IV. Organ weights :		Fetuses	
	LIG	106	
	HIG	50	

TABLE-13 (Contd.)

V. Effect of food supplementation :

Period of starting supplementation	Food supplemented pregnant women	Control pregnant women
I trimester	32	37
II trimester	31	60
III trimester	13	49

Newborn infants of	
Supplemented women	Control women
23	15

(iii) somatic growth in terms of linear measurements such as length, circumference of head, chest, abdomen, thigh and arm, shoulder to elbow length, elbow to finger tip length, hip to knee length, knee to heel length and sole length.

(iv) analysis of maternal and cord blood and serum for

(a) blood hemoglobin
(b) serum iron
(c) serum iron binding capacity
(d) serum magnesium.

The methods used for chemical estimations are given in Table-14.

Methodology on weight and somatic measurements

Weight : The infants were placed on the infant weighing balance and the weight at birth was noted.

Height : Height or length are best measured by laying the infant in the supine position and measuring the entire length from head to heel.

Head circumference : The tape was applied firmly over the glabella and supraorbital ridges anteriorly and that part of the occiput posteriorly which gives maximal circumference.

TABLE-14 : Methods used for chemical estimations.

Parameters	Material studied	Method used
Iron	Fetal liver, spleen, kidney	Dipyridal method A.O.A.C. (1955)
Iron and iron binding capacity	Maternal and cord serum	Ramsay (1957)
Hemoglobin	Maternal and cord blood	Cyanmethaemoglobin method, International Committee for Standardisation in Hematology (1965).
Magnesium	Fetal spleen, liver, kidney, muscle, heart	Atomic absorption spectroscopy using Perkin-Elmer model No. 373.
Magnesium	Maternal and cord serum	Orange and Rhein (1951)

Chest circumference : The measurement of chest circumference was made in midaspiration, at the level of xiphoid cartilage or substernal notch, in a plane at right angles to the vertebral column.

Abdomen circumference : Infant was placed in supine position and tape measure was passed around the abdomen at the level of the naval.

Mid-upper arm circumference : The measurement of the circumference of the left arm was taken midway between shoulder and elbow, i.e. over the triceps muscle with the arm hanging freely.

Shoulder to elbow length : The length from the shoulders to the elbow was measured.

Lower arm length : The length from the elbow to the finger tips was measured.

Hip to knee length : The length from hip to knee was measured.

Knee to heel length : The length from middle of knee to heel was measured.

Foot length : The length of the left foot from the tip of second toe to heel was measured.

Thigh circumference : The measurement of the cicumference of left thigh was taken midway between hip and knee.

Collection of blood

For blood hemoglobin estimation, cord and maternal venous blood were collected in a bulb containing 15 mg/ml of EDTA.

For serum, blood was collected in a plain test tube and allowed to clot. The clot shrinks and expresses serum that was obtained by centrifuging.

Blood hemoglobin

Hemoglobin was estimated by cyanmethemoglobin method as described by the International Committee for Standardisation in Haematology (1965).

Blood is treated with reagent containing potassium ferricynide and potassium cyanide at a slightly alkaline pH. The ferricynide forms methemoglobin which is converted to cyanmethemoglobin by the cyanide added.

20 µl of whole blood was mixed with 5 ml of Drabkin solution in a test tube. After waiting for 10 minutes at room temperature, the orange color developed was read at 540 nm in a spectrophotometer.

TABLE-15 : Reagents and standards used for various estimations.

Reagent	Procedure
Acetate buffer pH 5,0, 1M	83g of anhydrous sodium acetate was dissolved in about 500 ml of distilled water. The pH was adjusted with glacial acid to 5, and the volume made up to 1 litre with water. Buffer was stored in the refrigerator.
Chloroform	
α-α'-dipyridyl (0.1%)	0.1g α-α'-dipyridyl dissolved in a few ml of absolute alcohol and made up to 100 ml with water and stored in a dark brown bottle in refrigerator.
α-α'-dipyridyl (0.2%)	0.2g of α-α'-dipyridyl in acetic acid, 0.3% v/v.
Drabkin solution	200 mg of potassium ferricyanide and 50 mg of potassium cyanide were dissolved in a litre of distilled water. The solution should be clear and pale yellow in colour and measured against water at 540 nm. The instrument checked to give a reading of zero with water. If stored at room temperature in brown bottle, the solution keeps for several months.

Table-15 (Contd.)

Reagents	Procedure
Stock ferric chloride solution (5 ug iron per ml in 0.005 N HCl)	145 mg of ferric chloride were dissolved in 0.5 N hydrochloric acid.
Working ferric chloride solution	1 ml of the stock ferric chloride solution diluted and made up to 100 ml with water.
Cyanmethemoglobin solution	Obtained from CSIR centre of biochemicals, VP Chest Institute, Delhi.
Hydrochloric acid (1:1)	One volume of hydrochloric acid and one volume of water.
Hydroquinone solution (2.5%)	2.5g hydroquinone dissolved in 1 ml of 1:1 hydrochloric acid and made up to 100 ml with water. The reagent was prepared just before use.
Standard iron solution	To either 0.702g of ferrous ammonium sulphate or 0.498g of ferrous sulphate, 1 ml of concentrated sulphuric acid was added and the volume were made up to 1 litre with water.
Working iron solution	5 ml of the stock solution diluted to 100 ml with water.

Table-15 (Contd.)

Reagent	Procedure
Magnesium carbonate (LIGHT)	
Standard magnesium solution (1 mg/ml)	8.45g of magnesium chloride, 6H20 dissolved in 1 litre of water.
Working magnesium solution	0.5 ml of stock magnesium solution diluted to 100 ml with water.
Polyvinyl alcohol (0.2%)	0.2g of polyvinyl alcohol dissolved in 5 ml of water with the aid of heat and stirring. The volume made upto 100 ml with water and few drops of chloroform was added.
Sodium hydroxide (15%)	15g of reagent grade sodium hydroxide dissolved in 100 ml of water.
Sodium sulphite (0.2M)	2.52g of anhydrous salt dissolved in 100 ml of water.
Titan yellow (7.5 mg/100 ml)	7.5 mg of the dye dissolved in 100 ml of water. Stored in a refrigerator for not longer than three months.

In all cases double glass distilled water was used.

Cyanmethemoglobin solution having a concentration of 57.9 mg cyanmethemoglobin per 100 ml of Drabkin solution was used as a standard to calculate hemoglobin content of sample. (57.9 mg/dl standard corresponds to 14.5 g hemoglobin per 100 ml of blood).

Serum iron

Serum iron was estimated using the method described by Ramsay (1957).

The method is based on the reaction of ferrous iron with α,α'-dipyridyl so as to form a complex with a characteristic pink colour.

One ml of serum was made up to 2 ml with water in a glass stopper tube. To this was added 0.5 ml of 0.2 M sodium sulphite and 0.5 ml of 0.2% dipyridyl prepared in acetic acid, heated in boiling water bath for 5 minutes, cooled, and 1 ml of chloroform added. The tube was stoppered and shaken vigorously for thirty seconds. The stopper was removed and the contents were centrifuged for 5 minutes at 3000rpm. The procedure was repeated, if necessary, till a clear supernatant was obtained. The colour of the solution was read at 520 nm using water as a blank instead of serum.

A standard graph was obtained using solutions containing different concentrations of standard iron (100-500 µg) in the form of $FeSO_4$.

The tubes used were cleaned by placing them in boiling 5N hydrochloric acid. They were then washed with glass distilled water and solely used for this estimation.

For all the dilutions double glass distilled water was used.

Serum total iron binding capacity

Two ml of the ferric chloride solution were added to 1 ml of serum and allowed to stand for five minutes, 200 mg of magnesium carbonate (light) were then added and the contents were shaken frequently and vigorously for thirty to sixty minutes. The tubes were centrifuged and 2 ml of the supernatant fluid used for iron determination. 0.5 ml each of 0.2M sulphite and 0.2% α, α'-dipyridyl was added in glass stoppered tubes and kept in a boiling water bath for 5 minutes. The tubes were then cooled and 1 ml of chloroform added. The tubes were then stoppered and shaken vigorously for thirty seconds. The stoppers were removed and the tubes were centrifuged for five minutes at 300 rpm and the colour read at 520 nm, using water as a blank instead of serum.

A standard graph was obtained using different concentrations (100-500 µg) of standard iron.

Serum magnesium

Magnesium in serum was estimated using the method of Orange and Rhein (1951).

Titan yellow dye complexes with magnesium in a protein-free filtrate of plasma or serum in an alkaline medium to form an orange pink magnesium hydroxide titan yellow lake.

To 1 ml of serum were added 5 ml of 10% of TCA, and the precipitated proteins removed by centrifugation. To 2 ml of the supernatant were added 1 ml of 0.2% polyvinyl alcohol, 2 ml of titan yellow and 1 ml of 15% of sodium hydroxide, care being taken to mix the contents in the tube after each reagent is added on a vortex mixer. The colour developed was read at 535 nm in a spectrophotometer against a blank containing 1 ml of water instead of a sample.

A standard graph was obtained using different concentrations (10 ug to 50 µg) of standard magnesium.

Tissue iron

This was done by the method described by Official Methods of Analysis of the association of Official Agricultural Chemists (1955).

About 2-3g of the tissue sample were weighed, dried at 60°C, incinerated over a flame until it was charred and then kept in a muffle furnace overnight at 500°C. If the sample was not completely ashed, if it appeared greyish, 4 ml of concentrated nitric acid were added and the sample returned to the muffle furnace until completely ashed. The ash was dissolved in 0.4 ml of 1:1 hydrochloric acid and placed in a water bath (60-80°C) for 10 minutes and the final volume was made upto 5 ml with water. Aliquots (1 to 3 ml) were taken and the volume made upto 5 ml with double distilled water. 5 ml of acetate butter, 1 ml of 2.5% hydroquinone and 1 ml of 0.1% α,α'-dipyridyl were added and the contents mixed thoroughly. The blank contained 5 ml of water instead of the sample. The tubes were kept at room temperature for 30 minutes and then read at 540 nm using spectrophotometer. A standard graph was obtained using different concentrations (10-50 µg) of standard iron solution.

Tissue magnesium

This was done using Perkin-Elmer-373 Atomic Absorption Spectroscopy. About 2-3g of the tissue sample was weighed, dried at 60°C and incinerated over a flame until it was completely charred and then kept in muffle furnace overnight at 500°C. If the sample was not completely ashed, few drops of concentrated nitric acid were added and the sample returned to the muffle furnace until completely ashed. The ash was dissolved in a known quantity of 1N nitric acid. Magnesium was analysed by Atomic Absorption Spectroscopy from this solution.

A standard graph was obtained using different concentrations (0.1-0.5 µg) of standard magnesium solutions.

Effects of food supplementation on the pregnancy weight gains and outcome

Studies were conducted in an urban slum area, namely, Nava Yard, situated within the municipal limits of Baroda. A demographic survey of 100 families residing in the target area was first carried out. The average family size was found to be 5 and the per capita income to be Rs.100 per month. Majority of them resided in a 'semi pakka' house. They had to get water from outside and had no sanitory facilities. Forty six per cent of the women were illeterate, 26 per cent had primary education and 28 per cent had secondary or higher education, whereas 64 per cent of men had secondary or higher education. While only 15 per cent were illeterate. Typically, the men in these families were industrial workers and the women mostly housewives except for about 1% who worked either as factory workers or domestic helpers.

Married women in selected families in the target area not practicing family planning were monitored once a month and the date of last menstrual period recorded. For this purpose, one hundred and forty two non-pregnant women were monitored. Out of 142 women monitored, 37 women became pregnant during the course of the study period. The reliability of last menstrual period by prospective and retrospective

investigations on a selected sample was checked. Those who became pregnant and others at different stages of pregnancy were monitored throughout pregnancy and both the infants and mothers were monitored for atleast six months after delivery. However, because of the practice of women going to their parental home in late pregnancy for delivery and returning only a few months after delivery, serial observations could not be made in all cases.

Pregnant women identified in the target families described above were supplemented at different stages of gestation and those who did not come for supplementation served as controls. Information was recorded on their family size, dietary intake at home and clinical status at different stages of pregnancy. The information on dietary intake was derived using number of approaches so as to ensure the reliability of the information obtained. Record of foods purchased by the family and general dietary pattern in the family was obtained by the oral questionnaire method. In a few families all the raw ingredients used for cooking and the cooked food were weighed and the per capita availability of different foodstuffs was calculated therefrom. When the data in the two groups were examined, they were found not to differ with regard to either economic status, family

size or initial nutritional status. The critical factor involved in their participation or otherwise was personal. Reasons for not coming included refusal or permission to do so by either the husband or mother-in-law or domestic chores which had to be done at the time of supplementation. The body weights of the mother was recorded every week. The food supplement was provided in the form of a fermented food, Dhokla, Dhokla was prepared from wheat and bengal gram 'dal' (dehusked and split legume) in the proportion of 2:1 with leafy vegetables and lime added as source of iron, vitamin A and calcium. Each 100g of raw ingredients contained 40g of wheat, 20g of bengal gram dal, 30g of leafy vegetables, 10g of oil and 340 mg of lime powder (a mixture of $CaCo_3$ and $Ca(OH)_2$). Dhokla batter was prepared from coarsely ground wheat and bengal gram dal, allowed to ferment overnight at room temperature and chopped leafy vegetables, usually, fenugreek/spinach or amaranth were added. The pH of the fermented batter which is about 4.5-5.0 was brought to 6.0-6.5 by the addition of 0.34g of lime per 100g of batter, steamed in a pie-dish, cut into squares and seasoned with oil. This recipe is designed so as to make up to some extent for the deficits in the home diet of calories, protein, carotene, riboflavin, iron and calcium. The supplement provided per 100g of the cooked product, 109 kcals; 3.5g of protein; 3 mg iron, 175 mg calcium and 475 µg of β-carotene. This product has been subject to

through evaluation in previous studies over the last two decades in this laboratory both by animal experimentation and feeding trials on pre-school children and school children (Rajalakshmi and Ramachandran, 1967; Ramachandran, 1968; Rajalakshmi, 1976; Rajalakshmi and Ramakrishnan, 1977; Ramakrishnan, 1979). Fermentation of dhokla batter is found to be associated with increase in free sugar, amino nitrogen, ionisable iron, inorganic P (associated with phytate breakdown) riboflavin and total and free niacin. These changes are associated with improved digestability and nutritive value.

Subjects were allowed to eat according to appetite at the feeding site. The supplement was given at mid-morning well before lunch in order to ensure that it formed an addition rather than a substitution to the home diet. The amount consumed varied with an average intake of 250g of dhokla providing about 275 calories, 8.8g protein, 7.5 mg of iron, 434 mg of calcium and 1188 ug of carotene.

Birth weights of the infants born to these mothers were recorded. Information was obtained on the place of delivery, type of delivery, breast feeding and other feeding practices, age at the introduction of weaning foods for infants, and post partum practices for mothers. Growth of these infants was studied for six months in terms of weight.