Methods & Materials

METHODS AND MATERIALS

Diabetes mellitus is the most common metabolic disease worldwide. It is a global epidemic with devastating humanitarian, social and economic consequences. Diabetes is defined as a state in which homeostasis of carbohydrate and lipid metabolism is improperly regulated by insulin. Diabetes is the leading cause of blindness, renal failure, and lower limb amputations in adults and is a major risk factor for cardiovascular disease and stroke. Environmental factors, especially diet and physical activity interact with genetic predisposition to affect disease prevalence. Around 50 % of the people with diabetes remain undiagnosed (Harris et al 1998). Because Type 2 diabetes remains undiagnosed for several years, at the time of clinical diagnoses, many people have one or more micro or macro vascular complications (Rajala et al 1998).

The rising prevalence of diabetes in India (Ramchandran et al 2004) and other developing countries (Choi et al 2006) is chiefly attributed to urbanization. India will continue to have the largest number of diabetic subjects as a result of the rapid urbanization and economic development (Sicree et al 2006). Thus earliest detection would help to reduce the development of secondary complications in the diabetic individuals.

Various approaches have been used in the management of diabetes. The most popular approaches are drug therapy, dietary therapy, lifestyle modifications and recently the natural products therapy. A number of medicinal plants have been used for their anti-hyperglycemic properties since ancient times and are being increasingly sought after by diabetics and health care professionals. India is the largest producer of medicinal herbs and is called as botanical garden of the world (Seth and Sharma 2004).

The present study was thus planned with the following objectives:

1. To study the metabolic alterations in T2DM subjects and to map the prevalence of microalbuminuria.
2. To study the risk factors for diabetes in an industrial population of Vadodara and to track glycemic and lipemic levels over a period of four years.
3. To develop functional food products incorporating Barley Grass Powder (BGP).
4. To determine the acceptable level of BGP incorporation in the developed food products by sensory evaluation.
5. To analyze the nutrient composition of BGP.
6. To study the impact of BGP supplementation in the form of capsules for a period of 60 days on the carbohydrate and lipid metabolism of T2DM subjects.
7. To determine the feasibility of scaling up BGP Khakhra as a functional food.

The study was carried out in 4 phases as given below:

PHASE I	Metabolic alterations in T2DM subjects and the prevalence of microalbuminuria
PHASE II	Risk factor analysis and trends of dyslipidemia in T2DM subjects of an industrial population
PHASE III (a)	Nutritional analysis of BGP
(b)	Product development using BGP
(c)	Sensory evaluation of the developed products
PHASE IV (a)	Impact of BGP supplementation on the FBG, HbA1C and lipid profile of stable T2DM subjects
(b)	Scaling up of BGP khakhra for consumers at large

Medical Ethics: The study was approved by the departmental ethical committee (Approval No: FCSC/FND/ME 35).

PHASE I - METABOLIC ALTERATIONS IN TYPE 2 DIABETES MELLITUS SUBJECTS AND THE PREVALENCE OF MICROALBUMINURIA.

Enrollment of Subjects

For the study, 102 stable T2DM subjects, who gave verbal consent, were enrolled from pathological laboratories of Vadodara city. Two pathological laboratories were purposively selected. Out of these 102 diabetics, 53 were males and 49 were female subjects. For the study, subjects were enrolled based on the following criteria:

- Resident of Vadodara city
- Confirmed stable diabetics
- No apparent complication

Data Collection

Information regarding socio economic status, educational status, anthropometric measurements, family history, lifestyle factors, medical history, dietary habits and 24-hour dietary recall were recorded using a structured pre-tested questionnaire (Appendix I). Biochemical indicators including fasting blood glucose (FBG), HbA1C, lipid parameters and renal function tests were also studied. The details of all the above mentioned parameters and their methodology are summarised in Table 3.1 and the experimental plan is given in Figure 3.1.

The Major Outcome Measures for this Phase were:

a) Status of glycemic control
b) Prevalence of dyslipidemia
c) Prevalence of microalbuminuria
d) Risk factor analysis
e) Determinants of microalbuminuria

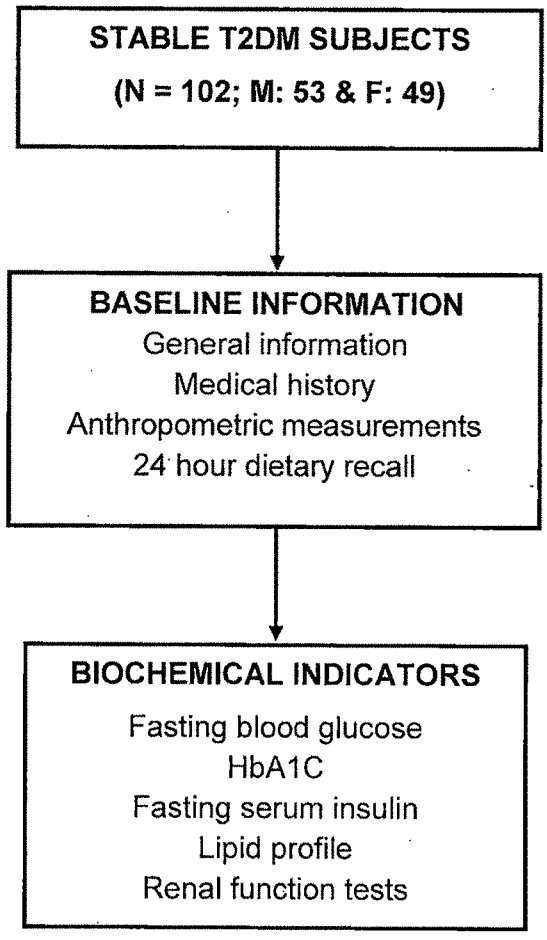
TABLE 3.1

TOOLS AND TECHNIQUES FOR DATA COLLECTION

Variable	**Methodology**
SES, lifestyle factors, family history and medical history	Pre-tested structured questionnaire
Weight, height, WC and hip circumference	Standard anthropometric measurement methods
Nutrient consumption	24 hr dietary recall
FBG, HbA1C, TC, TG, HDL-C, serum insulin, serum urea, urine urea, serum creatinine, urine creatinine, urine microalbumin	Diagnostic kits
% Body fat, HOMA-IR, HOMA-BF, BMI, WHR, WSR and WWR	Calculations

FIGURE 3.1

EXPERIMENTAL DESIGN FOR PHASE I



PHASE II - RISK FACTOR ANALYSIS AND TRENDS OF DYSLIPIDEMIA IN T2DM SUBJECTS OF AN INDUSTRIAL POPULATION

Place of Study

A petrochemical industry located on the outskirts of Vadodara city was purposively selected for the study. It was a large scale industry with a workforce consisting predominantly of males. Out of 10 units, 3 work units from the industry were purposively selected.

Sample Selection

From the selected work units, employees who were confirmed diabetics and willing to participate were enrolled for the study. A total of 54 subjects were enrolled for this phase of the study.

Evaluation of Diabetes Risk Profile

For this part, the following information was collected from the 54 enrolled diabetic subjects using a pre-tested structured questionnaire (Appendix II) and medical records (Anthropometric measurements, blood pressure (BP), FBG and lipid profile values).

a) *Anthropometric Measurements*- Height, weight and BMI

These measures were used to map the prevalence of overweight and obesity in accordance with the Asia Pacific classification (Overweight: BMI 23-24.9; Obesity: BMI ≥ 25).

b) *Medical History*

Medical history information was collected regarding any medical condition the employees had. Details about the family history of the medical conditions were also taken. Age at diagnosis of diabetes was noted.

c) *Lifestyle Factors*

Personal habit data on smoking, tobacco chewing and alcohol consumption was recorded. Frequency of exercising (< or > 3 times per week) was also noted.

d) *Fruit and Vegetable Consumption*

Information on the frequency of consuming fruits and vegetables (< or > 3 times per week) was collected.

e) *Information on Routine Tests*

Data on whether the diabetic subjects underwent necessary routine tests like HbA1C, kidney function tests, lipid profile, foot examination, eye examination and ECG/Stress test in the past year was recorded.

f) *Biochemical indicators and blood pressure measurements*

Fasting blood glucose, lipid profile values and blood pressure measurements for the diabetic subjects were obtained from medical records based on yearly checkups. These were used to assess the glycemic and lipemic control of the diabetic subjects and to find out the prevalence of hypertension.

Tracking Data

Retrospective data on fasting blood glucose, lipid profile and blood pressure for the past four years (2005-2008) could be obtained for 43 of the 54 diabetic subjects from the medical records. Keeping the 2005 data as the baseline, this data was used to decipher the trends of dyslipidemia and hypertension over a period of four years (Figure 3.2).

The Major Outcome Measures for this Phase of the Study were:

a) Risk factor analysis
b) Prevalence of dyslipidemia
c) Trends of dyslipidemia and hypertension over a period of four years.

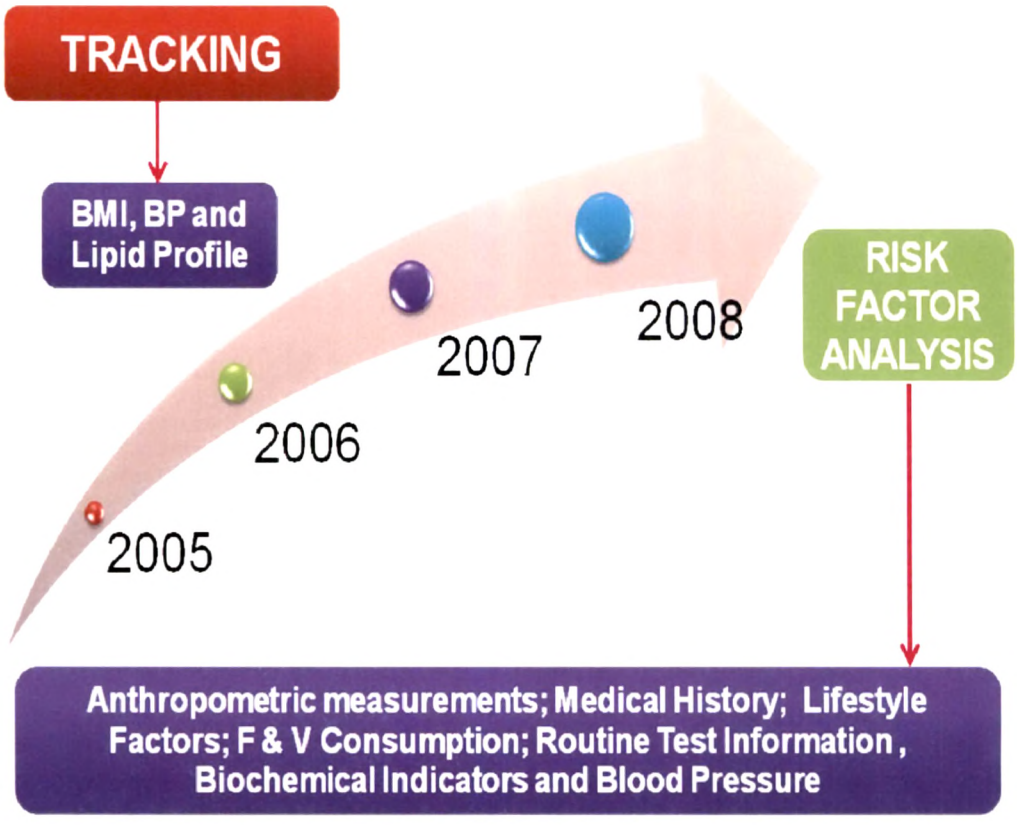
PHASE III (a) - NUTRITIONAL ANALYSIS OF BGP

Preparation of Barley Grass Powder

Barley grass (*Hordeum Vulgare*) consists of the young green leaves of the barley plant. Barley grass is purported to be a rich source of chlorophyll, antioxidants, antioxidant enzymes, and other phytochemicals that neutralize free radicals. Also, a wide spectrum of vitamins, minerals and amino acids

FIGURE 3.2

EXPERIMENTAL DESIGN FOR PHASE II



has been isolated from barley grass. Various health benefits have been attributed to the use of barley grass. The procedure adopted for the preparation of barley grass powder was as follows:

- Barley grain was procured from the local market and was authenticated by a botanist from The M.S.University of Baroda.
- Barley grass was cultivated on a large scale in a farm. The grass was cut when it was around 10-12 inches in height.
- The entire lot of grass was then transported to a food industry (Aum Agri Freeze Foods, Vadodara) where it underwent the following procedure :-
  - ♣ Barley grass was manually cut into 5 mm length with the use of scissors.
  - ♣ The grass was washed in a tray with clean water at 25-30°C.
  - ♣ Barley grass was then subjected to cold dehydration (5-10°C) for a period of 24-30 hours.
  - ♣ The dried product was ground in a grind mill with cold water jacketing (15-17°C in order to prevent generation of heat which in turn helps in retention of colour).
  - ♣ The powder was sieved using a mesh (No. 40).
  - ♣ The powder was packed with nitrogen gas.

Nutritional Analysis

A sample of barley grass powder was sent to an analysis centre (Analytical & Environmental Sciences, Baroda) for determination of nutritional value. The entire sample was mixed properly and homogenized and the representative sample was subjected to analysis for nutritional value. The sample was reported to be:

a) Free from insect infestation and fungal growth.
b) Conforming to the general standards of PFA rules (1955).

The methods used for analysis of various nutrients (conforming to BIS) is given in Table 3.2.

TABLE 3.2

METHODS USED FOR NUTRITIONAL ANALYSIS OF BGP

Test	Method
Moisture	Hot oven
Ash	Dry ashing (muffle furnace)
Fat	Soxhlet
Protein	Kjeldahl
Carbohydrate	Difference method
Energy	Calculation
Calcium	Flame photometric method
Potassium	Flame photometric method
Sodium	Flame photometric method
Iron	Colorimetric
Vitamin C	Titremetric
Vitamin B2	ELISA
Phytosterol	TLC

PHASE III (b) - PRODUCT DEVELOPMENT USING BGP

The four food products developed were Khakhra, Thepla, Muthiya and Cutlet which are commonly consumed food preparations in this region. Standardised recipes and procedures were adopted for preparation, the details of which are given in Appendix III along with the nutritive value of the recipes. The BGP was incorporated at 3 levels i.e 0.5g, 1g and 1.5g.

PHASE III (c) - SENSORY EVALUATION

Selection of Panel Members

12 semi trained judges (students from the department; third year to doctoral students) who gave their oral consent for evaluation were selected as panel members.

Sensory evaluation of the developed products- Khakhra, Thepla, Muthiya and Cutlet was carried out using a composite rating scale and the ranking test. All the developed food products were presented to the panel of 12 judges for organoleptic evaluation. As mentioned earlier, BGP was incorporated at different levels (0.5g, 1g, and 1.5g per piece) in the products for finding out the acceptable level of incorporation and for observing the changes in other sensory attributes, if any when BGP was added to the products.

Composite Rating Scale

This test was used so that specific characteristics of the product could be rated separately. This test helps to point out which attribute is at fault or not acceptable. The resulting total scores for different attributes were compared for each sample and different attributes. The panellists were asked to compare the attributes mentioned for a particular sample to an ideal standard product and rate each attribute on a scale of 1-10; with a score of 10 being the highest for each attribute. In order to facilitate the scoring the panellists were provided with a graded scoring system ranging from poor to liked extremely which was as follows:

Score range	Grade
0-2	Poor
3-4	Fair
5-6	Good
7-8	Very good
9-10	Excellent

Ranking test

In this, the panellists were presented with the four products and were asked to rank the products based on the overall acceptability in a descending order i.e. Rank 1 (liked most) .....Rank 4 (least liked).

The evaluation criteria for all the products was decided according to the expected organoleptic qualities for a standard product and included the following-

Variable	Quality
Appearance	Colour, size, shape, texture
Taste	Mouth feel, aroma, aftertaste
Overall acceptability	
Comments	

The specific attributes (composite rating scale) studied for each of the products, are mentioned in appendix IV. The study components and flow sheet for product development and sensory evaluation is given in Table 3.3 & Figure 3.3.

The major outcome of this phase of the study was to develop functional foods incorporating barley grass powder.

PHASE IV (a) - IMPACT OF BGP SUPPLEMENTATION ON THE FBG, HbA1C AND LIPID PROFILE OF STABLE T2DM SUBJECTS.

TABLE 3.3

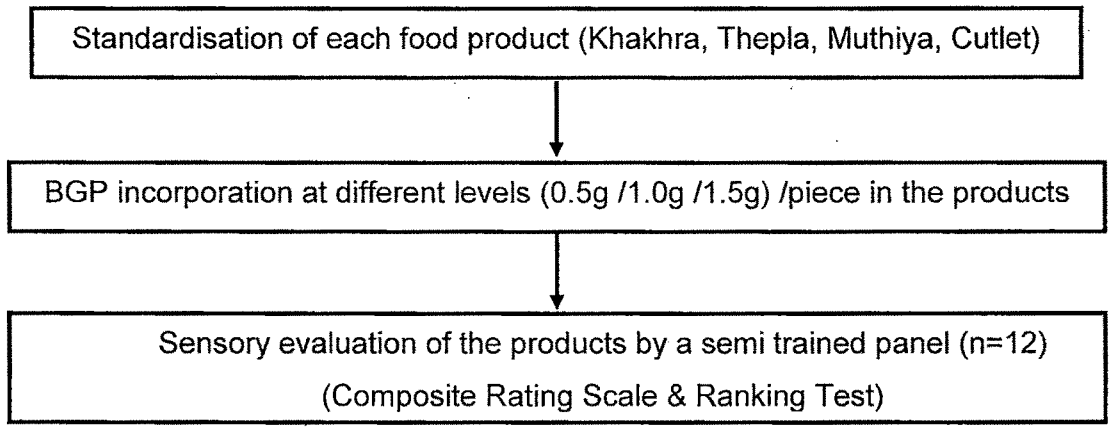
COMPONENTS OF PRODUCT DEVELOPMENT

AND SENSORY EVALUATION IN PHASE III A & III B

Component of study	Details
Food Products	Khakhra, Cutlet, Muthiya, Thepla
Level of BGP	(0.5g /1.0g /1.5g) /piece
Sensory Evaluation	Composite rating test Ranking test

FIGURE 3.3

PRODUCT DEVELOPMENT & SENSORY EVALUATION



Enrollment of Subjects

In this phase, 59 stable T2DM subjects who gave verbal consent were enrolled from pathology laboratories in Vadodara city. Subjects were enrolled with the following criteria for selection of subjects:

- Resident of Vadodara city
- Confirmed stable diabetics
- No apparent complication
- Willingness to participate

The subjects were divided into two groups – Control and Experimental based on their willingness to consume BGP capsules. The experimental group had 36 diabetic subjects and the control group had 23 subjects.

Formulation of Barley Grass Powder Capsules

The barley grass powder prepared and packed under nitrogen gas was given to a local pharmaceutical industry (AIMCO Pharmaceutical Manufacturing Company, Vadodara) for preparation of barley grass powder capsules. Trials at the pharmaceutical industry showed that a maximum of 300 mg of barley grass powder could be filled per capsule.

Level of Supplementation

Sensory evaluation results of barley grass powder incorporated recipes showed that the recipes were acceptable at all levels i.e. 0.5g-1.5g of barley grass powder. Therefore for supplementation it was decided to supplement 1.2 g/day i.e. 4 capsules per day. The subjects were asked to consume two capsules after lunch and two capsules after dinner.

Data Collection

Baseline Data

Pre data was collected for both the groups regarding socio-economic data, medical history, family history, lifestyle pattern, dietary habits and 24-hour dietary recall using a pre-tested structured questionnaire (Appendix I). Anthropometric measurements were taken using standard procedures. The

various biochemical parameters included were fasting blood glucose, HbA1C and lipid profile (total cholesterol, HDL-C, triglycerides).

Supplementation

The impact of supplementation of barley grass powder in the form of capsules was studied for a duration of 60 days (2 months) on various parameters in subjects of the experimental group. During the course of supplementation no modification in the diet or medication was made. During the study period none of the subjects took other complementary or alternative medications. In order to check compliance BGP capsules were supplied once in ten days and the number of remaining capsules from the previous lot, if any were counted. The control group received no supplementation.

Impact analysis (Post data)

Post data was collected for subjects in both the groups at the end of 60 days of BGP supplementation in relation to anthropometric measurements, biochemical parameters and dietary information using the same methodology as for baseline data collection. The detailed experimental plan is given in Figure 3.4.

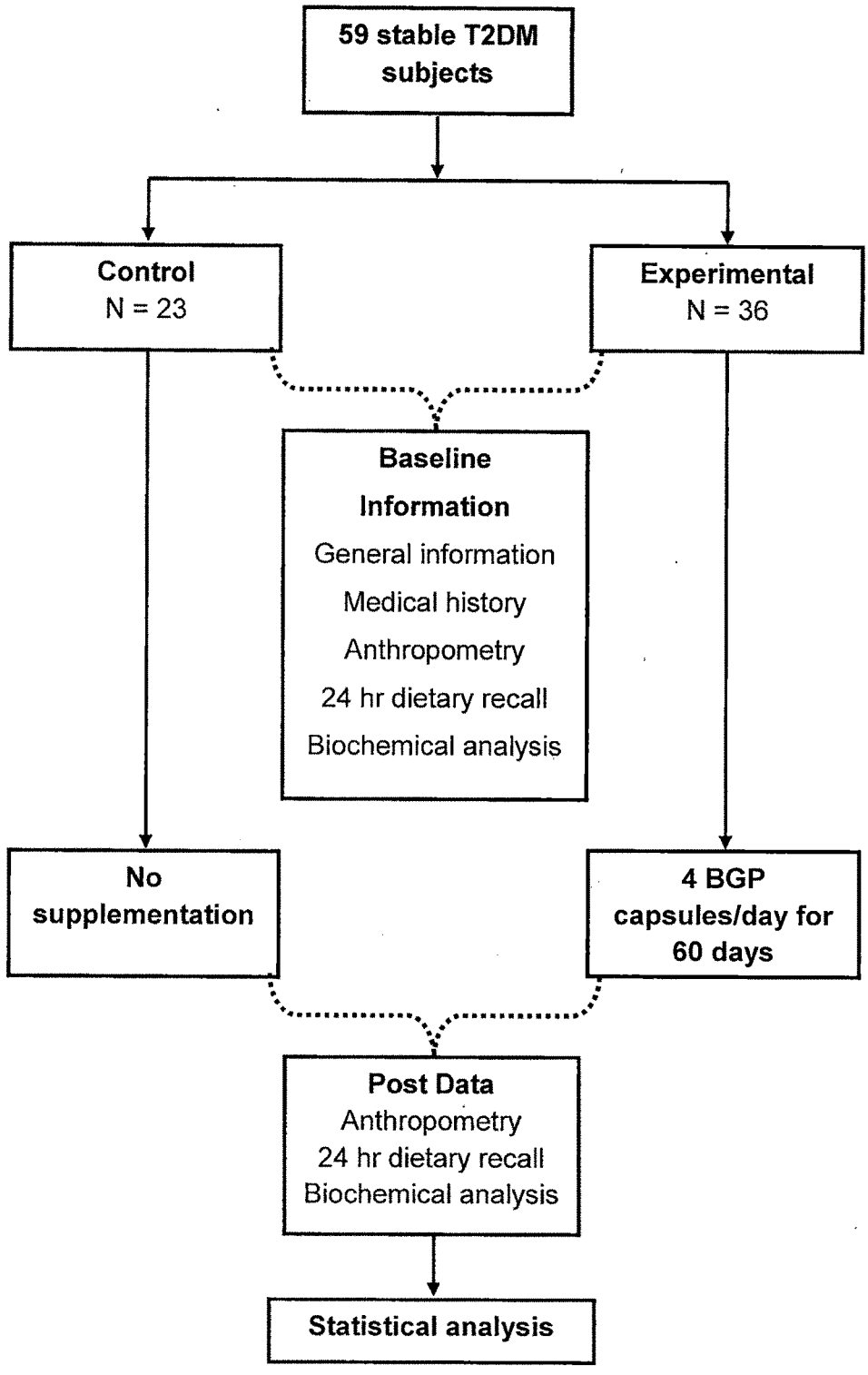
The major outcome of this phase of the study was to assess the compliance and test the efficacy of barley grass powder as an alternative therapy in the management of T2DM.

PHASE IV (b) – TRANSLATIONAL RESEARCH: PILOT STUDY ON SCALING UP OF BGP KHAKHRA FOR CONSUMERS AT LARGE.

Khakhra was selected as a product for scaling up because it was found to be easily acceptable for consumption as a snack. Also the product has a longer shelf life as compared to the other products i.e. about 2 weeks without any change in flavour. The BGP khakhras were prepared in bulk and kept in one of the health clubs in Vadodara (VLCC). The khakhras were distributed to 45 members of the club for sensory evaluation and acceptability. The evaluation criteria for BGP khakhra were on the following aspects:

FIGURE 3.4

IMPACT OF BGP SUPPLEMENTATION (STUDY DESIGN)



1. Taste of the BGP khakhra
2. Colour acceptability
3. Willingness to buy the product (if available in the market)
4. Maximum number that can be eaten at one time

The feedback from the consumers was recorded using a structured questionnaire. Any other suggestions or comments were also welcomed.

METHODOLOGY FOR DATA COLLECTION

Baseline Information

Socio-economic data (age, level of education, family information, income level/occupation), medical history, family history, lifestyle pattern (physical activity and addictions), general dietary habits were collected using a structured pre-tested questionnaire.

Anthropometric Measurements

They were measured using standard techniques.

- Height
- Weight
- Waist Circumference
- Hip Circumference

These anthropometric measurements were then used to derive various obesity measures such as:

- BMI
- Waist Hip Ratio
- Waist Stature Ratio
- Waist Weight Ratio

In addition to the above obesity measures % body fat was also calculated according to the method of Lean et al (1996) using the following formula:

Body fat % for men =

[(0.567 × waist circumference in cm) + (0.101 × age in years)] − 31.8

Body fat %for women =

[(0.439 × waist circumference in cm) + (0.221 × age in years)] − 9.4

Dietary Information

Information on dietary intake was ascertained using the 24 hour dietary recall method. Accordingly the calorie, fat, protein, carbohydrate, fibre, β-carotene, iron and vitamin C intake were calculated using the nutritive value of Indian foods by Gopalan (1989).

Biochemical Analysis

Biochemical analysis included testing blood sample of each subject for the following parameters: Fasting blood glucose, HbA1C, lipid profile (total cholesterol, triglycerides, HDL-C, LDL-C and VLDL-C), fasting serum insulin and renal function tests (Serum creatinine, serum urea, urine creatinine, urine urea and urine microalbumin). The subjects were asked to observe an overnight fast and the blood was drawn by a trained technician and then the serum was separated for further analysis.

Methods for Biochemical Analysis

1. *Estimation of Blood Glucose*

It was estimated using the GOD/POD method using an enzymatic kit from Ecoline. Glucose is oxidised by glucose oxidase (GOD) into gluconic acid and hydrogen peroxide (H_2O_2). H_2O_2 in the presence of peroxidase (POD) oxidises the chromogen, 4-aminoantipyrine/phenolic compound to a red coloured compound. The intensity of the red coloured compound is proportional to the glucose concentration and is measured at 500nm (490 – 530nm).

2. *Estimation of Total Cholesterol*

Cholesterol was estimated using enzymatic kits (Roche) by the enzymatic, colorimetric method. Cholesterol esters are hydrolysed to free cholesterol and fatty acids by cholesterol esterase (CE). The free cholesterol is then oxidised by cholesterol oxidase (CHOD) to cholest-4-en-3-one and hydrogen peroxide (H_2O_2). The liberated H_2O_2 reacts with 4-aminoantipyrine and phenol in the presence of peroxidase (POD) to produce a red quinine-imine dye. The colour intensity of the dye formed is directly proportional to the total cholesterol

concentration. It is determined by measuring the increase in absorbance at 512 nm.

Cholesterol esters $\xrightarrow{CE}$ cholesterol + RCOOH

Cholesterol + O_2 $\xrightarrow{CHOD}$ cholest-4en-3-one + H_2O_2

$2H_2O_2$ + 4-AAP + phenol $\xrightarrow{POD}$ quinine-imine dye +$4H_20$

3. *Estimation of Serum Triglycerides.*

Enzymatic kits using GPO/PAP method (Roche) were used for estimations of triglyceride. Triglycerides (TG) are hydrolysed by lipoprotein lipase (LPL) to produce glycerol and fatty acids. The glycerol is phosphorylated by ATP in presence of glycerol kinase (GK) to glycerol-3-phosphate which is oxidised by the enzyme glycerol phosphate oxidase (GPO) producing dihydroxyacetone phosphate and hydrogen peroxide. H_2O_2, in the presence of peroxidase (POD) effects the oxidative coupling of 4-chlorophenol and 4-aminophenazone to form a red coloured quinonimine dye, which is measured at 512 nm. The increase in absorbance is directly proportional to the concentration of TG in the sample.

Triglycerides $\xrightarrow{LPL}$ glycerol + fatty acids

Glycerol + ATP $\xrightarrow{GK}$ glycerol-3-phosphate +ADP

Glycerol-3-phosphate + O2 $\xrightarrow{GPO}$ dihydroxyacetone phosphate + H_2O_2

$2H_2O_2$ + 4-aminophenazone + 4-chlorophenol $\xrightarrow{POD}$ Quinoneimine dye + $4H_2O$

4 *Estimation of High Density Lipoprotein (HDL) Cholesterol*

Enzymatic kits from Roche were used. In the presence of magnesium sulphate and dextran sulfate, water soluble complexes with LDL, VLDL, and chylomicrons are formed which are resistant to PEG-modified enzymes. The cholesterol concentration of HDL-cholesterol is determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups (approx 40%). Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of

oxygen, cholesterol is oxidised by cholesterol oxidase to △4-cholestnone and hydrogen peroxide. The colour intensity of the blue quinoneimine dye formed is directly proportional to the HDL-cholesterol concentration. It is determined by measuring the increase in absorbance at 583 nm.

HDL-cholesterol esters + H_2O	PEG-CE →	Cholesterol + RCOOH
HDL-cholesterol + O2	PEG-CHOD →	△4 Cholestenone + H_2O_2
$2H_2O_2$ + 4-aminoantipyrine + HSDA+ H^+ + H_2O	Peroxidase →	purple blue pigment + $5H_20$

5. *Estimation of Low Density Lipoprotein (LDL) Cholesterol*

The value for LDL C were calculated using the formula

LDL= TC- [HDL + (TG/5)]

6. *Estimation of Very Low Density Lipoprotein (VLDL) Cholesterol*

VLDL-C was calculated by dividing triglyceride values by five.

VLDL = TG/5

7. *HbA1C*

Estimations for HbA1C were performed by the borate affinity assay using kit from Nycocard. As soon as blood sample is added to the reagent used in this assay, the reagent lyses the erythrocytes immediately and precipitates haemoglobin specifically. The blue boronic acid conjugate binds to the cis-diol configuration of glycated haemoglobin. An aliquot of this reaction mixture is added to the test device which contains a porous membrane filter. All the precipitated Hb, conjugate- bound and unbound remain on the filter top. Excess coloured conjugate is removed with the washing solution. The precipitate is evaluated by measuring the blue (HbA1C) and red (total Hb) colour intensity with the NycoCard READER II, The ratio between them being proportional to the percentage of HbA1C in the sample. The analysis for HbA1C was performed by a pathologist in a laboratory.

8. *Urea*

Kinetic test with urease and glutamate dehydrogenase. Urea is hydrolyzed by urease to form ammonium and carbonate. In the second reaction 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrgenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD for each mole of urea hydrolyzed. The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen. It is determined by measuring the absorbance at 340 nm.

$$\text{Urea} + 2\,H_2O \xrightarrow{\text{Urease}} 2\,NH_4^+ + CO_3^{2-}$$

$$NH_4^+ + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{\text{GLDH}} \text{L-glutamate} + NAD^+ + H_2O$$

9. *Creatinine*

Enzymatic, colorimetric method. The enzymatic method is based on the established determination of hydrogen peroxide after conversion of creatinine with the aid of creatininase, creatinase and sarcosine oxidase. The liberated hydrogen peroxide reacts with 4-aminophenazone and HT1B to form a quinine imine chromogen.

$$\text{Creatinine} + H_2O \xrightarrow{\text{creatininase}} \text{creatine}$$

$$\text{Creatine} + H_2O \xrightarrow{\text{creatinase}} \text{sarcosine} + \text{urea}$$

$$\text{sarcosine} + O_2 + H_2O \xrightarrow{\text{SOD}} \text{glycine} + HCHO + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{HT1B} \xrightarrow{\text{POD}} \text{quinine imine chromogen} + H_2O + HI$$

10. *Microalbumin*

Estimations for microalbumin were carried out using a solid phase, sandwich-format, immunometric assay using NycoCard U-Albumin kit. When the diluted sample is applied to the test device, the sample flows through the membrane, and immobilized albumin specific monoclonal antibodies on the membrane capture the albumin molecules. Albumin trapped on the membrane will bindthe gold-antibody conjugate then added, in a sandwich type reaction. Unbound conjugate is removed from the membrane by the washing solution. The paper layer underneath the membrane absorbs excess liquid. Due to the

bound gold particles the membrane appears purple with color intensity proportional to the albumin concentration of the sample. The colour intensity is measured quantitatively by using the colour densitometer NycoCard READER II.

11. *Serum Insulin*

The estimations for serum insulin were performed using a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplates. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of insulin. Calibrators and samples react with the capture monoclonal antibody (MAb 1) coated on microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP). After an incubation period allowing the formation of a sandwich: coated MAb 1 – human insulin – MAb 2 – HRP, the microtiterplate is washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody is measured through a chromogenic reaction. Chromogenic solution (Tetramethylbenzydine ready for use) is added and incubated. The reaction is stopped with the addition of Stop Solution and the microtiterplate is then read at 450 nm. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is proportional to the insulin concentration. A calibration curve is plotted and INS concentration in samples is determined by interpolation from the calibration curve.

Insulin resistance and β cell function were derived using the HOMA method (Matthews et al 1985) using the following formulae:

Insulin resistance (HOMA-IR) =

$$\frac{\text{Fasting insulin (μ/ml)} \times \text{Fasting glucose (mmol/l)}}{22.5}$$

Beta cell function (HOMA-BF) =

$$\frac{20 \times \text{Fasting insulin (μ/ml)}}{\text{Fasting glucose (mmol/l)} - 3.5}$$

STATISTICAL ANALYSIS

The data was entered in Microsoft® Office Excel 2003 spreadsheets. The data entered was checked for errors, if any and the data was subjected to appropriate statistical analysis such as:

- Means and standard deviations
- Frequency distributions
- Percentages
- Student's 't' test, Paired 't' test and 'F' test to find out statistical significance between the groups.
- Correlation analysis
- Odds ratio
- Logistic regression analysis
- Incidence

A result was declared to be statistically significant only if the *p* value of an analysis was less than 0.05. Confidence interval (CI) limits are specified at 95%.