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Study Protocol

The Effects of Inorganic Nitrate on Carbohydrate and Lipid Metabolism in Type 2 Diabetes: The Protocol of a Randomized Placebo-Controlled Clinical Trial

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Abstract

Background and Aim: Decreased bioavailability of nitric oxide (NO) in type 2 diabetes contributes to disrupted pathways of glucose/insulin homeostasis and progression of long-term complications. Due to its ability to convert to NO, inorganic nitrate (NO_3) has been recently highlighted as a potential therapeutic agent in type 2 diabetes.

Materials and Methods: This research entails a double-blind, randomized, placebo-controlled, phase II clinical trial that will be conducted on 62 type 2 diabetic patients. The patients will be randomized to receive a 6-month daily dose of NO_3-rich beetroot powder (5 g/d, contains ~250 mg NO_3) or placebo (5 g/d, contains <25 mg NO_3). The primary outcome is glycosylated hemoglobin A1c (HbA1c). The study is powered to detect a 0.75% reduction in HbA1c levels between the groups. Fasting serum glucose, serum insulin, lipid parameters, liver enzymes, thyroid function tests and complete blood count will be measured as secondary outcomes. The measurements will be done at baseline, and will be repeated in the fourth, twelfth and twenty-fourth weeks. Protocol of the study was approved by the ethical committee of the Research Institute for Endocrine Sciences of Shahid Beheshti University of Medical Sciences (IR.SBMU.ENDOCRINE.REC.1395.322). The trial was registered in the Iranian Registry of Clinical Trials with the following identification: IRCT20180409039246N1.

Keywords: Inorganic nitrate, Beetroot, Type 2 diabetes, Clinical trial

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Introduction

Type 2 diabetes mellitus (T2DM) is a prevalent metabolic disorder characterized by hyperglycemia, development of insulin resistance, insufficient insulin production, and pancreatic β-cell dysfunction (1). Nitric oxide (NO), a biologically active hormone with multiple critical function in the body (2), is now highlighted as a signaling molecule involved in the

homeostatic pathways of glucose and insulin (3). Reduced NO production and NO bioavailability have been introduced as risk factors for the development of cardiometabolic disorders, especially insulin resistance, metabolic syndrome and T2DM (4-6). Inorganic nitrate (NO_3)/nitrite (NO_2) have recently been highlighted as potential therapeutic agents in T2DM due to their ability to potentiate NO_3-NO_2-NO pathway (3). Several beneficial properties, including the regulation of glucose hemostasis and insulin signaling pathways, improvement of insulin resistance and vascular function, hypotensive, hypolipidemic as well as anti-inflammatory and anti-oxidative effects have been observed following the administration of inorganic NO_3 (7). Although a number of animal studies have confirmed the beneficial effects of NO_3/NO_2 supplementation in diabetes (8), there is no evidence regarding long-term outcomes following inorganic NO_3 therapy in T2DM in human subjects.

Beetroot (*Beta vulgaris L.*) and its byproducts have been recently considered as simple, safe and popular dietary vehicles for inorganic NO_3, and beetroot supplementation has been indicated to improve endogenous NO production and its bioavailability. Beetroot supplementation is mainly used as an ergogenic compound, and a multi-targeted complementary treatment in cardio-metabolic disorders such as hypertension, vascular dysfunction, atherosclerosis, cardiorespiratory disorders and diabetes (9-13).

This trial is designed to test whether long-term complementary treatment with inorganic NO_3 in the context of beetroot powder as a natural NO_3-rich product has beneficial effects on glycemic control in T2DM patients. We also aim to investigate whether inorganic NO_3 might improve lipid parameters as well as systolic and diastolic blood pressures. Furthermore, the potential effects of long-term NO_3 supplementation on liver function tests (LFT) and thyroid function tests (TFT) will be assessed during the study follow-up. Nitric oxide metabolites (NO_3+NO_2) in serum, saliva and urine samples will be measured at baseline and during the follow-up measurements.

Study Design and Methods

Participant Recruitments

This research is a double-blind, randomized, placebo-controlled, phase II clinical trial that will be conducted on 62 type 2 diabetic patients. Men and women, aged 18-50, with clinical diagnoses of type 2 diabetes for at least one year, will be recruited from the Iranian Diabetes Society. The purpose, eligibility recruitments and the study protocol will be explained for the patients. Informed written consents will be obtained from all participants.

Eligible patients will be randomly assigned to receive one of the two study interventions in a 1:1 ratio. Randomization and allocation sequence will be performed by principle investigator. Allocation will be implemented using a simple randomization approach within three HbA1c strata ($\leq$ 7.4%, 7.5% - 8.9% and $\geq$ 9.0%) and based on the patients' serial identification numbers with a treatment code (A and B) created by sealed envelope online software (14). Randomization will be concealed, and boxes will be labeled according to the random allocation list. All trial group members and participants will be blinded for the type of intervention until the trial will be completed.

Figure 1 shows CONSORT (Consolidated Standards of Reporting Trials) flowchart of the study.

Ethical Consideration

Ethics approval for the trial was obtained from the ethical committee of the Research Institute for Endocrine Sciences of Shahid Beheshti University of Medical Sciences (IR.SBMU.ENDOCRINE.REC.1395.322). The trial was registered in the Iranian Registry of Clinical Trials with the following identification: IRCT20180409039246N1.

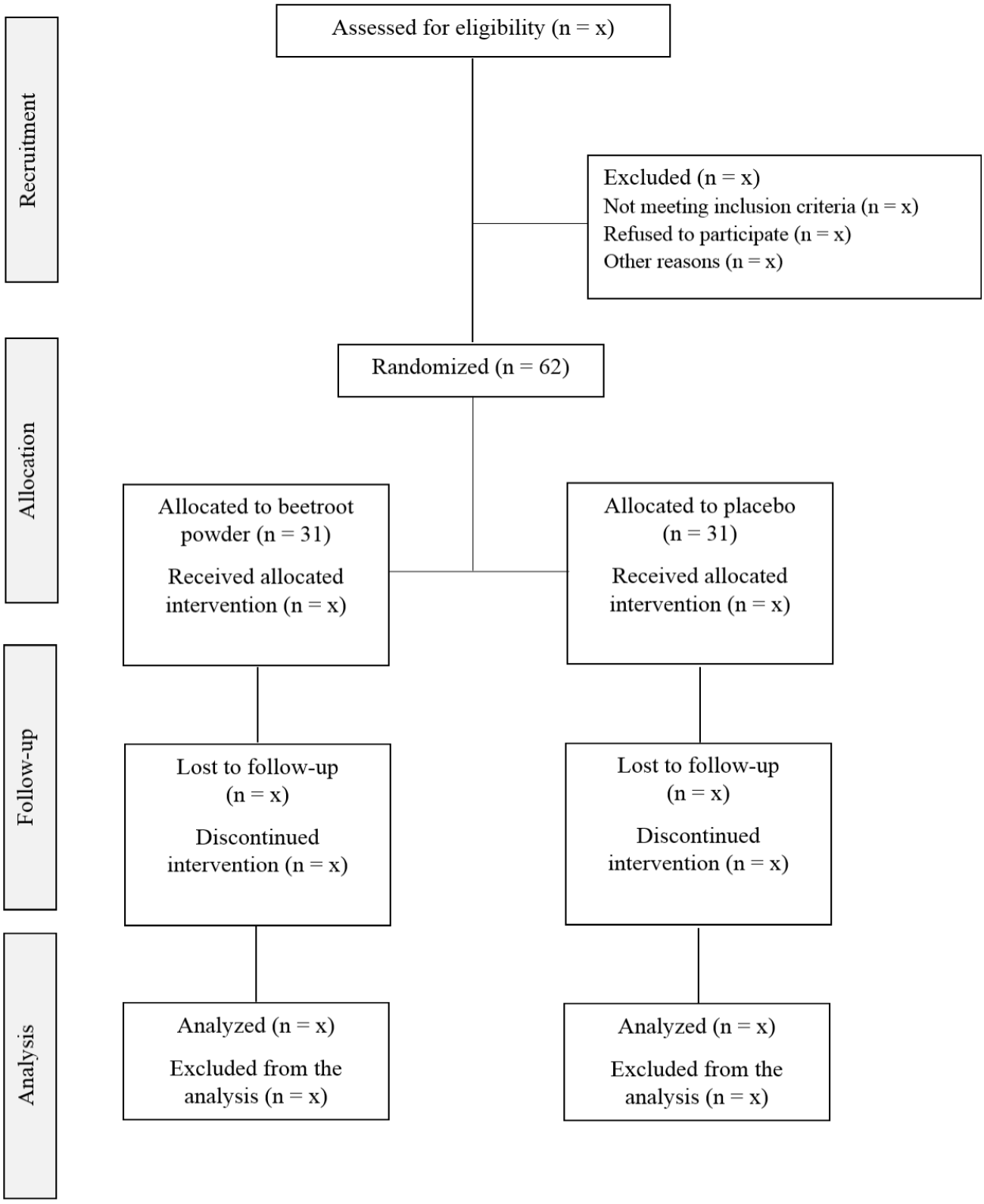

Figure 1. CONSORT flowchart of the trial.

Inclusion and Exclusion Criteria

The following inclusion and exclusion criteria will be followed in the recruitment of the patients:

Inclusion criteria: men and women aged 18-50,

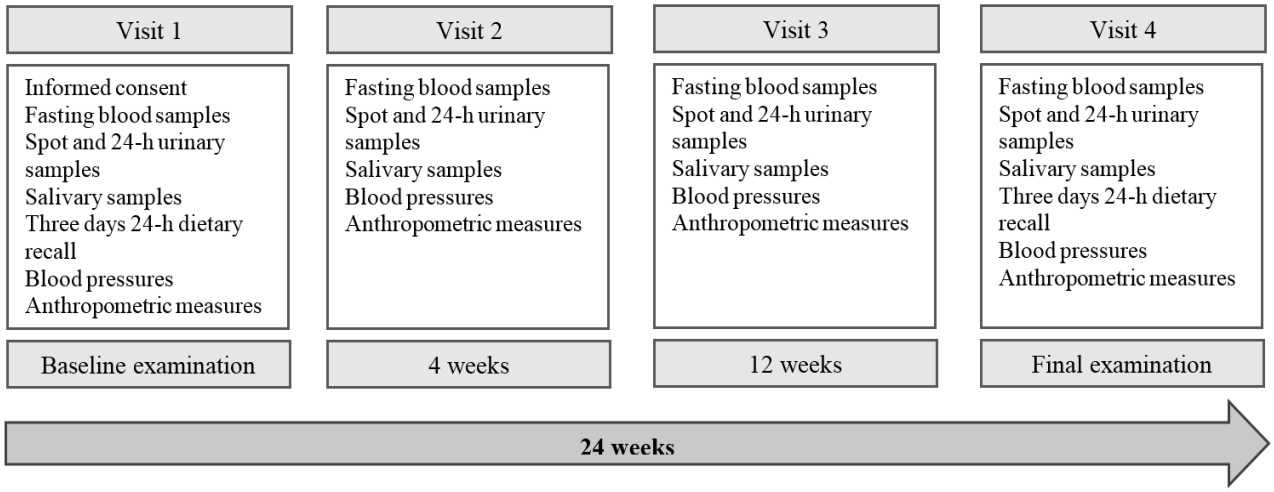

Figure 2. Flow diagram of the trial.

clinically diagnosed T2DM (fasting serum glucose ≥126 mg/dL, or 2-hours serum glucose ≥200 mg/dL, or taking oral anti-diabetic medications) for at least 1 year, willingness and ability to participate and complete the study

Exclusion criteria: the use of insulin, known allergy to beetroot, HbA1c ≥ 11% or fasting blood glucose ≥ 216 mg/dL (12 mmol/L), smoking or alcohol consumption, pregnancy or lactation, self-report of acute or chronic diseases (cardiovascular, renal or liver diseases), systemic autoimmune disease such as rheumatoid arthritis, chronic inflammation such as inflammatory bowel disease, and thyroid dysfunction, taking medications for migraine like sumatriptan, allopurinol, tricyclic antidepressants, antihistamines, nitrates, meperidine, sedative medications, and phosphodiesterase-5 inhibitor drugs.

Intervention

Patients will be randomly allocated in equal proportions to one of the following treatment groups: 5 g/d beetroot powder (contains ~ 250 mg NO_3) or 5 g/d placebo (rice powder colored with beetroot, contains < 25 mg NO_3). Rice powder will be selected as placebo because rice is a staple food among Iranian opulation and has no dietary limitation in this amount (4.5 g/day) in type 2 diabetic patients. Moreover, compared to other components that are commonly used as placebo (e.g. corn powder), colored rice powder provides a more similar color to beetroot powder. The total energy and carbohydrate content of 4.5 g rice powder is 16.5 kcal and 3.6 g respectively. A similar amount of beetroot powder contains 18 kcal energy and 4.2 g total carbohydrate.

Beetroot powder was produced from fresh beetroot (*Beta vulgaris* var. *esculenta*, *chenopodiaceae* family) using a wet-controlled drying method. The method of beetroot powder preparation was recorded as a patent (No. 95415/7-04/2018) in the Iran Intellectual Property Office.

The quality of beetroot powder that was assessed for its macronutrient composition, microbial load, and heavy metals (Table 1) was confirmed by Food and Drug Organization, Shahid Beheshti University of Medical Sciences (1396/S/42196 and 1396/S/43435). Furthermore, measurements of 110 aflatoxins and pesticides residue in beetroot powder samples (n=10) showed values < limit of detection (LOD) (Supplementary Table 1). The NO_3 content of beetroot powder was determined as ~ 50 mg/g (mean ± SD was 52.9±0.8 mg/g, n=3) using a validated spectrophotometric method (15). Placebo, a mixture of rice powder and beetroot powder (with a 9:1 proportion), contains < 25 mg/g NO_3. All supplements will be stored and dispensed to the patients in identical boxes at the trial cite.

Baseline and Follow-up Examinations

Fig.2 shows the flow diagram of the trial. After the initial screening for inclusion and exclusion criteria, patients will be invited to refer to our trial site (Samin Health & Diabetes Clinic) to complete baseline examination and measurement as well as

Table 1: Macronutrient composition, microbial load and heavy metals concentrations of beetroot powder samples (n=10)*

	Result	Limit
Nutritional composition		
Carbohydrate *(%)*	33.3	-
Protein *(%)*	15.3	-
Total Ash *(%)*	8.9	-
Moisture *(%)*	3.8	-
Microbial analysis		
Total microorganisms *(Cfu/g)*	10^4	5×10^3
Salmonella *(per 25 g)*	Negative	Negative
Staphylococcus aureus *(Cfu/g)*	Negative	Negative
Escherichia coli *(Cfu/g)*	Negative	Negative
Clostridium botulinum *(Cfu/g)*	Negative	<10
Mildew *(Cfu/g)*	Negative	10^2
Yeast *(Cfu/g)*	Negative	10^2
Heavy metals		
Plumbum *(mg/kg)*	0.027	0.10
Cadmium *(mg/kg)*	0.044	0.05

* All analyses were performed by Food and Drug Administration, Shahid Beheshti University of Medical Sciences (1396/S/42196 and 1396/S/43435)

randomization at visit (V1). Demographic, anthropometric and clinical measurements will be performed at V1. Trained interviewers will collect information using the questionnaires (a supplementary file). Information on age, educational levels, medical history and medications, duration of diabetes and oral anti-diabetic drugs will be collected. All the data collected during the study will be entered into the Case. Anthropometric measurements including height, body weight, and waist as well as wrist circumference will be assessed by trained staffs. Blood pressure will be measured after a minimum 5-min rest in the sitting position using BM60 (Beurer) Automatic Blood Pressure Monitor. In addition to a 12-h fasting blood sample, spot salivary sample, spot and 24-h urine samples will be collected for biochemical measurements. Similar measurements will be repeated at the following visits in the fourth, twelfth and twenty-fourth weeks (V2, V3, V4).

Usual dietary intakes of the participants will be assessed using 3 consecutive days 24-h recalls at V1 and V4. Total intakes of NO_3 from usual diet will be estimated using a recently developed NO_3 database of frequently consumed food items among Iranians (15).

Physical activity will be assessed using the Modifiable Activity Questionnaire (MAQ) at V1 and V4. The frequency and time spent on light, moderate, hard and very hard intensity activities according to the list of common activities of daily life over the past year will be obtained.

The patients will start taking daily doses after V1 and continued daily for 24 weeks. They will be recommended to take their doses (beetroot powder or placebo) at the same time each day. All patients will be advised to avoid lifestyle modification (including dietary habits and physical activity), or using mouth wash during the study follow-up.

Primary and Secondary Endpoints

The primary outcome is the concentration of HbA1c. Fasting serum glucose, serum insulin, and lipid

parameters including serum total cholesterol, triglycerides, HDL-C and LDL-C, systolic and diastolic blood pressures will be assessed as secondary endpoints.

The potential effects of long-term NO_3 supplementation on liver enzymes (alanine transaminase; ALT, aspartate transaminase; AST, alkaline phosphatase; ALP, and γ-glutamyl transpeptidase; GGT), and thyroid function (thyroid-stimulating hormone; TSH, thyroxine; T4, and triiodothyronine; T3, anti-thyroid peroxidase antibodies, anti-TPO), will be checked during the study follow-up as secondary outcomes. Urinary levels of creatinine and microalbumin will be also assessed.

Concentration of NO metabolites in serum, saliva and urine samples will be measured by a validated spectrophotometric method (using Griess reaction) developed by Miranda et al., for the simultaneous detection of NO_3 and NO_2 (16) at baseline and during the follow-up measurements.

Calculation of Sample Size and Statistical Analysis

The study is powered to detect a 0.75% reduction in HbA1c concentrations in the treatment group compared with the placebo group. The reduction by 0.75% in A1C was selected as effect size value because it is in the range (0.5-1.25%) of the effectiveness reported by most oral anti-diabetic agents (OAD). To achieve an 80% probability, the study will detect a treatment difference at a two-sided 5% significance level, and considering a 1.0% standard deviation (SD) of the response variable from the previous studies, sample size calculation determined a total 56 participants. Considering the potential drop-out, withdrawal or non-compliance, an additional 10% recruitment will be taken into account. The final sample size includes therefore, 62 subjects.

All statistical analyses will adhere to the intention-to-treat principle and included all randomized eligible participants. The Kolmogorov-Smirnov test will be utilized to carry out the test for a normal distribution. Repeated measures analysis of variance (ANOVA) will be used to assess the change of repeated values during the study follow-up. To compare the means of the variables after the treatment and obtain the main effect of each treatment, general linear models will be adjusted for baseline values and oral anti-diabetic drugs will be used. Mean changes of variables from baseline were calculated [(after-treatment values - baseline values)/baseline values) ×100], and independent sample t test will be used to compare these changes between the groups. Statistical analysis will be performed using SPSS (version 20.0; SPSS, Inc., Chicago, IL, USA). A P value <0.05 will be considered significant.

Safety Consideration

Both intervention (beetroot powder) and placebo (rice powder colored with beetroot) are classified as foodstuffs. The content and safety of beetroot powder was assessed by Food and Drug Organization, Shahid Beheshti University of Medical Sciences (1396/S/42196 and 1396/S/43435). Furthermore, a wide range of beetroot juice (70-500 mL) with different contents of inorganic NO_3 (316-860 mg/d) was well tolerated in both short- and long-term trials (12). Some trials with longer duration (24-36 weeks) of beetroot supplementation that are also ongoing, have not reported any side effect or severe complication (17, 18).

Patients will be contacted every week to evaluate the compliance to intervention and also to enquire regarding possible complications. A trial monitoring committee, composed of principal investigators and an endocrinologist, will closely monitor the implementation process of the trial. All potential adverse events (AEs) and serious AEs (SAEs) will be documented and assessed by the committee.

The reports of potential side effects, such as headache or gastrointestinal complications (nausea, vomiting, and gastroesophageal reflux) will be assessed by the trial monitoring committee. The trial will be stopped for the patients with severe side effects distinguished as the direct result of intervention. Interim analysis will be conducted in a timely manner (the fourth, twelfth and twenty-fourth weeks) for glycemic parameters (FBS, HbA1C), TFT and LFT. In the case of clinically significant abnormal value in these parameters, the study will be stopped and the patients will be referred to the endocrinologist of our study group.

Dissemination

Data collection will be completed by July, 2019. Primary and secondary analyses will start following the accomplishment of data collection and data monitoring. The results of the trial will be reported according to the CONSORT statement (19). The manuscript will be ready to be submitted to peer-reviewed journals by October, 2019.

Acknowledgment

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supplementary Table 1. Aflatoxines and pesticides residue in beetroot powder samples (n=10)*

Compound	Quantity	Limit of detection (LOD)	Limit of quantification (LOQ)
2,2-DDE	< LOD	14	40
2,4-DDD	< LOD	14	40
2,4-DDE	< LOD	14	40
2,4-DDT	< LOD	14	40
4,4-DDD	< LOD	14	40
4,4-DDE	< LOD	14	40
4,4-DDT	< LOD	14	40
Alachlor	< LOD	14	40
Aldrin	< LOD	14	40
Allethrin	< LOD	14	40
Alpha-endosulphan	< LOD	14	40
Alpha-HCH	< LOD	14	40
Amitraz	< LOD	14	40
Atrazine	< LOD	14	40
Azinphos-ethyl	< LOD	14	40
Benalaxyl	< LOD	14	40
Beta-endisulfan	< LOD	14	40
Bifenthrin	< LOD	14	40
Bioallethrin	< LOD	14	40
Biteranol	< LOD	14	40
Bromopropylate	< LOD	14	40
Buprofezin	< LOD	14	40
Butachlor	< LOD	14	40
Carbaryl	< LOD	14	40
Chlorothalonil	< LOD	14	40
Chlorpyrifos	< LOD	14	40
Chlorpyrifos-methyl	< LOD	14	40
cyhalothrin	< LOD	14	40

* All analyses were performed by Food and Drug Administration, Shahid Beheshti University of Medical Sciences (1396/S/42196 and 1396/S/43435)

Supplementary Table 1 (cont'd). Aflatoxines and pesticides residue in beetroot powder samples (n=10)*

Compound	Quantity	Limit of detection (LOD)	Limit of quantification (LOQ)
Cypermethrin I	< LOD	14	40
Cypermethrin II	< LOD	14	40
Cyprodinil	< LOD	14	40
Delta-HCH	< LOD	14	40
Deltamethrin	< LOD	14	40
Diazinon	< LOD	14	40
Diclofluanid	< LOD	14	40
Dicofol	< LOD	14	40
Dieldrin	< LOD	14	40
Difenoconazole I	< LOD	14	40
Difenoconazole II	< LOD	14	40
Dimethoate	< LOD	14	40
Diniconazole	< LOD	14	40
Diphenylamine	< LOD	14	40
Disulfoton	< LOD	14	40
Edifenphos	< LOD	14	40
Endosulphan-sulphate	< LOD	14	40
Esenvalerate	< LOD	14	40
Ethion	< LOD	14	40
Etoxazole	< LOD	14	40
Fenamiphos	< LOD	14	40
Fenarimol	< LOD	14	40
Fenbuconazole	< LOD	14	40
Fenitrothion	< LOD	14	40
Fenpropathrin	< LOD	14	40
Fenthion	< LOD	14	40
Fenva;erate	< LOD	14	40
Fipronil	< LOD	14	40

* All analyses were performed by Food and Drug Administration, Shahid Beheshti University of Medical Sciences (1396/S/42196 and 1396/S/43435)

Supplementary Table 1(cont'd). Aflatoxines and pesticides residue in beetroot powder samples (n=10)*

Compound	Quantity	Limit of detection (LOD)	Limit of quantification (LOQ)
Fludioxonil	< LOD	14	40
Gamma-HCH (Lindan)	< LOD	14	40
Heptachlor	< LOD	14	40
Heptachlor-endo-epoxide	< LOD	14	40
Heptachlor-exo-epoxide	< LOD	14	40
Hexaconazole	< LOD	14	40
Imazalil	< LOD	14	40
Indoxacarb	< LOD	14	40
Iprodione	< LOD	14	40
Kresoxim-methyl	< LOD	14	40
Lambda-cyhalothrin	< LOD	14	40
Malathion	< LOD	14	40
Metalaxyl	< LOD	14	40
Methidation	< LOD	14	40
Mthoxychlor	< LOD	14	40
Metribuzin	< LOD	14	40
Molinate	< LOD	14	40
Monocrotophos	< LOD	14	40
Oxadiazon	< LOD	14	40
Penconazole	< LOD	14	40
Permethrin I	< LOD	14	40
Permethrin II	< LOD	14	40
Pertilachlor	< LOD	14	40
Phenothrin	< LOD	14	40
Phorate	< LOD	14	40
Phosalone	< LOD	14	40
Phosmet	< LOD	14	40
Piperonyl butoxide	< LOD	14	40

* All analyses were performed by Food and Drug Administration, Shahid Beheshti University of Medical Sciences (1396/S/42196 and 1396/S/43435)

Supplementary Table 1(cont'd). Aflatoxines and pesticides residue in beetroot powder samples (n=10)*

Compound	Quantity	Limit of detection (LOD)	Limit of quantification (LOQ)
Quaintozen	< LOD	14	40
Tebuconazole	< LOD	14	40
Tetramethrin	< LOD	14	40
Tetradifon	< LOD	14	40
Thiometon	< LOD	14	40
Triadimefon	< LOD	14	40
Triadimenol	< LOD	14	40
Triazophos	< LOD	14	40
Tricyclazole	< LOD	14	40
Triflumizole	< LOD	14	40
Trifluxystrobin	< LOD	14	40
Vinclozolin	< LOD	14	40

* All analyses were performed by Food and Drug Administration, Shahid Beheshti University of Medical Sciences (1396/S/42196 and 1396/S/43435)

Research Institute for Endocrine Sciences

Information Sheet

Beetroot Trial

In this trial, we are doing research on type 2 diabetes, which is common chronic disease in Iran. I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

This trial is designed to investigate whether long-term complementary treatment with beetroot powder as a natural nitrate-rich product, has beneficial effects on glycemic control in diabetic patients. In this trial, the patients will be randomized to receive beetroot powder or placebo for 6 months. Beetroot powder was produced in the Research Institute for Endocrine Sciences and approved by Food and Drug Administration, Shahid Beheshti University of Medical Sciences. Beetroot powder is classified as foodstuffs and there is no report for any side effect or sever complication following consumption of beetroot powder.

During the study, a mild to severe beeturia (red or pink urine) and/or red/pink stool, may be occurred following consumption of beetroot powder. This situation is usually harmless.

The research takes place 6 months in total and you will be asked to come 4 times to the clinic. We will take fasting blood samples, urine and salivary samples from you in each visit (baseline, 4, 12, and 24 weeks).

During the study period, you will continue common medications, and you will be asked to keep regular diet and lifestyle. Our research group will contact you every week to ensure your compliance for treatment or record any side effect.

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. You may also stop participating in the research at any time you choose. It is your choice and all of your rights will still be respected.

Travel costs of the study visiting will be refunded. If you participate in this research, you will have the following benefits: You will be informed of the results of your clinical assessments (including HbA1c, blood glucose and lipid parameters, thyroid and liver tests) in each visit.

The information that we collect from this research project will be kept confidential. Information about you that will be collected during the research will be put away and no-one but the researchers will be able to see it. Any information about you will have a number on it instead of your name.

Protocol of the study was approved by the ethical committee of the Research Institute for Endocrine Sciences of the Shahid Beheshti University of Medical Sciences (IR.SBMU.ENDOCRINE.REC.1395.322).

Research Institute for Endocrine Sciences

Informed Consent Form

Beetroot Trial

1.	I have read and understood the information about the project, as provided in the Information Sheet	☐
2.	I have been given the opportunity to ask questions about the project and my participation.	☐
3.	I voluntarily agree to participate in the project.	☐
4.	I understand I can withdraw at any time without giving reasons and that I will not be penalized for withdrawing nor will I be questioned on why I have withdrawn.	☐
5.	The procedures regarding confidentiality have been clearly explained (e.g. use of names, pseudonyms, anonymisation of data, etc.) to me.	☐
6.	The procedures of intervention and data collection have been explained and provided to me.	☐
7.	The use of the data in research, publications, sharing and archiving has been explained to me.	☐
8.	I understand that other researchers will have access to this data only if they agree to preserve the confidentiality of the data and if they agree to the terms I have specified in this form.	☐
9.	Select only **one** of the following: • I would like my name used and understand what I have said or written as part of this study will be used in reports, publications and other research outputs so that anything I have contributed to this project can be recognised. • I do not want my name used in this project.	☐ ☐
10.	I, along with the Researcher, agree to sign and date this informed consent form.	☐

Participant:

_____ _____ _____
Name of Participant Signature Date

Researcher:

_____ _____ _____
Name of Researcher Signature Date

Research Institute for Endocrine Sciences

Case Report Form

Beetroot Trial

Code: Given name: Family name:

Age: Sex:

Contact number:

Medical history

Anti-diabetic drugs: Metformin □ Glibenclamide □ Acarbose □ Gliclazide □ Zipmet □ Sitagliptin □ Others □

Lipid lowering drugs: Atorvastatin □ Other statins □ Gemfibrozil □ Clofibrate □ Others □

Anti-hypertensive drugs: Losartan □ Amlodipine □ Metoral □ Furosemide □ Hydrochlorothiazide □ Others □

Supplements:

Others:

Duration of type 2 diabetes:

History of other diseases:

Baseline visit (V1) Date:

Height:

Weight:

Body mass index:

Waist circumference:

Wrist circumference:

Systolic blood pressure:

Diastolic blood pressure:

V2 Date:

Weight:

Body mass index:

Waist circumference:

Systolic blood pressure:

Diastolic blood pressure:

Potential complication of intervention:

V3 Date:

Weight:

Body mass index:

Waist circumference:

Systolic blood pressure:

Diastolic blood pressure:

Potential complication of intervention:

V4 Date:

Weight:

Body mass index:

Waist circumference:

Systolic blood pressure:

Diastolic blood pressure:

Potential complication of intervention:
