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The effect of resveratrol supplementation on the expression levels of factors associated with cellular senescence and sCD163/sTWEAK ratio in Patients with Type 2 Diabetes Mellitus: Study Protocol for a double blind Controlled Randomized Clinical Trial

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Abstract

Introduction: Type 2 Diabetes Mellitus (T2DM) is one of the challenges of the health care system. Over the past decades, the numbers of people with diabetes has increased, globally. One of the major complication in these patients is cardiovascular disease; it seems that the cell cycle arrest can improve vascular function in these patients. Resveratrol is a natural polyphenol and appears to improve the vascular function through several cellular pathways. We will aim to evaluate the effects of resveratrol supplementation on mRNA expression of peroxisome proliferator activated receptor alpha (PPARα), tumor suppressor protein p53, cyclin-dependent kinase inhibitor 1 (p21) and Cyclin-dependent kinase inhibitor 2A (p16) in patients with T2DM. We will also measure serum levels of Cluster of Differentiation 163 (CD163) and TNF-like weak inducer of apoptosis (TWEAK) as the indicators of cardiovascular status.

Methods and analysis: Seventy-two subjects suffering from T2DM will be participated in this double blind randomized controlled clinical trial. Participants will be randomly assigned to receive 1000 mg/day trans-resveratrol or placebo (methyl cellulose) for 8 weeks. The mRNA expression levels of PPARα, p53, p21 and p16 genes will be assessed using real-time polymerase chain reaction (PCR) and serum CD163 and TWEAK levels will be measured using commercially available ELISA kits at the baseline and the end of the study.

Ethics and dissemination: The study is performed in agreement with the Declaration of Helsinki and is approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences (no: ir.ssu.sph.rec.1396.120). The results will be published in scientific journals. We will also use conferences and social media to disseminate our findings.

Trial registration number: IRCT20171118037528N1, Registered 29 December 2017)

Keywords: *Type 2 diabetes mellitus, Cardiovascular Diseases, Resveratrol, Peroxisome Proliferator Activated Receptors, p53, p16, p21, TWEAK, CD163*

'Strengths and limitations of this study'

- To our knowledge, this is the first human study to investigate the effects of resveratrol supplementation on the cellular factors associated with intima hyperplasia through the cellular pathways.
- This study is a first trial that uses resveratrol as a natural ligand for PPARα.
- The study is not designed to follow up the patients to determine the long-term effects of resveratrol supplementation.

Introduction

With an increasing trend in its prevalence, type 2 diabetes mellitus (T2DM) has become one of the important causes of mortality and morbidity worldwide (1-3). uncontrolled T2DM might lead to a broad range of micro- and macro-vascular complications including vascular dysfunction and cardiovascular disease (CVD) (4-6). Atherosclerosis is one of the most important causes of CVD which leads to intima hyperplasia (IH) (4). IH is associated with CVD and recently, it has been suggested to be added to the Framingham risk factors (7). In addition, IH might lead to the restenosis after percutaneous transluminal angioplasty or vascular graft and it is known as one of the major complications during the treatment of CVD (8-10). Proliferation of Vascular Smooth Muscle Cells (VSMCs) is increased during IH and is in accordance with vascular stenosis and heart attack (11). Recent animal studies indicated that the VSMCs' proliferation inhibition through cell cycle arrest can reduce the IH levels (12, 13).

Some cellular studies suggest that peroxisome proliferator activated receptors (PPARs) also play a key role in VSMCs' proliferation (14, 15). PPARs are a group of nuclear receptors with various isoforms, including α, β/δ and γ that are involved in transcription regulation of a broad range of genes (16, 17). PPARα is one of the members of this family and has a critical role in the regulation of genes involved in fatty acid oxidation, glucose metabolism, vascular function, obesity, cell proliferation, plaque stability and inflammation (18, 19). Some body of evidences showed that PPARα activation might arrest the cell cycle progression in G_1/S phase through induction of the p16INK4a (6, 20). Cyclin-dependent kinase inhibitor 2A, which is also known as p16INK4a, is a tumor suppressor that inhibits CDK4-mediated phosphorylation of retinoblastoma and inhibits induction of E2F-dependent genes and therefore suppresses cell cycle progression (21-23).

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), which is structurally known as stilbenoid and phytoalexin, is a type of natural polyphenol found mostly in red grapes; it has been introduced as a ligand of PPARα and it seems to stimulate cellular senescence through the above mentioned pathways (24-26). It also has been proposed that resveratrol has the potential to activate p53, another important tumor suppressor, via phosphorylating the serine residue in p53 protein through extracellular kinases (27, 28); phosphorylated p53 is proposed to be able to upregulate the cyclin-dependent kinase inhibitor 1 (p21) gene, which thereby inhibits CDK2 activity and induces the cell cycle arrest in S and G2 phase (29, 30). Animal studies indicated that p53 plays a key role in decreasing the intima thickness (31-34).

Insulin resistance induces chronic inflammation via increased macrophage activity and overexpression of pro-inflammatory cytokines (35). TNF-related weak inducer of apoptosis (TWEAK) is a member of TNF superfamily which is mainly produced by macrophages and is released into the circulation in its soluble form (sTWEAK) (36). Studies have found that sTWEAK levels are reduced in T1DM, T2DM as well as in the presence of CVD risk factors (37-39). The main cause of reduced sTWEAK levels is its binding to fibroblast growth factor-inducible 14 (Fn14) receptor, which therefore can results in inflammatory responses (40).

Intraplaque hemorrhage is a common feature of atherosclerotic plaques that is prevalent in patients with T2DM (41, 42); studies have shown that Intraplaque hemorrhage is most likely to occur in unstable plaques and is associated with ischemic stroke (43-45). On the other hand, cluster of differentiation 163 (CD163) is a macrophage scavenger receptor that is involved in the uptake of hemoglobin-haptoglobin complexes and also known as a scavenger of sTWEAK (46, 47); sCD163 is the soluble form of this receptor and it has been proposed that sCD163/sTWEAK ratio can be used as an indicator of the severity and progression of vascular disease (37, 48,

49). Resveratrol, as an antioxidant, can reduce inflammatory responses and macrophages activity (50) and thus, it seems that resveratrol might affect sCD163/sTWEAK ratio in patients with T2DM.

We proposed to start a randomized clinical trial (RCT) with the following objectives:

i. To investigate the effect of resveratrol supplementation on the changes in PPARα, p16, p53 and p21 gene expression as well as serum level of sCD163 and sTWEAK in patients with T2DM.

ii. To compare the changes in serum levels of lipid profile, including triglyceride, total cholesterol, high density of lipoprotein cholesterol (HDL-C) and low density of lipoprotein cholesterol (LDL-C) as well as glycemic control indices including fasting blood sugar (FBS), fasting insulin, glycosylated hemoglobin (HBA1c) and pancreatic beta cell function and atherogenic index of plasma between the intervention and control groups.

Methods and analysis

Study design

We designed a double-blind, randomized placebo controlled clinical trial among patients with T2DM; we randomly assigned patients to receive either 1000 mg resveratrol or placebo in a daily manner for two months. This monocentral study will be conducted in Diabetes Clinic Center in Yazd, Iran. The overall overview of the study is presented in Figure 1. Any methodological changes in the study design or sample size, which may potentially affect the patients' safety or study procedures, will be discussed in the committee of ethics before implementation.

Randomization

The present study will be an 8-week double-blind parallel RCT; patients will be randomized 1:1 according to the method of stratified block randomization based on sex (male and female) and age (30-45 and 45-60 years).

Computer-generated random numbers will be used to randomly allocate eligible participants into the intervention group to receive two 500-mg capsules of resveratrol per day, or placebo group to receive two 500-mg capsules of methylcellulose per day; resveratrol supplements and placebo will be provided in the same shape, color and appearance and will be packed in the same bottles and a person, who is not involved in this project, will label the containers as A or B. Participants and administrators will be unaware about the content of the bottles. Each bottle contains 60 capsules (providing supplement for one month). All participants will be requested to bring back the first bottle after first month and then will be given the second bottle. At the end of the study, if the remained capsules of every patient exceed 10% of the total administered capsules (12 capsules), that patient will be categorized as non-adherent. There will be some advices for enhancing the participant's compliance such as taking capsules with meals. Moreover, patients who complete the intervention will have an 8-hour nutrition education program for free. All randomized patients, including those who will complete the study or those who will not complete due to any reasons, will follow the same schedule.

Eligibility criteria

30 to 60 year-old male and female, who have been diagnosed with established T2DM for at least three months prior to the intervention, will be invited to participate in the study; participants with the following criteria will be excluded: 1) diagnosis of any liver, kidney, cancer and Alzheimer's diseases or gastrointestinal ulcer; 2) pregnancy or lactation; 3) insulin therapy or the HbA1c levels at or above 8% at any point of study;

4) consumption of supplements containing fish oil, vitamin E or C and red wine in the previous six months; 5) a history of allergic reaction to grapes; 6) consumption of anticoagulants, fibrates and anti-inflammatory agents; 7) a history of myocardial infraction or the presence of stent or battery in patients's heart.

Sample size

Sample size is calculated based on a pervious human study regarding the PPARα expression in peripheral blood nuclear cells (PBMCs) as the primary variable (51); the participant numbers needed in each group is calculated using a proposed formula for parallel clinical trials via considering $\alpha=0.05$ and a power of 80% (52). Assuming a 20% of dropout rate, the final sample size is set to be 36 participants in each group.

Intervention

Two trained researchers will introduce the study protocol to the participants. Written consent form will be obtained from all individuals who will decide to participate in the study. Patients will also receive information sheets. The related questionnaires will be reviewed and approved by the ethical committee members.

Participants in the intervention group will take two capsules of resveratrol per day (one at breakfast and another at dinner) and individuals in the placebo group will take two capsules of 500 mg methylcellulose per day, at the same time for 8 weeks; each capsule of resveratrol contains 500 mg of 99.71% micronized trans-resveratrol (particle size: <1.9 μm) which provides 495 mg trans-resveratrol without any inactive ingredients, fillers, additives or preservatives (Mega-Resveratrol, USA). Moreover, taking routine drugs prescribed by doctors, due to diabetes, will be permitted until their dosages remain unchanged during the study.

Any possible adverse event will be reported to the medical ethics committee within a week and Shahid Sadoughi University of Medical Sciences will be responsible for any participation related problems. Some of the participants may withdraw from the study for any reason at any time, before or after signing the consent form; the investigator also may terminate an individual's participation in the study in order to keep the safety and protect the participant from excessive risks and/or to maintain the integrity of data due to the improper follow-up of the procedures by participant.

General information including age, diseases, medications and supplements will be recorded through interviews at the beginning of the study. In order to obtain the physical activity level, metabolic equivalents (METs) will be calculated through a questionnaire at the beginning and the end of study (53). For assessing the dietary intakes, patients will be asked to complete a three-dietary record form (two weekdays and one weekend day), once at the first week and another at the last week of the intervention; collected data will be analyzed using Nutritionist IV software (The Hearst Corporation, San Bruno, CA). All of the study related data will be stored confidentially.

Anthropometric measurements

Anthropometric parameters will be measured at the beginning and end of the intervention by the same person; body weight will measured with light clothing with an accuracy level of 100 gram and height will be measured using Seca stadiometer with an accuracy level of 0.5 cm; waist and hip circumferences will be measured to the nearest 0.5 cm according to the standard methods, using a flexible tape (54). Body mass index (BMI) will be calculated by dividing body weight (kg) by the height squared (m^2), waist-to hip-ratio (WHR) and waist–to-height ratio (WHtR) will be calculated via standard equations (6). Body composition analysis will be performed via InBody (USA)

analyzer for estimating the measures of total body fat, abdominal fat, fat free mass and body liquids, before and after the intervention.

Biochemical measurements

After 12 hours of fasting, 10 ml of venous blood will be collected. Biochemical analyses including fasting blood glucose, total cholesterol, triglycerides, HDL-C and LDL-C will be measured using automated enzymatic methods. Circulating insulin level and the percent of HbA1c will be assessed by enzyme-linked immunosorbent assay (ELISA) and high pressure liquid chromatography (HPLC), respectively. Commercially available ELISA kits will be used for estimating serum levels of sCD163 and sTWEAK. Homeostatic model assessment of insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI) as an insulin sensitivity index and homeostasis model assessment of beta-cell function (HOMA-B) as well as atherogenic index will be calculated using the suggested formulas (55, 56). All laboratory data will be identified by an ID number to maintain the confidentiality of every participant.

Gene expression assay

After isolating PBMCs from whole blood by Ficoll-paque method (57), total mRNA will be extracted using Blood RNA kit (GeneAll Biotechnology, Korea); total extracted mRNA will be reverse transcribed to cDNA by cDNA synthesis kit (GeneAll Biotechnology, Korea). Real-time polymerase chain reaction (PCR) will be applied to assess the mRNA expression levels of PPARα, p53, p21 and p16. Glyceraldehyde phosphate dehydrogenase (GAPDH) will be the housekeeping gene in real-time PCR assessments.

Statistical analysis

Principal researchers will have full access to the final data sets. Data entry and statistical analyses will be performed using SPSS for Windows (SPSS, Chicago, IL, USA), version 23.0; categorical data will be presented as number and percentages in study groups. The intervention and the control arms will be compared with each other for primary analysis. One-sample Kolmogorov-Smirnov test will be conducted to check normal distribution of continuous data; continuous variable will be expressed as means ± SD or median and interquartile range. Independent sample t-test will be carried out for comparing parametric continuous data and Mann-Whitney U test will be used to test the differences in asymmetric variables between the two groups. Pearson's correlation coefficient will be applied to show the correlation between biochemical and anthropometric indices. General linear models will be used to assess the effects of resveratrol relative to placebo after adjustment for baseline values. P-value ≤0.05 will be defined as statistically significance for all tests.

Data analysis will be performed on two sets including the intention-to-treat (ITT) and the "per protocol" analysis; ITT analysis considers all patients in the intervention or control groups as originally allocated by randomization, independently of their actual adherence to the determined treatment and it ignores anything that happens after randomization including misallocation, noncompliance, withdrawal or protocol deviations; In the case that researchers observe a significant difference between those who will be allocated to receive the intervention and those who actually will adhere to the intervention, additional analysis will be performed considering the actual adherence to the treatment (per protocol analysis). The results of the two mentioned analyses will be compared with each other.

Ethics and dissemination

Written consent form will be obtained from all patients before the study initiation. This protocol is approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences (no: ir.ssu.sph.rec.1396.120). This study is registered at the Iranian Registry of Clinical Trials (IRCT20171118037528N1).

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Competing interests statement

Part of the cost of this study will be borne by INSF. The funding body had no role in the design of the study or in the writing of this manuscript or decision to submit the manuscript for publication. They will also have no role in any aspect of the described data management, analysis or the reporting of study results. The authors declare that they have no competing interests.

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Authors' contributions

Ash: initial idea of this study, Study design, grant, major contributor in writing the manuscript, reviewed all version of the paper. SA: Study design, grant, reviewed and contributed to manuscript. MH: Study design, grant, reviewed and contributed to

manuscript, submission. FH: provided statistical expertise in clinical trial design, sample size calculation. ShM and EK: Contributed to design of biochemical procedures, reviewed and contributed to manuscript. RH: Contributed to study design, reviewed and contributed to manuscript. TO: manuscript revisions and edit the final edition of the manuscript. TM: contributed to manuscript and contributed to the edit of the manuscript. All authors read and approved the final manuscript.

List of abbreviations

PPARα: peroxisome proliferator activated receptor alpha; p21: cyclin-dependent kinase inhibitor 1; p16: Cyclin-dependent kinase inhibitor 2A; CD163: Cluster of Differentiation 163; TWEAK: TNF-like weak inducer of apoptosis; BMI: Body mass index; GAPDH: Glyceraldehyde phosphate dehydrogenase; WHtR: waist–to-height ratio; WHR: waist-to hip-ratio; ELISA: enzyme-linked immunosorbent assay; HPLC: high pressure liquid chromatography; METs: metabolic equivalents; HDL: high density lipoprotein; LDL: low density lipoprotein; FBS: fasting blood sugar; HBA1c: glycosylated hemoglobin; T2DM: type 2 diabetes mellitus; CVD: cardiovascular disease; ITT: intention-to-treat.

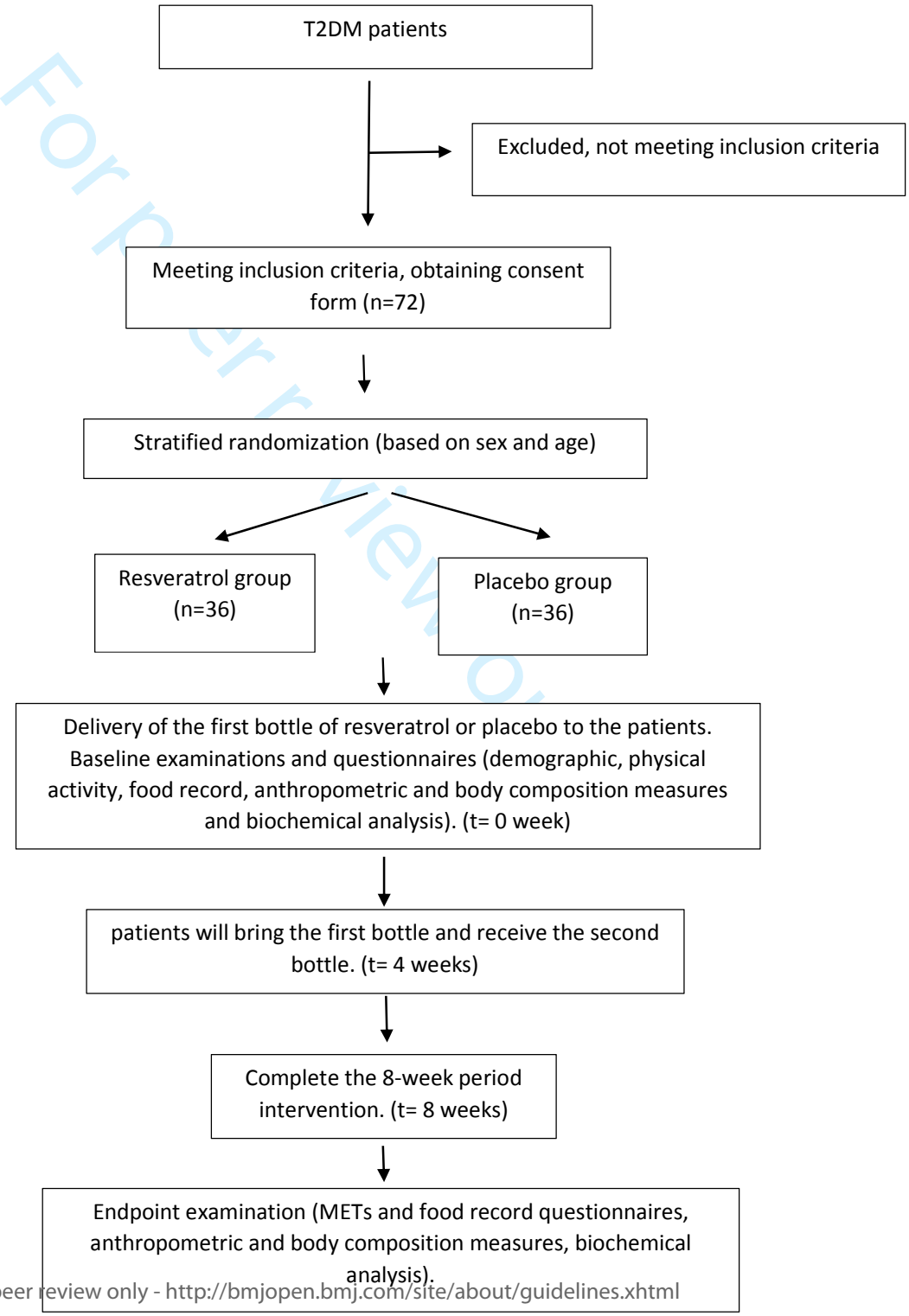

Figure 1: Overview of the Study

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
	2b		
Protocol version	3	Date and version identifier	-
Funding	4	Sources and types of financial, material, and other support	13
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	2 & 13
	5b	Name and contact information for the trial sponsor	-
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	13
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	-
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5-7
	6b	Explanation for choice of comparators	-
Objectives	7	Specific objectives or hypotheses	7
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	7
Methods: Participants, interventions, and outcomes			

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	7
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	8 & 9
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	8
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	-
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	7
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	15
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	9
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	-
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	8
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	-
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	-
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors,	8

		data analysts), and how	
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	-
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	-
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	8
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	10
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	11
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	12
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	_____
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	12
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	9
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	_____

Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	7
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	10
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	11
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	9
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	_____
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

The effect of resveratrol supplementation on the expression levels of factors associated with cellular senescence and sCD163/sTWEAK ratio in patients with type 2 diabetes mellitus: study protocol for a double-blind controlled randomized clinical trial

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The effect of resveratrol supplementation on the expression levels of factors associated with cellular senescence and sCD163/sTWEAK ratio in patients with type 2 diabetes mellitus: study protocol for a double-blind controlled randomized clinical trial

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ABSTRACT

Introduction: Over the past decades, the number of people with type 2 diabetes (T2D) has increased, globally. One of the major complications in these patients is cardiovascular disease; it seems that the cell proliferation inhibition can improve vascular function in these patients. It is proposed that peroxisome proliferator activated receptor alpha (PPARα), can induce cell cycle arrest via cyclin-dependent kinase inhibitor 2A (p16) activation. Also, it has been shown that phosphorylated tumor suppressor protein p53 is involved in cell senescence by cyclin-dependent kinase inhibitor 1 (p21) upregulation. Resveratrol is a natural polyphenol and appears to improve the vascular function through the mentioned pathways. We will aim to evaluate the effects of resveratrol supplementation on mRNA expression of PPARα, p53, p21and p16 in patients with T2D. We will also measure serum levels of cluster of differentiation 163 (CD163) and TNF-like weak inducer of apoptosis (TWEAK) as the indicators of cardiovascular status.

Methods and Analysis: Seventy-two subjects suffering from T2D will participate in this double blind randomized parallel placebo-controlled clinical trial. Participants will be randomly assigned to receive 1000 mg/day trans-resveratrol or placebo (methyl cellulose) for 8 weeks. The mRNA expression levels of PPARα, p53, p21 and p16 genes will be assessed using real-time polymerase chain reaction (PCR) and serum CD163 and TWEAK levels will be measured using commercially available ELISA kits at baseline and the end of the study. Clinical outcomes parameters (glycemic and lipid profiles and body composition) will also be measured before and after study duration.

Ethics and dissemination: The study is performed in agreement with the Declaration of Helsinki and is approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences (no: ir.ssu.sph.rec.1396.120). The results will be published in scientific journals.

Trial registration number: IRCT20171118037528N1, Registered 29 December 2017)

Keywords: *Type 2 diabetes mellitus, Cardiovascular Diseases, Resveratrol, Peroxisome Proliferator Activated Receptors, p53, p16, p21, TWEAK, CD163*

'Strengths and limitations of this study'

- To our knowledge, this is the first human study to investigate the effects of resveratrol supplementation on the cellular factors associated with intimal hyperplasia through the cellular pathways.
- This study is a first trial that uses resveratrol as a natural ligand for PPARα.
- The study is not designed to follow up the patients to determine the long-term effects of resveratrol supplementation.

INTRODUCTION

With an increasing trend in its prevalence, type 2 diabetes mellitus (T2DM) has become one of the important causes of mortality and morbidity worldwide (1-3). Uncontrolled T2DM might lead to a broad range of micro- and macro-vascular complications such as vascular dysfunction and cardiovascular disease (CVD) (4-6). Atherosclerosis is one of the most important causes of CVD which leads to intimal hyperplasia (IH) (4). IH is a cardinal manifestation of atherosclerosis which is associated with CVD and recently, it has been suggested to be added to the Framingham risk factors (7). In addition, IH might lead to restenosis after percutaneous transluminal angioplasty or vascular graft and it is known as one of the major complications during the treatment of CVD (8-10). Proliferation of vascular smooth muscle cells (VSMCs) is increased during IH and is in accordance with vascular stenosis and heart attack (11). Recent animal studies indicated that the VSMCs' proliferation inhibition through cell cycle arrest can reduce the IH levels (12, 13).

Some cellular studies suggest that peroxisome proliferator activated receptors (PPARs) also play a key role in VSMCs' proliferation (14, 15). PPARs are a group of nuclear receptors with various isoforms, including α, β/δ and γ that are involved in transcription regulation of a broad range of genes (16, 17). PPARα is one of the members of this family and has a critical role in the regulation of genes involved in fatty acid oxidation, glucose metabolism, vascular function, obesity, cell proliferation, plaque stability and inflammation (18, 19). Some bodies of evidence showed that PPARα activation might arrest the cell cycle progression in G_1/S phase through induction of the p16INK4a (6, 20). Cyclin-dependent kinase inhibitor 2A, which is also known as p16INK4a, is a tumor suppressor that inhibits CDK4-mediated phosphorylation of retinoblastoma and inhibits induction of E2F-dependent genes and therefore suppresses cell cycle progression (21-23).

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), which is structurally known as stilbenoid and phytoalexin, is a type of natural polyphenol found mostly in red grapes; it has been introduced as a ligand of PPARα and it seems to stimulate cellular senescence via the above mentioned pathways (24-26). It has also been proposed that resveratrol has the potential to activate p53, another important tumor suppressor, by phosphorylating the serine residue in p53 protein through extracellular kinases (27, 28). Phosphorylated p53 is proposed to be able to upregulate the cyclin-dependent kinase inhibitor 1 (p21) gene, thereby inhibiting CDK2 activity and induces the cell cycle arrest in S to G2 phase (29, 30). Animal studies indicated that p53 plays a key role in decreasing the intimal thickness (31-34).

Insulin resistance induces chronic inflammation via increased macrophage activity and overexpression of pro-inflammatory cytokines (35). TNF-related weak inducer of apoptosis (TWEAK) is a member of TNF superfamily which is mainly produced by macrophages and is released into the circulation in its soluble form (sTWEAK) (36). Studies have shown that sTWEAK levels are reduced in T1DM, T2DM as well as in the presence of CVD risk factors (37-39). The main cause of reduced sTWEAK levels is its binding to fibroblast growth factor-inducible 14 (Fn14) receptor, which therefore can result in inflammatory responses (40).

Intraplaque hemorrhage-common feature of atherosclerotic plaques- is prevalent in patients with T2DM (41, 42). Studies have shown that intraplaque hemorrhage is most likely to occur in unstable plaques and is associated with ischemic stroke (43-45). On the other hand, cluster of differentiation 163 (CD163) is a macrophage scavenger receptor that is involved in the uptake of hemoglobin-haptoglobin complexes and is also known as a scavenger of sTWEAK (46, 47); sCD163 is the soluble form of this receptor and it has been proposed that sCD163/sTWEAK ratio can be used as an indicator of the severity and progression of vascular diseases (37, 48, 49). Resveratrol, as an antioxidant, can

reduce inflammatory responses and macrophages activity (50) and thus, it seems that resveratrol might affect sCD163/sTWEAK ratio in patients with T2DM. Given that no study has investigated this issue, this has prompted us to design a randomized clinical trial (RCT) with the following objectives:

i. To investigate the effect of resveratrol supplementation on the changes in PPARα, p16, p53 and p21 gene expression as well as serum levels of sCD163 and sTWEAK in patients with T2DM.

ii. To compare the changes in serum levels of lipid profile, including triglyceride, total cholesterol, high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) as well as glycemic control indices including fasting blood sugar (FBS), fasting insulin, glycosylated hemoglobin (HbA1c), pancreatic beta cell function and atherogenic index of plasma between the intervention and control groups.

METHODS AND ANALYSIS

Study design

We designed a double-blind, randomized parallel placebo-controlled clinical trial among patients with T2DM; we will randomly assign patients to receive either 1000 mg resveratrol or placebo in a daily manner for two months. This monocentral study will be conducted in Diabetes Clinic Center in Yazd, Iran. The overall overview of the study is presented in **Figure 1**. Any methodological changes in the study design or sample size, which may potentially affect the patients' safety or study procedures, will be discussed in the committee of ethics before implementation.

Randomization

The present study will be an 8-week double-blind parallel RCT; patients will be randomized 1:1 according to the method of stratified block randomization based on sex (male and female) and age (30-45 and 45-60 years).

Computer-generated random numbers will be used to randomly allocate eligible participants into the one of the two trial groups by an independent statistician. Participants will be allocated to one of the two arms, using sealed envelope by a researcher who will not be involved in participant's enrollment (EKN) and assignment of intervention will be carried out by principal investigator (SA) who will be blinded to allocation. Resveratrol supplements and placebo will be provided in the same shape, color and appearance and will be packed in the same bottles and a person, who is not involved in this project, will label the containers as A or B. Participants and administrator will be unaware about the content of the bottles until data analyses.

Eligibility criteria

Thirty to sixty-year-old male and female, who have been diagnosed with established T2DM for at least three months prior to the intervention and are taking medication for diabetes, will be invited to participate in the study. Participants with the following criteria will be excluded: 1) diagnosis of any liver, kidney, cancer and Alzheimer's diseases or gastrointestinal ulcer; 2) pregnancy or lactation; 3) insulin therapy or the HbA1c levels at or above 8% at any point of study; 4) consumption of supplements containing fish oil, vitamin E or C in the previous six months; 5) a history of allergic reaction to grapes; 6) consumption of anticoagulants, fibrates and anti-inflammatory agents; 7) a history of myocardial infarction or the presence of stent or battery in patients's heart, and 8) consumption of red wine or supplements containing resveratrol in 6 months prior to intervention.

Sample size

Sample size is calculated based on a previous human study regarding the PPARα expression in peripheral blood mononuclear cells (PBMCs) as the primary variable (51). The participant numbers needed in each group is calculated using a proposed formula for parallel clinical trials by considering α=0.05 and a power of 80% (52). Assuming a 20% of dropout rate, the final sample size is set to be 36 participants in each group.

Intervention

Recruitment of participants will take place through installing announcements at Diabetes Clinic Center in Yazd, Iran. Interested patients will be invited to a screening session and two trained researchers (SA and MT) will introduce the study protocol to them and assess eligibility criteria. Written consent form will be obtained from all eligible patients who will decide to participate in the study. Participants will also receive information sheets. Blood sample will be also obtained to assess HbA1c and eligible patients will be included in the study. It should be mentioned that; ineligible patients will be excluded from the study after receiving nutrition recommendations for diabetes.

General information including age, parity, education, medical information, duration of the disease, etc. will be recorded through interviews at the beginning of the study. In order to obtain the physical activity level, metabolic equivalents (METs) will be calculated through a questionnaire at the beginning and the end of the study (53). To assess the dietary intakes, participants will be asked to complete a three-dietary record form (two weekdays and one weekend day), one at the first week and another at the last week of the intervention; collected data will be analyzed using Nutritionist IV software (The Hearst Corporation, San Bruno, CA). The questionnaires will be reviewed and

approved by the ethical committee members. All the study related data will be stored confidentially.

Participants in the intervention group will take two capsules of resveratrol per day (one at breakfast and another at dinner) and individuals in the placebo group will take two capsules of 500 mg methylcellulose per day, at the same time for 8 weeks; each capsule of resveratrol contains 500 mg of 99.71% micronized trans-resveratrol (particle size: <1.9 μm) which provides 495 mg trans-resveratrol without any inactive ingredients, fillers, additives or preservatives (Mega-Resveratrol, USA). Moreover, participants will continue taking diabetic medication prescribed by doctor during the study. Each bottle contains 60 capsules (providing supplement for one month). All participants will be requested to bring back the first bottle after first month and then they will be given the second bottle. At the end of the study, if the remaining capsules of every patient exceed 10% of the total administered capsules (12 capsules), that patient will be categorized as non-adherent. There will be some advices for enhancing the participant's compliance such as taking capsules with meals. Moreover, patients who complete the intervention will have an 8-hour nutrition education program for free. All randomized patients, including those who will complete the study or those who will not complete due to any reasons, will follow the same schedule.

Any possible adverse event will be reported to the medical ethics committee within a week and Shahid Sadoughi University of Medical Sciences will be responsible for any participation related problems. Some of the participants may withdraw from the study for any reason at any time, before or after signing the consent form; the investigator may also terminate an individual's participation in the study in order to keep the safety and protect the participant from excessive risks and/or to maintain the integrity of data due to the improper follow-up of the procedures by participant.

Data collection

Anthropometric measurements

Anthropometric parameters will be taken at the beginning and end of the intervention by the same person. Height will be measured using a stadiometer (Seca, Hamburg, Germany) with an accuracy level of 0.5 cm; waist and hip circumferences will be measured to the nearest 0.5 cm according to the standard methods, using a flexible tape (54). Weight estimate and body composition analysis (% fat mass, % fat free mass and visceral fat) will be performed via InBody (USA) analyzer, with light clothing before and after the intervention. Body mass index (BMI) will be calculated by dividing body weight (kg) by the height squared (m^2), waist-to hip-ratio (WHR) and waist–to-height ratio (WHtR) will be calculated via standard equations (6).

Biochemical measurements

After 12 hours of fasting, 10 ml of venous blood will be taken at baseline and week 8 of the study. A 6 ml of blood sample will be collected in the colt-activator tubes and centrifuged after 30 minutes clotting time (3000 g, 10 minutes at room temperature; Eppendorf AG, Hamburg, Germany) for serum isolation. Serum samples will be stored at -70°C until analyses. Remaining blood will be obtained in two EDTA-coated tubes for gene expression and HbA1c assessment, separately. Biochemical analyses including fasting blood glucose, total cholesterol, triglycerides, HDL-C and LDL-C will be measured using automated enzymatic methods and commercial kits (Pars Azmoon, Tehran, Iran). Laboratory kits will be used to assess circulating insulin levels and the percent of HbA1c will be assessed by enzyme-linked immunosorbent assay (ELISA, Monobind, USA) and high pressure liquid chromatography (HPLC, Pars Azmoon, Tehran, Iran), respectively. Commercially available ELISA kits will be used for

estimating serum levels of sCD163 and sTWEAK. Homeostatic model assessment of insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI) as an insulin sensitivity index and homeostasis model assessment of beta-cell function (HOMA-B) as well as atherogenic index will be calculated using the suggested formulas (55, 56). All laboratory data will be identified by an identification number to maintain the confidentiality of participants.

Gene expression assay

Total RNA will be extracted directly from whole blood using GeneAll Hybrid-R purification kit protocol (GeneAll Biotechnology Co., Seoul, South Korea). The quality and purity (260/280 nm ratio between 1.8 to 2.2) of the RNA will be checked using spectrophotometer (NanoDrop, Thermo Scientific, USA). After normalization, high quality mRNA will be reverse transcribed to cDNA by cDNA synthesis kit (GeneAll Biotechnology Co., Seoul, South Korea) and according to the manufacturer's instruction. Three primer designing tools (Primer Blast, Oligocalc, and Gene runner 5.0.99) are applied for sequencing the study primers (**Table 1**). Real-time polymerase chain reaction (PCR) and SYBR Green method (Takara Bio Inc., Japan) will be applied to assess the mRNA expression levels of PPARα, p53, p21 and p16 in the StepOne system (Applied Biosystems, Foster City, California, USA). To this aim, 1 µL of cDNA, 10 µL of SYBR Green, 1 µL of primers (revers and forward) and 0.4 µL of Rox, will be mixed together. Final volume of solution will be reached to 20 µL by adding ddH_2O (7/6 µL). Real-time PCR will be adjusted for initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of 90 °C for 15 seconds. The optimal annealing temperature for primers will be set at a range of 55-60 °C for 20 seconds. The final step will be set-up at 72 °C for 20 seconds, for primer extension. Glyceraldehyde phosphate dehydrogenase (GAPDH) will be the housekeeping gene in real-time PCR

assessments. Real-time PCR efficacy and changes in expression levels will be tested using LinRegPCR software (57) and Pfaffl equation, respectively (58).

Statistical analysis

Principal researchers will have full access to the final data sets. Data entry and statistical analyses will be performed using SPSS for Windows (SPSS, Chicago, IL, USA), version 23.0. The intervention and the control arms will be compared with each other for primary analysis. One-sample Kolmogorov-Smirnov test will be conducted to check normal distribution of data. Continuous variable will be expressed as means ± SD or median and interquartile range and categorical data will be presented as number and percentages in study groups. Independent sample t-test will be carried out for comparing parametric continuous data and Mann-Whitney U test will be used to test the differences in asymmetric variables between the two groups. Pearson's correlation coefficient will be applied to show the correlation between biochemical and anthropometric indices. General linear models will be used to assess the effects of resveratrol relative to placebo after adjustment for baseline values and participant's characteristics. P-value ≤0.05 will be defined as statistically significance for all tests.

Data analysis will be performed on two sets including the intention-to-treat (ITT) and the "per protocol" analysis; ITT analysis considers all patients in the intervention or control groups as originally allocated by randomization, independently of their actual adherence to the determined treatment and it ignores anything that happens after randomization including misallocation, noncompliance, withdrawal or protocol deviations. In the case that researchers observe a significant difference between those who will be allocated to receive the intervention and those who will actually adhere to the intervention, additional

analysis will be performed considering the actual adherence to the treatment (per protocol analysis). The results of the two mentioned analyses will be compared with each other.

Strengths and limitations

This study has been designed as a double-blind randomized controlled clinical trial, which will investigate the effects of resveratrol supplementation on the cellular factors associated with intimal hyperplasia for the first time. It will also be the first to use high-bioavailable resveratrol supplement as a natural ligand for PPARα in human. However, as a limitation, the labeling of the containers as A and B can result in unblinding entire group when unblinding is necessary; although, resveratrol has not shown serious adverse events in previous studies. Another limitation in this study is the surrogate markers that will be used for endothelial function assessment instead of gold-standard methods such as flow-mediated dilation (FMD) or peripheral arterial tonometry (PAT). Moreover, we will not perform oral glucose tolerance test (OGTT) or hyperinsulinemic clamp to evaluate glycemic control effects of resveratrol. Finally, this study is designed for short term assessment of resveratrol supplementation effects in patients with T2DM.

Patient and public involvement

Patients or the public will not involve in the setting of the research question, outcome measures or study design and implementation. In this study, the intervention will involve taking daily supplement, and participants will not receive any lifestyle changes, so, participants will not be asked to assess the benefits and burdens of participating. The summary results of the trial will be presented in a grouped form to scientific journals. Participants will be provided individual body composition report, as well as individual glycemic and lipid profile results, on request, when study is completed.

Ethical consideration

Written consent form will be obtained from all patients before the study initiation. This protocol is approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences (no: ir.ssu.sph.rec.1396.120). This study is registered at the Iranian Registry of Clinical Trials (IRCT20171118037528N1).

Acknowledgments

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Competing interests statement

Part of the cost of this study will be borne by INSF. The funding body had no role in the design of the study or in the writing of this manuscript or decision to submit the manuscript for publication. They will also have no role in any aspect of the described data management, analysis or the reporting of study results. The authors declare that they have no competing interests.

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Authors' contributions

SA, ASA, OT, MHS, MR and HMK were involved in initial idea of this study and designing the trial. SA, ASA and HMK were contributed in writing the manuscript and getting grant. MT and EKN are co-investigator and will involve in collecting data, concealment procedure and counseling patients. HF provided statistical expertise in clinical trial design, sample size calculation and blinding. MHS and EKN were contributed to design of biochemical procedures. All authors read and approved the final manuscript.

List of abbreviations

PPARα, peroxisome proliferator activated receptor alpha; p21, cyclin-dependent kinase inhibitor 1; p16, cyclin-dependent kinase inhibitor 2A; CD163, cluster of differentiation 163; TWEAK, TNF-like weak inducer of apoptosis; BMI, Body mass index; GAPDH, glyceraldehyde phosphate dehydrogenase; WHtR, waist–to-height ratio; WHR, waist-to hip-ratio; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography; METs, metabolic equivalents; HDL, high density lipoprotein; LDL, low density lipoprotein; FBS, fasting blood sugar; HbA1c, glycosylated hemoglobin; T2DM, type 2 diabetes mellitus; CVD, cardiovascular disease; ITT, intention-to-treat.

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Table 1: Real-time PCR primer sequences

	Forward	Reverse
p53	GAGCTGAATGAGGCCTTGGA	CTGAGTCAGGCCCTTCTGTCTT
p21	TGGAGACTCTCAGGGTCGAAA	GGCGTTTGGAGTGGTAGAAATC
p16	CTTCCTGGACACGCTGGTG	GCATGGTTACTGCCTCTGGTG
PPARα	CTATCATTTGCTGTGGAGATCG	AAGATATCGTCCGGGTGGTT
GAPDH (Reference gene)	TGGTATCGTGGAAGGACTCATG	GCTTCACCACCTTCTTGATGTC

GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; PCR, Polymerase chain reaction; PPARα, Peroxisome proliferator activated receptor alpha

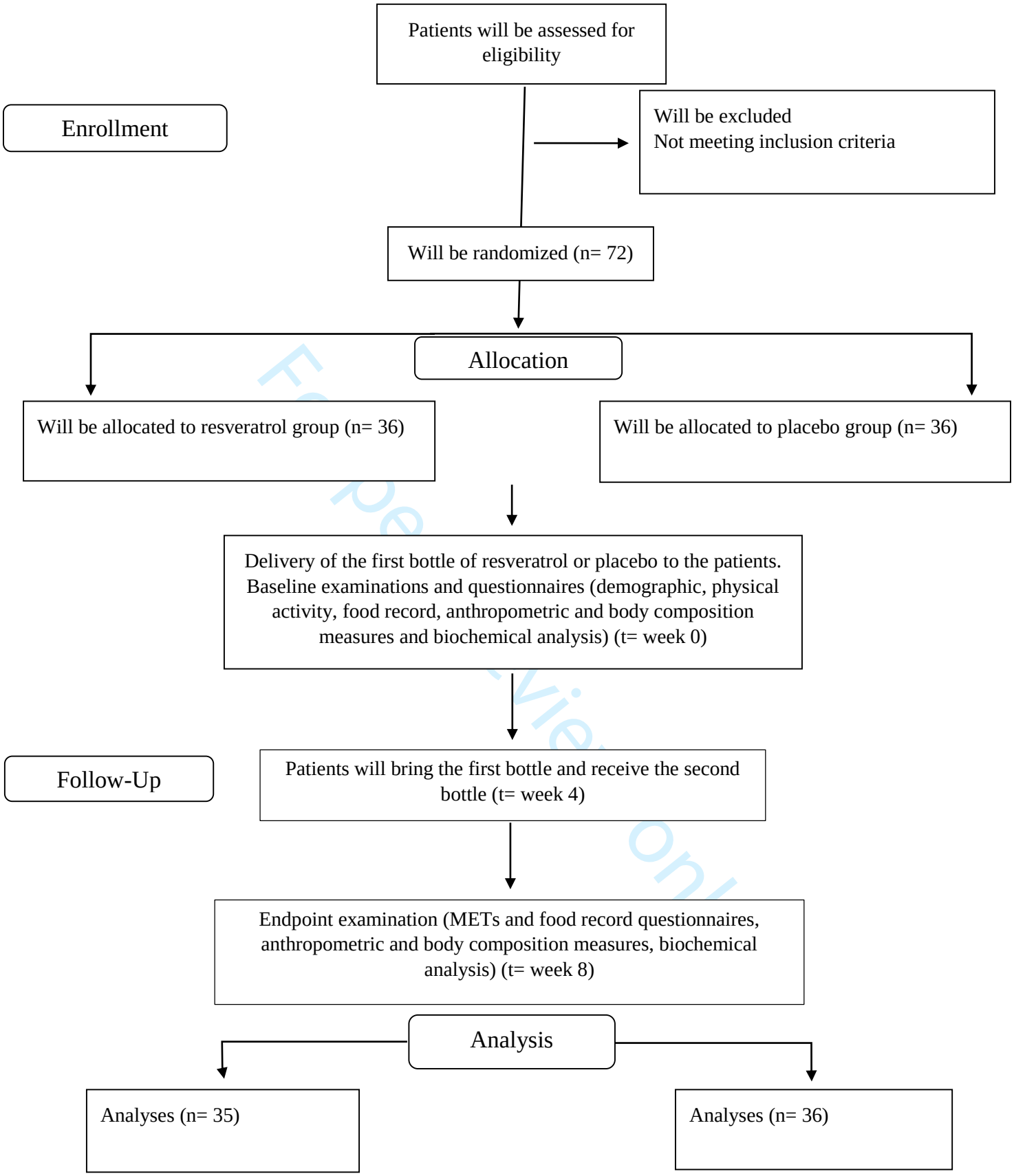

Figure 1: Overview of the study

SPIRIT

STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
	2b		
Protocol version	3	Date and version identifier	-
Funding	4	Sources and types of financial, material, and other support	13
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	2 & 13
	5b	Name and contact information for the trial sponsor	-
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	13
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	-
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5-7
	6b	Explanation for choice of comparators	-
Objectives	7	Specific objectives or hypotheses	7
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	7
Methods: Participants, interventions, and outcomes			

Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	7
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	8 & 9
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	8
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	-
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	7
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	15
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	9
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	-
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	8
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	-
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	-
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors,	8

		data analysts), and how	
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	-
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	-
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	8
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	10
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	11
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	12
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	_____
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	12
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	9
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	_____

Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	7
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	10
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	11
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	9
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	_____
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.