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Study protocol Article

Formulation of Canola Oil with γ-Oryzanol and Evaluation of the Effectiveness of Its Consumption Compared with Unfortified Canola and Sunflower Oils on Certain Cardiometabolic, Oxidative Stress and Immunity Indicators of Adults with Type 2 Diabetes: A Randomized Controlled Clinical Trial Study Protocol

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ABSTRACT

Background and Objectives: Diabetes mellitus is the most common endocrine disorder worldwide. Cardiometabolic risk factors like truncal obesity and unhealthy life style including unhealthy diet contribute to development of type 2 diabetes (T2D) and its complications and attributable deaths. Therefore, modification of the patient's diet and lifestyle is a core of T2D treatment. γ-Oryzanol (ORZ) is a phytochemical found in rice bran oil. An increasing body of evidence has demonstrated health benefits of ORZ including decreasing oxidative stress and insulin resistance. This study will be conducted to examine possible effects of ORZ-fortified canola oil, as compared with plain canola and sunflower oils, on certain cardiometabolic indicators.

Materials and Methods: A total of 90 adult subjects aged 20-65 y with confirmed diagnosis of T2D will randomly be allocated in one to the three groups to receive: (i) ORZ-fortified canola oil (14 mg ORZ/14g); (ii) plain canola oil (without ORZ); and (iii) sunflower oil. All oils will have equal amounts of vitamins D (4.5 µg =180 IU) and A (240 µg =800 IU) per serving, i.e. 14 g. The subjects will be instructed to use only the given oils for cooking during 12 weeks intervention period. Dietary, anthropometric and laboratory evaluations will be done for all participants before and after the intervention.

Conclusions: The findings of this study will shed a light to our current knowledge of health aspects of cooking oils and also will input information to our future dietary recommendations to T2D patients and, probably, the general population.

Keywords: Type 2 diabetes, γ-oryzanol, canola oil, sunflower oil, clinical trial

Introduction

Diabetes mellitus (DM) is the most common endocrine disorder worldwide. In 2019, it was estimated that about 9.3% of the general population (463 million) were affected by DM and this number would rise to 10.2% (578 million) and to 10.9% (700 million) by the years 2030 and 2045, respectively (1). In addition, the global prevalence of impaired glucose tolerance (IGT) in 2019 was estimated to be 7.5% (374 million) with a probable increase up to 8.0% (454 million) and 8.6% (548 million) by 2030 and 2045, respectively (1). Cardiometabolic risk factors like truncal obesity and unhealthy life style including unhealthy diet, sedentary life and smoking all contribute to development of type 2 diabetes (T2D), the commonest form of DM, and its complications and attributable deaths (2). Therefore, modification of the patient's diet and lifestyle is a core of T2D treatment (3).

Oils and fats considerably contribute to the total energy intake of humans. Dietary guidelines from scientific bodies recommend that 20-35% of total energy intake must be provided by dietary fats (4). Observational studies have shown that replacement of saturated fats with liquid plant

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oils containing mono- and polyunsaturated fatty acids (MUFAs and PUFAs, respectively) is associated with lower risk of cardiovascular disease (CVD) (5), the major cause of death in T2D (6). Plant oils may naturally contain bioactive compounds, phytochemicals, with a wide range of health effects including antidiabetic properties (7). Some phytochemicals may be added to the edible oils as fortificants (8). γ-oryzanol (ORZ) is a phytochemical found in rice bran oil (RBO). Early studies have demonstrated health benefits of RBO consumption especially on serum cholesterol concentrations and attributed these effects to the naturally occurring ORZ and tocotrienols (9, 10). Presence of about 26% saturated fatty acids (mostly palmitic acid, ~23%) in RBO has made healthy aspects of its consumption somehow challenging (11). An increasing body of evidence, on the other hand, has demonstrated health benefits of ORZ (12, 13). Antioxidant and anti-inflammatory properties of ORZ have been already documented (13-15) and its therapeutic effect in murine model of hepatic fibrosis has been recently reported (16). ORZ may also reduce insulin resistance (17, 18) and hence it has been considered as a potential therapeutic agent in DM and the related complications including dyslipidemia (19-21). Considering the association between appetite hormones (leptin and ghrelin) and blood glucose hemoestasis (22, 23), the effect of ORZ on blood glucose may be mediated through these hormones.

This study will be conducted to examine possible effects of ORZ-fortified canola oil on certain cardiometabolic indicators. The specific aims of this study include (i) to formulate ORZ-canola oil; and (ii) to evaluate the effects of daily consumption of ORZ-fortified canola oil, as compared with unfortified canola and sunflower oils, in adult subjects with T2D on some selected biomarkers including anthropometric, inflammatory, immunity, appetite and metabolic. As most cooking oils available in the Iran market are voluntarily fortified with vitamins A and D, all oils to be used in this study will contain equal amounts of these two vitamins.

Materials and Methods

This study comprises two steps: (i) formulation and production of ORZ-canola oil; and (ii) a randomized clinical trial.

(a) Formulation and Production of ORZ-fortified canola oil

The steps of ORZ-fortified canola oil are neutralization, decoloration, winterization, deodorization, fortification and packaging. The dosages to be used in this study are as follows: ORZ 14 mg, vitamin D 4.5 μg (180 IU) and vitamin A 240 μg (800 IU) for each oil serving, i.e. 14 g. The amounts of the fortificants in the fortified oil will be monitored using national standard protocols as follows:

ORZ: item 7 of national standard 6658 using spectrophotometry

Vitamins A and D: national standard 13579 using high performance liquid chromatography (HPLC)

All steps of formulation and production of the oils will be performed at Kourosh Food Industry, Tehran, Iran.

(b) Clinical trial

Study design

Subjects with T2D who meet inclusion criteria will be recruited from Iran Diabetes Society or general population using announcements. The whole protocol and objectives of the study will be explained to the subjects and those willing to participate will be randomly allocated to one of the three groups based on receiving one of the three cooking oils as follows: (a) ORZ-A-D-fortified canola oil; (b) A-D-fortified canola oil (without ORZ); and (c) A-D-fortified sunflower oil.

The inclusion criteria are:

i. Having T2D with confirmed diagnosis and with latest fasting serum glucose concentration ≥ 100 mg/dL and/or HbA1c ≥ 6%
ii. Age 20-65 years
iii. Not using insulin

The exclusion criteria include:

i. Any changes in medications, diet or life style that may affect the results of the intervention
ii. Eat out for three consecutive days or more than ten days during the intervention period

All participants will be given enough edible oils for their whole household members (based on 30 g/person) and they will be instructed to use only the given oils for all cooking purposes but frying. The packaging of the oils in the intervention groups will be similar and only the main supervisor will be aware of the group allocations. Duration of the study will be twelve weeks and all assessments (dietary, anthropometric and biochemical) will be done at the beginning and in the end of the intervention period.

Sample size

Considering the effect size of 0.35, α error = 5% and power = 80%, a total of eighty four subjects (24 in each group) will suffice. Nevertheless, with the probability of 10% attrition in each group, thirty eligible subjects per group will be enrolled.

Dietary assessment

Dietary intake will be assessed using 24 hour dietary recall for two days, including one holiday. To translate dietary intake into energy and nutrients, Nutritionist IV (version 4.1, 1997; First DataBank, The Hearst Corporation, San Bruno, CA) will be employed. Per capita oil intake will be estimated by dividing the whole amount

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of consumed oils by the number of household members and the number of oil consumption days.

Anthropometric and blood pressure measurements

Weight and height will be measured with light clothing (without coat and shoes) using a digital scale (Seca 808, Hamburg, Germany) to the nearest of 0.1 kg and a stadiometer to the nearest of 0.1 cm, respectively. Body mass index (BMI) will be calculated by dividing weight (kg) by height $(m)^2$.

Blood pressure will be measured two times after the subject has rested in sitting position for five minutes. The mean of two measurements will be considered as the subject's blood pressure value (mmHg).

Laboratory investigations

(a) Collecting and handling of specimens

Both blood and salivary specimens will be taken in the morning and after a whole night fasting (12-14 hours). Five milliliter of venous blood taken from each participant will be divided into two tubes either with or without anticoagulant (EDTA). Following 30 minutes at room temperature (RT), clotted blood samples will be centrifuged at 800 *g* at RT. The sera will then be aliquoted in several fresh microtubes. One of the microtubes will be used to determine glucose and lipid profile at the same day of sampling whereas the other microtubes will be preserved at -80° C until the day of analysis.

In order to take salivary samples, subjects will be instructed first to wash their mouth by gurgling tap water for 2-3 times and then transfer a salivary sample to a screw cap plastic tube given to them. Salivary samples will also be kept at -80° C.

(b) Biochemical measurements

Serum concentrations of glucose, triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), blood urea nitrogen (BUN) will be measured using enzymatic method while serum creatinine will be determined by colorimetric assay using commercial kits (all from Pars-Azmoon, Tehran, Iran) with the aid of an auto-analyzer (Selecta E; Vitalab, Holliston, Netherlands). According to the manufacturer, intra- and inter-assay variations for all kits is less than 5%.

Glycated hemoglobin (HbA1c) will be determined using enzymatic method (Pishtaz Teb, Tehran, Iran). According to the manufacture, limit of quantification (LOQ) was 3% and intra- and inter-assay variation coefficients are both less than 1.1%.

Serum concentrations of high-sensitivity C-reactive protein (hsCRP), an indicator of systemic inflammation, will be measured by immunoturbidometric method. According to the manufacturer, LOQ is 0.013 mg/L and intra- and inter-assay variation coefficients are less than 4.21% and 4.29%, respectively. Both HbA1c and hsCRP assays will be performed with the aid of an auto-analyzer (Selecta E; Vitalab, Holliston, Netherlands).

Oxidative stress biomarkers including serum total antioxidant capacity (TAC) and malondialdehyde (MDA) will be evaluated using commercial kits (both from Cayman Chemical, MI, US). TAC will be assayed based on inhibition of 2, 2´-azino-di-3-ethylbenzhiazoline sulphonate (ABTS) oxidation by metmyoglobin against Torolox standard. According to the manufacturer, LOQ is 0.068 nM Torolox equivalent and intra- and inter-assay variations are 3% and 3.4%, respectively. Serum MDA concentration will be determined based on colorogenic reaction resulting from thiobarbituric acid reacting substances (TBARS). According to the manufacturer, assay range is 0-50 μM and intra- and inter-assay variations are 5.5% and 5.9%, respectively.

All other biomarkers will be evaluated using enzyme immunoassay (EIA) method. Table 1 shows the biomarkers, method of determination and certain performance characteristics of the kits to be used.

Statistical analyses

Normal distribution of the data will be checked using Shapiro-Wilk's test. Descriptive and qualitative data will be expressed as mean ± standard deviation (SD) and number (percent), respectively. Within-group comparisons will be made by paired *t* test and Wilcoxon for data with and without normal distribution, respectively. Between-group comparisons will be performed by analysis of covariate (ANCOVA) with adjustment for basal (time zero) variables. In this study, $p<0.05$ will be considered statistically significant. All analyses will be done using Statistical Software for Data Science (STATA, version 17, StataCorp LLC, Texas, US).

Ethical issues

The protocol and objectives of the study will clearly be explained for all participants before they sign a written informed consent. The protocol of this study was approved by the Ethics Committee of National Nutrition and Food Technology Research Institute (IR.SBMU.NNFTRI.REC.1400.031). This clinical trial has been registered at clinicaltrials.gov (NCT05271045).

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Table 1. The biomarkers and certain performance characteristics of the EIA kits to be used

No	Biomarker*	Manufacturer	LOQ	Inter-assay	Intra-assay
1	ox-LDL	USCN, Wuhan, China	0.52 ng/mL	< 10%	< 12%
2	sIgA	USCN, Wuhan, China	0.07 ng/mL	< 10%	< 12%
3	IL-6	DIAsource ImmunoAssays S.A., Rue du Bosquet, Lonvain-la-Neuve, Belgium	2 pg/mL	4.2%	5.4%
4	IL-1β	DIAsource ImmunoAssays S.A., Rue du Bosquet, Lonvain-la-Neuve, Belgium	0.35 pg/mL	2.3%	4.9%
5	IFN-γ	DIAsource ImmunoAssays S.A., Rue du Bosquet, Lonvain-la-Neuve, Belgium	0.03 IU/mL	3.8%	8.8%
6	Leptin	DIAsource ImmunoAssays S.A., Rue du Bosquet, Lonvain-la-Neuve, Belgium	0.7 pg/mL	8.6%	9.1%
7	Ghrelin	USCN, Wuhan, China	4.87 pg/mL	< 10%	< 12%

* All analytes will be assayed in serum specimens except for sIgA which will be measured in saliva samples.
Abbreviations: EIA: enzyme immunoassay; IFN-γ: interferon gamma; IL: interleukin; LOQ: limit of quantification; ox-LDL: oxidized low density lipoprotein; sIgA: secretory immunoglobulin A

Discussion

Both canola and sunflower oils are considered healthy (24, 25). There are some evidence for more beneficial health effects of canola oil than sunflower oil (26, 27). Considering the bioactive properties and health promoting effects of ORZ (28, 29), it is plausible that fortification of canola oil with ORZ will boost its healthy effects. Nevertheless, this notion needs to be documented. To the best of our knowledge, this study will be the first clinical trial examining the effectiveness of daily consumption of ORZ-fortified canola oil, as compared with plain canola and sunflower oils, on a wide spectrum of health and nutrition biomarkers. The findings of this study will help answer the following questions:

i. What are the health effects of replacing common cooking oils and fats, including butter and animal oils, with solely sunflower or canola oils?
ii. Consumption of which cooking oil may confer more beneficial health effects, sunflower or canola? And finally and importantly
iii. Can addition of ORZ to canola oil potentiate its health effects in terms of cardiometabolic (including anthropometric, glycemic and lipidemic), oxidative stress and immunity biomarkers to be evaluated in adult subjects with T2D?

Having the answers to the above questions will shed a light to our current knowledge of cooking oils and also will input information into our future dietary recommendations to T2D patients and, probably, the general population.

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