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Original article

Proposal of a study protocol of a preliminary double-blind randomized controlled trial. Verifying effects of selenium supplementation on selenoprotein p and s genes expression in protein and mRNA levels in subjects with coronary artery disease: selenegene study

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Summary. *Background:* Selenium is the component of selenocystein amino acid, which itself is the building block of selenoproteins having diverse effects on various aspects of the human health. Among these proteins, selenoprotein P is the central to the distribution and homeostasis of selenium, and selenoprotein S as a transmembrane protein is associated with a range of inflammatory markers, particularly in the context of cardiovascular disease. It is known that selenium status outside of the normal range is considered to confer different benefits or adverse cardiovascular risk factors. Therefore, for the first time, we aimed to verify effects of Selenium supplementation on Selenoprotein P and S Genes Expression in Protein and mRNA Levels in Subjects with Coronary Artery Disease (CAD). *Methods:* This is the study protocol of a double blinded randomized clinical trial on 130 subjects with angiographically documented stenosis of more than 75% in one or more coronary artery vessels. In this 60-day study, 65 patients in each group received either a 200mg selenium yeast or placebo tablets once daily. During the study, subjects were followed by phone calls and visited our clinic twice to repeat baseline measurements. We hypothesized that our finding would enable a more basic and confirmed understanding for the effect of selenium supplementation by investigating its effect on gene expression levels in people with CAD. *Discussion:* Upon confirmation of this hypothesis, the beneficial effect of inflammation regulation by supplementation with micronutrients could be considered for subjects with CVD. (www.actabiomedica.it)

Key words: selenium, supplementation, gene expression, metabolic syndrome, coronary artery disease, randomized controlled trial

Background

Selenium is a vital trace element for humans and is part of the unusual amino acids selenocysteine and selenomethionine (1-4). It is involved in the mechanisms that form part of tissue inflammation and oxidative stress (5).

To date, almost 25 different selenocysteine-containing selenoproteins have been detected in human cells and tissues (6). Since a lack of selenium deprives

a cell of its ability to synthesize selenoproteins, many health effects associated with a low selenium intake are believed to be caused by the deficiency of one or more particular selenoproteins (7). On the other hand, it is important to note that too much selenium in the diet causes toxic effects and leads to selenium poisoning (8).

The threshold between essential and toxic concentrations of this element is rather narrow: the factor is in the range of 10-100 (8).

A noticeable geographical variety is found in its dietary intake from plants, which is low in Europe because of contrasts in its bioavailability. In a few sections of the world, for example, the UK, China, and Russia, its extensively decreased admission can be principally brought on by the brought down imports of wheat developed in the northern selenium-rich high-protein soils for making bread. Its inadequacy has been every now and again reported in areas where most nourishment is privately developed and expended (9).

Recently, the level of blood selenium has been determined by measuring the amount of selenoprotein P (SELP). The latter is the most common selenoprotein found in the plasma (10), which contains ten selenocysteine residues and functions as a selenium supply protein (11). In addition, it is suggested that there is an association between the level of SELP and an abnormal glucose metabolism. Moreover, previous studies have shown that SELP plays a part in the development of cardiovascular risk factors, such as obesity, diabetes, and atherosclerosis (12).

Another selenoprotein is selenoprotein S (SELS), which was recently, described as an endoplasmic reticulum (ER) and plasma membrane-located selenoprotein that is involved in the physiological adaptation to ER stress (13, 14). The SELS gene is known to be expressed in a wide variety of tissues and cell types, including those of the former that are important for glycemic control, such as adipose tissue, muscle, and liver (15, 16). In omental adipose tissue from diabetic subjects, gene expression of SELS was increased compared with that of non-diabetic controls, while in both groups, SELS expression correlated with the homeostasis model assessment of insulin resistance (HOMA-IR) (17). In HepG2 cells, SELS expression is inhibited by glucose; meanwhile, SELS has been suggested to be involved in glucose homeostasis in an animal model of type 2 diabetes mellitus (18) and in human diabetic subjects (19). Additionally, in HepG2 and intestinal epithelial cells, pro-inflammatory cytokines activate the transcription of SELS (20). In the past, genetic analyses of SELS in various cohorts have been performed (21) and associations between SELS polymorphisms and inflammation (22), and hard end points in cardiovascular disease (23,24), have been found. However, there is no previous study showing how selenium supplementation changes the expression of SELP and SELS genes in the level of mRNA and protein in subjects with documented coronary artery disease.

Main objective

Our hypothesis in the present study is that Se supplementation will alter the expression of SELP and SELS genes in mRNA and protein levels. Our aim is to estimate the effects of Se intake on changes in the expression of these genes.

Specific objectives

1. To compare the level of selenium in both intervention groups, before and after the study.
2. To compare the level of expression of SELP and SELS in both intervention groups.
3. To estimate the effect of selenium intake on quality of life.
4. To describe nutritional status and selenium feeding habits.

Methods

Ethical considerations

The trial was approved by the Isfahan University of Medical Sciences and Research Ethics Committee and conforms to the standards currently applied by the Iranian Registry of Clinical Trial (IRCT=10252). An external data safety monitoring board monitored the trial to guarantee its quality. An option was in place for the trial protocol to be altered, with confirmation from this committee, if interim analyses demonstrated statistically significant differences in the primary end point between the groups. If significantly beneficial ef-

fects of Se supplementation were found, Se treatment would have been offered to those patients who were randomized to the placebo group.

Study design and settings

This was a single-center; double-blind, placebo-controlled, superiority-randomized clinical trial. All participants had coronary artery disease, which was documented by angiography. In total, 160 patients were enrolled in the study after fulfilling inclusion and exclusion criteria. The subjects were referred to our research center from referral heart hospitals in Isfahan, Iran.

Participants

1. Having angiographically documented stenosis in one, two or three vessels. These should have confirmed coronary artery disease angiographic diagnosis, based on the stenosis, of more than 75% in each vessel.
2. Aged between 30 and 65 years.
3. Living in Isfahan province for more than 5 years.

Exclusion criteria

1. Pregnancy or breastfeeding.
2. Having diabetes mellitus or being under treatment by metformin.
3. History of hormone therapy, Cushing's syndrome, inflammatory bowel disease and other inflammatory disorders, gastrointestinal disease and lactose intolerance, and/or use of any type of selenium supplements.
4. History of liver disease, kidney disease, gout, rheumatoid artery, thyroid disorders, adrenal and parathyroid dysfunction, women's diseases, cancer, cardiovascular disease including arrhythmias, uncontrolled advanced congestive heart failure (CHF), severe valvular disease, pericarditis and/or myocarditis.
5. Under medical prescription of vitamins or supplements
6. Participating as a volunteer in other clinical investigations with interventions.
7. Having conditions that may result in low protocol adherence.

Patients who met the inclusion criteria were invited to participate in the study and informed consent was obtained from them. Participants underwent an initial interview. The initial interviews and tests included a questionnaire to collect demographic data, medical history, stress levels, physical activity, smoking habits, quality of life, anthropometric measurements, and detailed information for a nutritional profile, including selenium intake in diet and biochemical laboratory measurements.

Intervention

Patients received either 200 mg selenium yeast tablets or placebo tablets orally after a meal, once daily for 60 days. The placebo tablets had the same color, form and texture as the selenium tablets. At each visit, patients in both groups received the number of tablets needed to be taken until the next scheduled appointment. If, for any reason, a treatment interruption occurred, the treatment duration was to be extended until the volunteer had taken 60 tablets.

Selenium_yeast was provided in commercial form by the Nature company, while Amin pharmaceutical company was responsible for providing the placebos. Adverse events were to be recorded.

Measurement of Gene Expression in RNA level

Total RNA (2 μg) was treated with DNase I and reverse transcribed using random hexamers and Super Script II reverse transcriptase (Invitrogen Ltd, UK). Primers were designed using Primer3 software and synthesized by Sigma-Aldrich, Ireland. PCR was carried out in a 50 ml mix containing 0.5 ml of Taq polymerase (Invitrogen) and 1 ml of cDNA. PCR products were then run on 2% agarose gel with a parallel 100 bp DNA ladder (Promega, UK). Real-time PCR was carried out according to the manufacturer's instructions using the Light Cycler RNA SYBR Green 1 Amplification Kit (Roche Applied Science). All measurements were independently repeated six times (n=6). The maximum concentration of total RNA template used was 0.5 μg ml-1. Data are presented as cycle thresholds

(Ct), and in quantitative analysis, the Ct method was performed using the LightCycler version 4.0 software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels were used to normalize.

Determination of Selenoprotein P and S

Each selenoprotein was determined considering similar strategies described as follows: Total plasma selenoprotein concentration was measured at baseline and at the 8 week of follow-up in 130 participants who were participated in this study by commercial kit Hangzhou Eastbiopharm Co.,Ltd (China). This kit used a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human selenoprotein in samples. Then, the selenoprotein was added to the selenoprotein pre-coated monoclonal antibody Enzyme. Selenoprotein antibodies labeled with biotin were then added and combined with Streptavidin-HRP to form an immune complex. The incubation was carried out and washed again to remove the uncombined enzyme. Chromogen solutions were then added which the liquid color was finally turned from blue to yellow due to the effect of the acid. The chroma of color and the concentration of the Human selenoprotein of samples were positively correlated.

Outcomes

1. To compare the average concentration of serum protein products and mRNA levels of SELP and SELS in target groups before and after the trial.
2. To determine the selenium level before and after supplementation.

Secondary putcome

Changes in components of metabolic syndrome, such as diabetes, hypertension, and central obesity after supplementation.

Follow-up

Participants were followed every two weeks by phone calls and answered brief questions on the occurrence of any adverse events. Four weeks after the initial visit, they were invited to visit Isfahan Cardiovascular Research Institute and interviews and non-laboratory evaluations similar to the baseline were performed. Moreover, all evaluations including blood tests were repeated eight weeks after the baseline visit.

All examination results were recorded and stored in digital media to do later analysis at an EPI with software SPSS (Version 15)

Sample size

We estimated a minimum sample size of 130 volunteers (65 in the placebo group and 65 in the Se group). In this calculation, we considered anα error of 0.05, a β error of 0.20, and a difference in progression risk of 50%.

Randomization

The 130 subjects were randomly allocated into two groups in a 1:1 ratio. Placebo pills that looked similar to selenium were provided by the pharmacist. The tablets were packaged by pharmaceutical companies in the same numerical packages and were recognized with four different codes. Executives and interviewers were both unaware of the coding. The latter decided which set of participants would form the placebo group and which would be the intervention group by flipping a coin. As volunteers were recruited by the medical staff, they were assigned a number that sequentially corresponded to a treatment box. In this way, a strategy of numbered boxes was used for sequence concealment.

Blinding

Patients, interviewers, and staff involved in outcome assessment were blinded to treatment. The blinding was conducted using the same strategy of allocation concealment by numbered boxes. In brief, numbers were assigned to volunteers' treatments, but only one pharmacist, who was not involved in the tasks, was aware of what was in each numbered box.

Minimum sample size estimation

The type of analysis that was of the greatest interest was the intention-to-treat analysis. At baseline, both intervention groups were compared concerning information that may have modified disease progno-

Table 1. Clinical Characteristics of Iranian Population Participants in the Selengene Study

Demographic characteristics	Non MetS	MetS	P Value
Women (%)	11 (15.5)	21 (32.3)	0.021
Age	55.9±7.52	55.6±6.41	0.798
FBS (mg/dL) (Mean ±SD)	96.3±11.0	106.7±14.1	<0.001
Chol (mg/dL) (Mean ±SD	153.7±39.7	158.6±40.3	0.479
TG (mg/dL) (Mean ±SD)	139.1±86.5	198.5±122.0	0.003
HDL_C (mg/dL) (Mean ±SD)	43.0±9.52	37.8±9.67	0.003
LDL-C (mg/dl) (Mean ±SD)	81.4±32.2	80.8±32.9	0.913
BMI (Kg/m2), Mean +SD	26.8±3.67	28.8±4.01	0.003
Waist circumference\Mean +SD	97.9±9.78	106.7±14.1	<0.001
SBP (Mean ±SD)	125.8±17.6	141.4±19.7	<0.001
DBP (Mean ±SD)	78.4±9.78	83.0±10.3	0.009
Diabetic			<0.001
Normal	51 (71.8)	18 (29.0)	
Pre diabetic	18 (25.4)	27 (43.5)	
diabetic	2 (2.8)	17 (27.4)	
Hypertension			<0.001
Normal	17 (23.9)	5 (7.7)	
Pre hypertensive	34 (47.9)	23 (35.4)	
hypertensive	20 (28.3)	37 (56.9)	
Central Obesity			
Residency (Urban)	65 (91.5)	64 (98.5)	0.118
Education Years (%)			
illiterate(%)	7 (9.9)	10 (15.4)	0.754
<9	39 (54.9)	35 (53.8)	
9-12	14 (19.7)	10 (15.4)	
13<	11 (15.5)	10 (15.4)	
Family history of Cardiovascular disease (%)	6 (8.5)	8 (12.3)	0.460
Lifestyle			
Smoking (%)	14 (19.7)	11 (16.9)	0.674
Intake of food items			
Red Meat Intake (times/week)	6.58±2.39	7.23±4.23	0.269
Fats	2.01±3.68	1.51±2.59	0.583
Fruit and vegetables	49.0±28.0	43.5±19.2	0.182
Nuts	3.95±4.10	3.46±2.81	0.435
Beans	1.76±0.85	1.88±0.96	0.462
Diary	13.5±4.62	14.8±5.00	0.119
Cereals	21.8±6.67	23.1±6.06	0.244

SePP1: Selenoprotein P, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, Chol: Total Cholesterol, TG: Triglyceride, LDL-C low density Cholesterol, HDL_C: High density Cholesterol, FBS: Fasting Blood Suger

Table 2. SELS and SELP gene expression in subjects with MetS versus subjects without MetS

Gene	Variantes	MetS ΔCt	Non MetS ΔCt	2^(-ΔCt)	P value
SELS	VIMP I(MEDIAN (IQR))	8.85 (7.93-12.2)	8.99 (7.49-11.48)	1.02	0.948
	VIMP II(MEDIAN (IQR))	7.64 (4.06-9.05)	6.82 (5.28-9.56)	1.60	0.863
SELP	SELPI(Mean ± SD)	2.75±4.21	2.04±3.40	0.61	0.56

sis, to check whether random allocation was working properly at that time. Analysis was conducted by adjusted regression as per protocol. This analysis was mainly conducted on the understanding that random allocation did not fulfill its purposes at baseline.

Stopping rules

All important harmful or unintended effects in each group, such as nausea, vomiting, nail changes, loss of energy, and irritability.

Ethics

The study protocol was explained to all who agreed to enroll in the study and sign the agreement. All potential side effects were described.

Results

A total of 65 subjectswith MetS and 71 subjects without MetS were included in this study. No significant differences were observed between both groups in age 55.6±6.41 vs. 55.9±7.52 P=0.798) and sex (female, 32.3% vs. 15.5%, P=0.021), respectively. Mean of FBS is higher among subjects with MetS (106.7±14.1vs, 96.3±11.0, P<0.001). Mean of TG is higher among MetS+ 198.5±122.0 vs. 139.1±86.5, P=0.003) Mean of systolic and diastolic blood pressure, BMI, WC, were higher among subjects with MetS (P=0.05). There was no significant difference in the family history of cardiovascular disease between two groups (P=0.388). Also, there were no significant differences between smoking and nutritional habits (such as using beans, dairy, all types of meats, cereals, nuts, fruits, and vegetables) between the two groups (P<0.05) (Table 1). Median and interquartile for the expression of different variants in our study groups are shown in table 2. The level of VIMP II was lower in MetS+ compared to the MetS- subjects; however it was not a significant difference (P <0.05). We found no significant differences in quantitative expression of VIMPI in both groups. There are no significant mean differences between both variants in subjects with MetS (2.74±1.61, P=0.109). There is no significant difference in the quantitative expression of SELP in subjects with MetS versus subjects without MetS (2.75±4.21, 2.04±3.40, p=0.56).

Discussion

If the hypothesis is confirmed, it will constitute a new contribution to the improvement of the healthcare of the affected population; in Iran, this equates to approximately 1 million sufferers. Supplementation with micronutrients is simple and has been widely employed using oral administration (25). In many countries, including the US and France, selenium is considered a supplement rather than a medicine. According to the Brazilian Agency of Sanitary Inspection, 100 micrograms of selenium is considered medicinal, because it is up to 100% of the recommended 34 microgram daily intake. However, the daily level in our trial was within the limits considered to be safe (200 micrograms; Secretariat of Health Surveillance, Ministry of Health (SVS/MS) Ordinance 40/1998). The organic form of selenium (selenium-yeast) was chosen because it has been proven to prevent and improve cardiovascular disease. If the microgram treatment in this trial turns out to be beneficial, a new and affordable treatment strategy for cardiovascular disease would be suggested which require further multicentral trials to support and confirm our mentioned possible trial results.

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Conflict of interest: None to declare

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