

Effect of L-Arginine and Selenium Added to a Hypocaloric Diet Enriched with Legumes on Cardiovascular Disease Risk Factors in Women with Central Obesity: A Randomized, Double-Blind, Placebo-Controlled Trial

Mohammad Alizadeh^a Abdolrasoul Safaeiyan^b Alireza Ostadrahimi^c
Rassul Estakhri^d Sevana Daneghian^e Aida Ghaffari^e
Bahram Pourghassem Gargari^c

^aDepartment of Biochemistry and Nutrition, School of Medicine, Urmia University of Medical Sciences, Urmia, ^bDepartment of Vital Statistics and Epidemiology and ^cDepartment of Biochemistry and Nutrition, Faculty of Health and Nutrition, Nutrition Research Center, ^dLiver and Gastrointestinal Disease Research Center, and ^eStudent Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran

Key Words

Central obesity · Hypocaloric diet · Cardiovascular disease · Selenium · L-Arginine · Legumes

Abstract

Background/Aims: We aimed to discover if L-arginine and selenium alone or together can increase the effect of a hypocaloric diet enriched in legumes (HDEL) on central obesity and cardiovascular risk factors in women with central obesity. **Methods:** This randomized, double-blind, placebo-controlled trial was undertaken in 84 premenopausal women with central obesity. After a 2-week run-in period on an isocaloric diet, participants were randomly assigned to a control diet (HDEL), L-arginine (5 g/day) and HDEL, selenium (200 µg/day) and HDEL or L-arginine, selenium and HDEL for 6 weeks. Cardiovascular risk factors were assessed before intervention and 3 and 6 weeks afterwards. **Results:** After 6 weeks, L-arginine had significantly reduced waist circumference (WC); selenium had significantly lowered fasting

concentrations of serum insulin and the homeostasis model assessment of insulin resistance index; the interaction between L-arginine and selenium significantly reduced the fasting concentration of nitric oxides (NO_x), and HDEL lowered triglycerides (TG) and WC and significantly increased the fasting concentration of NO_x. HDEL reduced high-sensitivity C-reactive protein levels in the first half of the study and returned them to basal levels in the second half. **Conclusion:** These data indicate the beneficial effects of L-arginine on central obesity, selenium on insulin resistance and HDEL on serum concentrations of NO_x and TG.

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Introduction

Central obesity (also known as ‘abdominal obesity’) is a characteristic of male obesity. However, in the Middle East, central obesity is more prevalent in females than in males [1]. The prevalence of central obesity in females in

Iran is 54.4% according to criteria set by the Adult Treatment Panel III, and 73.4% according to criteria set by the International Diabetes Federation [2]. Abdominal obesity raises the risk of cardiovascular disease (CVD) through major risk factors (e.g. hypertension, hyperglycemia, hypercholesterolemia) and emerging risk factors (e.g. insulin resistance, atherogenic dyslipidemia, prothrombotic state, proinflammatory state) [3]. Strategies which can help to reduce abdominal adipose tissue and cardiovascular risk factors will be of obvious clinical importance.

Most of the studies focusing on oral supplementation with L-arginine have been conducted in patients with different types of heart disease and have investigated the function of coronary endothelial cells. Studies on the effects of such supplementation in females with central obesity are lacking. Oral consumption of high doses of L-arginine has been shown to reduce abdominal adipose tissue in animal models [4–6] and to reduce central obesity in subjects with type 2 diabetes mellitus [7].

L-Arginine produces nitric oxide (NO) by the action of NO synthase (NOS). Endothelial NOS (eNOS) and neuronal NOS enhance the serum concentration of NO. It appears that this level of NO in the serum has beneficial effects on health [8–10]. However, inducible NOS (iNOS) increases the NO concentration to an extent that produces deleterious effects upon health [8, 10–12]. In environments with high levels of reactive oxygen species, NO is oxidized and converted to peroxynitrite [13]. Antioxidants such as selenium can neutralize free radicals and increase the bioavailability of NO. In animal studies, iNOS^(-/-)/apolipoprotein E^(-/-) mice showed a reduction in the levels of markers of oxidant stress and improved atherogenesis [14, 15]. Conversely, selenium decreased lipopolysaccharide-induced NO production by iNOS to pathological levels in murine macrophage cultures in vitro [16], and selenium deficiency enhanced iNOS expression in RAW 264.7 macrophages [17]. Also, a contrary association between the cellular concentration of selenium and iNOS expression in RAW 264.7 cells stimulated by lipopolysaccharide was observed [17]. We selected participants from a population whose mean serum level of selenium was lower than normal values [18], so we accompanied selenium supplementation with L-arginine supplementation. We needed a basic diet for all groups to show the effects of supplementation with arginine and selenium. Hence, we used a basic hypocaloric diet in all groups. Apart from being a food associated with protection of the cardiovascular system, legumes are an inexpensive and rich source of L-arginine and selenium. Hence, we enriched the basic hypocaloric diets with 2

servings of legumes a day. With a hypocaloric diet enriched in legumes (HDEL) and supplementation with L-arginine, we could increase the total intake of L-arginine to approximately 9 g/day. This figure has been quoted as being the minimum amount that has a reducing effect on central obesity [7].

This is the first time that the effect of low doses of L-arginine, selenium and HDEL, each alone or together, on central obesity and CVD risk factors in females with central obesity has been studied. We wanted to find out if L-arginine and selenium, each alone or together, can increase the effect of HDEL. L-Arginine was administered to enhance the physiologic level of NO. Selenium was given to ameliorate antioxidant defenses, to increase NO availability and to prevent the production of NO to pathologic levels. HDEL was administered as a rich source of L-arginine and selenium and as the basis of treatment for central obesity.

Methods

Ethical Approval of the Study Protocol

The study was approved by the Ethics Committee of the Tabriz University of Medical Sciences (Tabriz, Iran). It is registered at www.irct.ir (Iranian Registry of Clinical Trials ID: IRCT138712101720N1). Written informed consent was obtained from all selected subjects.

Study Design

This was a randomized, double-blind, placebo-controlled study which lasted 8 weeks. Because of interaction between NO and estrogens, we could study only one sex and we selected females.

Participants

We placed advertisements for inclusion in the study in local newspapers. A total of 257 premenopausal women were screened.

Inclusion criteria were as follows: age (premenopausal women aged 20–50 years), waist circumference (WC) >88 cm, no participation in weight reduction programs and maintenance of a stable weight (± 2 kg) during the previous 6 months.

Exclusion criteria were as follows: any secondary cause of hyperglycemia (such as trauma) or hypertension (such as renal disease); treatment with oral hypoglycemic agents or insulin, antilipemic drugs or antihypertensive drugs; consumption of vitamin or mineral supplements, nitrate, L-arginine, selenium or antacids containing magnesium or calcium; psychiatric disorders; untreated hypothyroidism; cancer; hepatic, systemic, pulmonary or renal disease or CVD; inflammatory or infectious disease; smoking; alcoholism, and legume intolerance.

Finally, 84 premenopausal women were enrolled. Of these, 16 were excluded and did not complete the study, 3 patients because of transient skin dermatitis in the L-arginine group, 1 patient because of dysmenorrhea in the L-arginine plus selenium group and 12 patients because of poor compliance. Sixty-eight women re-

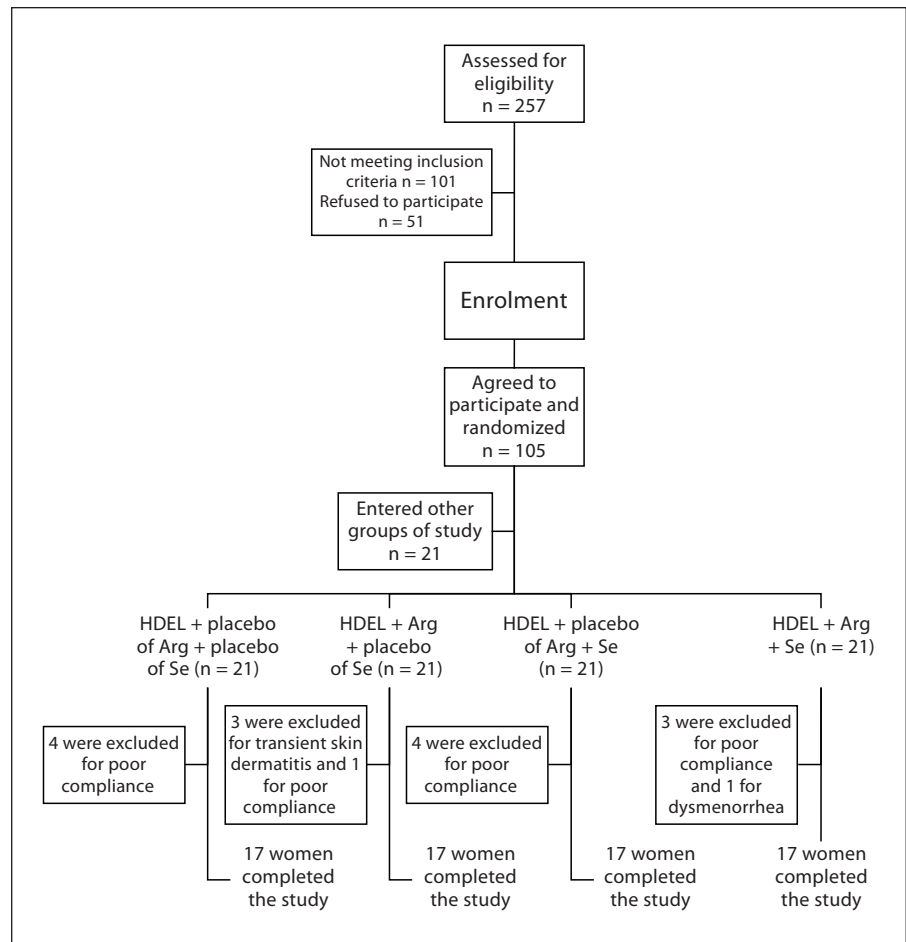


Fig. 1. Flowchart for enrolment of participants.

mained for analysis. Figure 1 shows the flowchart for enrolment of the participants in the study.

Diets

The caloric needs for each subject were determined individually using the equation from the Food and Nutrition Board of the Institute of Medicine [19]. In the run-in period, the participants consumed an isocaloric diet for 2 weeks. In the intervention period, members of all groups were on HDEL (which comprised two servings per day of legumes instead of meat). The composition of all diets was 55% carbohydrate, 30% fat and 15% protein. All participants in all groups were given a diet which contained 500 kcal less than their caloric needs in the intervention period. Participants were being visited every week; each session for each participant lasted 20–30 min. Behavioral counseling was instigated. The nutritionist described the advantages of the diets for the participants and told them that central obesity could be controlled by continuing the diets. Diets were prescribed individually using a calorie count system. Also, an ‘exchange list’ was given to each participant for calorie counting and exchanging food items. A nutritionist taught participants how to write ‘food diaries’. Each participant had to write 3-day diet and physical activity records before the run-in period as well as before, in the

middle and at the end of the intervention. The diaries were evaluated by the investigators. A menu for 7 days with 42 meals and snacks at 17 calorie levels (1,200–2,800) was developed for each diet. Participant compliance was evaluated by reviewing the 3-day food records and weekly visits. Each week, the number of reported food group exchanges from the 3-day food records was compared with prescribed exchanges. Participants who completed $\geq 80\%$ of the planned diet were encouraged to follow it diligently for the subsequent weeks. Volunteers who did not complete $\geq 80\%$ of the prescribed diet for 2 successive weeks were excluded from the study ($n = 12$). Supplements of L-arginine and selenium were delivered every week, and participants were encouraged to use them regularly. Unused tablets were counted at each visit and collected at the end of the treatment period to measure compliance. Subjects consumed $96 \pm 3.5\%$ of the pills assigned.

Study Procedures

We had contact with the subjects on a weekly basis during the 8 weeks of the study. The true isocaloric needs of some of the subjects were different from the amount determined by the equation from the Food and Nutrition Board of the Institute of Medicine. In such individuals, an isocaloric diet would cause reduction or

gain of weight rather than weight maintenance. Such individuals could cause biases in the study. Hence, among individuals eligible to enter the study, only those who maintained their weight at the end of the run-in period using an isocaloric diet calculated according to the equation were selected. Moreover, the dietary pattern of each participant was different and this could affect the results of interventions. Hence, in addition to getting detailed information about the study population, we planned a run-in period to standardize macronutrient consumption. After 2 weeks of the run-in period on an isocaloric diet, subjects were randomly assigned to one of four groups for the intervention period of 6 weeks: (1) HDEL supplemented with a placebo of L-arginine and a placebo of selenium; (2) HDEL supplemented with L-arginine (5 g/day) and a placebo of selenium (HDEL + Arg); (3) HDEL supplemented with selenium (200 µg/day) and a placebo of L-arginine (HDEL + Se), and (4) HDEL supplemented with L-arginine and selenium (HDEL + Arg + Se). We needed randomly allocated and matched groups, so first we divided all of the participants into four groups by factor analysis. Participants in each group were similar according to general characteristics and CVD risk factors. Participants in each group were then randomly allocated to four study groups. We repeated random allocation several times and selected the most homogenous groups. For allocation of the participants, a computer-generated list of random numbers was used. Measurements were obtained before, in the middle and at the end of the intervention.

L-Arginine (5 g/day) was administered as two 1-gram L-arginine hydrochloride tablets (Pooyan Nutrition Company, Tehran, Iran, a joint venture with Mass Global Nutrition, Toronto, Canada) twice a day with meals. Selenium (200 µg/day) was administered as a selenium-enriched yeast tablet (Nature Made, Pharmavite LLC, San Fernando, Calif., USA) given once a day, 2 h after one of the meals (and after L-arginine). The main components of the placebo tablets were starch and lactose. Participants were told not to vary their usual physical activity during the study.

Measurements

All measurements were conducted by the same researcher using the same instrument in the first and follow-up assessments. WC was measured (to the nearest 0.1 cm) at the narrowest point without pressure to the body surface over light clothing using a tape measure.

After a 12-hour fast, blood samples were taken. Samples were centrifuged at 500 g for 10 min at 4°C, and the serum was separated. All parameters except malondialdehyde (MDA), total antioxidant capacity (TAC) and nitric oxides (NO_x) were measured on the day of blood collection. Serum was frozen at -80°C until it was analyzed for assessment of the other parameters.

Levels of total cholesterol, high-density lipoprotein cholesterol and triglycerides (TG) were measured by enzymatic means (ParsAzmoon, Tehran, Iran). Levels of low-density lipoprotein cholesterol were calculated using the Friedewald formula. Plasma concentrations of high-sensitivity C-reactive protein (hs-CRP) were measured using an immunoturbidimetric assay with an enzymatic kit (ParsAzmoon) [20]. Plasma levels of insulin were measured by a human insulin enzyme-linked immunosorbent assay test kit (Diaplus, San Francisco, Calif., USA) [21] according to the manufacturer's instructions. Insulin resistance was calculated on the basis of the homeostasis model assessment of insulin resistance (HOMA-IR) [22].

Levels of nitrites/nitrates were measured concurrently using the Griess reaction [23]. Briefly, nitrates were reduced to nitrites by vanadium (III), and then the level of total nitrites was measured. MDA was measured using a modified version of the Yagi [24] protocol based on the thiobarbituric acid reaction. We used the ferric reducing ability of plasma assay for measuring TAC based on the protocol devised by Benzie and Strain [25].

Inter- and intra-assay coefficients of variation were, respectively, 1.22 and 0.61% for cholesterol, 1.8 and 0.73% for high-density lipoprotein cholesterol, 1.04 and 1.47% for TG, 1.7 and 1% for hs-CRP, and 8 and 8% for insulin.

Additional covariate information (age; age of onset of obesity; education level; income; family history of overweight and metabolic syndrome; economic status of family; dieting history; number of diets undertaken in the past; dieting duration in the past; weight loss in previously completed diets; time of dieting, and weight maintenance in past diets) was obtained by questionnaires. 'Chronic dieters' would have less weight loss with hypocaloric diets. Hence, if they were not distributed evenly among the groups, they could cause errors in the study. Hence, dieting history, number of diets completed in the past and dieting duration in the past were considered as covariates. Subjects were stratified into three groups for each of the following: education (had not obtained a high school diploma, had obtained a high school diploma and university graduates); personal income [no income (housewife), <USD 350 per month and >USD 350 per month]; family income (<USD 350 per month, USD 350–700 per month, and >USD 700 per month), and family history of overweight and metabolic syndrome (any relative, first-degree relative and second-degree relative). A participant was characterized as overweight if the body mass index was >25. Metabolic syndrome was defined according to criteria set by the Adult Treatment Panel III [26].

Statistical Analysis

The sample size for each group was estimated with regard to other studies conducted on obese women [27, 28]. With $1 - \beta = 95\%$ and $1 - \alpha = 95\%$, the maximum sample size was achieved from WC indicators using the following formula:

$$n = a\sigma^2 \times \sigma^2 / \Sigma t_i^2 = 16.49 = 17$$

in which σ^2 (the indicator curve) = 2.5, $\sigma^2 = 59.9$, $\Sigma t_i^2 = 36.46$ and $a = 4$.

Finally, samples for each group were evaluated to be 17 persons. Values are means \pm SE at each time interval.

Two methods were used for data analysis. In the first method, we used nested multivariate analysis of variance (M-ANOVA) for repeated measurements of a multifactor model with the form shown below [this was analyzed with the Minitab package (version 13) to recognize the effects of time and treatments as well as the interaction between treatments on the different variables]:

$$\text{variation of dependent variables} = \text{intraindividual variation} + \text{time} + \text{L-arginine (time)} + \text{selenium (time)} + \text{L-arginine} \times \text{selenium (time)} + \text{error}.$$

In this method, we also used another model for controlling the effect of reduction in WC:

$$\text{variation of dependent variables} = \text{intraindividual variation} + \text{time} + \text{L-arginine (time)} + \text{selenium (time)} + \text{L-arginine} \times \text{selenium (time)} + \text{error} + (B_1 \times \text{WC}).$$

Table 1. Baseline characteristics of the four treatment groups

	Treatment group				p
	HDEL	HDEL + Arg	HDEL + Se	HDEL + Arg + Se	
Number	17	17	17	17	–
Age, years	36.6 ± 8.6	33.8 ± 9.1	36.7 ± 8.3	33.9 ± 8.5	NS
Height, cm	158.7 ± 7	159.7 ± 6	157.1 ± 7	156.4 ± 6	NS
Age at obesity onset, years	17.5 ± 8.7	18.2 ± 10.4	19.6 ± 8.7	18.5 ± 8.1	NS
Education					
No high school diploma	8 (47)	10 (59)	7 (41)	5 (30)	NS
High school diploma	4 (23)	3 (18)	6 (35)	7 (41)	
University graduate	5 (29)	4 (23)	4 (23)	5 (29)	
Income status					
Without income (housewife)	12 (70)	16 (94)	15 (88)	13 (76)	NS
<USD 350 per month	2 (12)	0	0	3 (18)	
>USD 350 per month	3 (18)	1 (6)	2 (12)	1 (6)	
Overweight subjects in family					
Any relative	1 (6)	2 (12)	2 (12)	1 (6)	NS
First-degree relatives	13 (76)	14 (82)	15 (88)	14 (82)	
Second-degree relatives	3 (18)	1 (6)	0	2 (12)	
Metabolic syndrome in family					
Any relative	9 (53)	8 (47)	6 (35)	7 (41)	NS
First-degree relatives	7 (41)	6 (35)	10 (59)	8 (47)	
Second-degree relatives	1 (6)	3 (18)	1 (6)	2 (12)	
Family economic status					
<USD 350 per month	4 (23)	5 (29)	1 (6)	4 (23)	NS
USD 350–700 per month	7 (41)	6 (35)	6 (35)	10 (59)	
>USD 700 per month	6 (35)	6 (35)	10 (59)	3 (18)	
Dieting history					
Yes	9 (53)	10 (59)	6 (35)	7 (41)	NS
No	8 (47)	7 (41)	11 (65)	10 (59)	
Number of diets completed	1.1 ± 1.6	0.6 ± 0.9	1.1 ± 1.4	0.8 ± 0.8	NS
Dieting duration, days	253 ± 877	477 ± 1239	509 ± 1871	248 ± 863	NS
Weight loss in dieting periods, kg	5.3 ± 8.3	3.7 ± 6.6	4.4 ± 5	5.7 ± 6.6	NS
Time of dieting					
Any time	8 (47)	10 (59)	6 (35)	7 (41)	NS
6 months to 1 year ago	3 (18)	1 (6)	6 (35)	4 (23)	
1–5 years ago	3 (18)	4 (23)	3 (18)	4 (23)	
>5 years ago	3 (18)	2 (12)	2 (12)	2 (12)	
Weight maintenance in past diets					
No dieting	9 (53)	9 (53)	6 (35)	7 (41)	NS
Maintenance of reduction	0	1 (6)	1 (6)	2 (12)	
Some maintenance	0	1 (6)	2 (12)	0	
No maintenance	8 (47)	6 (35)	8 (47)	8 (47)	

Values are shown as means ± SE or numbers of patients with percentages in parentheses, as appropriate. NS = Not significant.

In the model described above, intraindividual variability in participants was separated from the effect of interventions. ‘Time’ simultaneously determines and compares HDEL-related changes in outcomes before, at the midpoint and at the endpoint of the intervention. The other three parameters of the model [L-arginine (time), selenium (time) and L-arginine × selenium (time)] simultaneously determine the effect of L-arginine, selenium and their interaction on outcomes at different times during the study

by the analysis of variance. ‘Error’ represents the random changes during the study, and these changes were separated from the effects of interventions. ‘B₁’ is the regression coefficient. ‘B₁ × WC’ represents the effect of WC reduction on outcomes, and its covariate effect was separated from the effects of the interventions. The concurrency of all analyses in this model minimized the probability of false-positive results due to multiple comparisons.

In the second method, we used a paired t test or the Wilcoxon test for comparing the value of variables at different times in the study groups. Also, we used an independent t test or the Mann-Whitney U test for comparing the percentage changes in variables between different time points (T3-T1, T2-T1 and T3-T2) within groups with changes in the HDEL group. Histograms were used to recognize normal distributions. These analyses were conducted using SPSS 13.0 (SPSS, Chicago, Ill., USA).

We used one-way ANOVA and χ^2 tests to assess significant differences in baseline values between the diet groups. For appropriate variables, we merged subclasses of variables and then used the χ^2 test. $p < 0.05$ (two-tailed) was considered significant.

Results

The general characteristics of the four groups are shown in table 1. The mean age of obesity onset in all participants was 18.5 years. With regard to education, 44% of participants had not obtained a high school diploma, 29% had obtained a high school diploma and 27% were university graduates. Eighty-two percent of participants were housewives. Family income per month for 20% of the participants was <USD 350; for 43% it was USD 350–

Table 2. Intake of food, calorie intake and calories expended in activity before the run-in period

	Treatment group				p
	HDEL	HDEL + Arg	HDEL + Se	HDEL + Arg + Se	
Milk, servings	0.3 ± 0.6	0.4 ± 0.5	0.7 ± 0.4	0.6 ± 0.4	NS
Vegetables, servings	2.3 ± 1.2	2.1 ± 1.3	1.9 ± 1	2.1 ± 1.5	NS
Fruit, servings	1.5 ± 1.2	1.1 ± 1	1.4 ± 1	1.2 ± 0.8	NS
Meat, servings	2.8 ± 1.6	3.2 ± 1.5	3.3 ± 1.8	3.8 ± 2.1	NS
Cereal, servings	10.3 ± 3.8	10.6 ± 3.9	10.7 ± 4.1	11 ± 4	NS
Legumes, servings	0.5 ± 0.5	0.3 ± 0.3	0.5 ± 0.4	0.4 ± 0.5	NS
Sugar, servings	2.7 ± 1.1	3.2 ± 2	3.2 ± 1.9	3.4 ± 1.4	NS
Fat, servings	13.3 ± 7.6	10.6 ± 4.1	15.3 ± 7.8	14.9 ± 12.3	NS
Calorie expenditure through activity, kcal	337 ± 186	299 ± 123	440 ± 284	310 ± 233	NS
Calorie intake, kcal	2,046 ± 725	1,970 ± 615	2,262 ± 792	2,279 ± 901	NS

Values are means ± SE. NS = Not significant.

Table 3. Effect of interventions on risk factors of cardiovascular disease assessed by nested M-ANOVA for repeated measurements of a multifactor model

	Treatment								
	HDEL			HDEL + Arg			HDEL + Se		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
WC, cm	92.7 ± 1.7	89.8 ± 1.9	88.4 ± 1.8	92.2 ± 2.2	86.9 ± 2.3	84.8 ± 2.5	91.5 ± 1.8	87.9 ± 1.6	86.1 ± 1.5
Cholesterol, mg/dl	188.4 ± 7.6	197.9 ± 8.4	191 ± 10.2	187.6 ± 6.6	188.8 ± 8	193.6 ± 7.8	186.6 ± 7.2	183.8 ± 8.8	187.8 ± 9.2
LDL, mg/dl	111.6 ± 7.3	120.9 ± 7.8	117.5 ± 9.7	109.7 ± 6.7	114.8 ± 7.6	116.8 ± 8.3	109.1 ± 6.7	105.9 ± 7.8	110.9 ± 8.4
HDL, mg/dl	44.6 ± 1.2	46.2 ± 1.3	45.3 ± 1.4	45.8 ± 1.1	46.5 ± 1.1	46.9 ± 1.3	45.5 ± 1.6	46.1 ± 1.3	45 ± 1.4
TG, mg/dl	160.6 ± 13	154 ± 14.3	141 ± 13.6	160.3 ± 20.1	137.6 ± 20.6	150 ± 19.9	160.2 ± 16.8	158.9 ± 12.8	159.2 ± 14.2
hs-CRP, mg/l	2.5 ± 0.4	1.2 ± 0.3	2.2 ± 0.4	2.5 ± 0.4	2.1 ± 0.3	2.4 ± 0.3	2.5 ± 0.4	2.1 ± 0.2	2.5 ± 0.2
Insulin, μ IU/ml	18.8 ± 1.1	23.9 ± 2.3	19 ± 1.5	18.3 ± 2.9	16.3 ± 2.9	17.1 ± 3.4	18.5 ± 2.8	15 ± 1.9	15.1 ± 1.4
HOMA-IR	4.3 ± 0.3	5.6 ± 0.5	4.4 ± 0.4	4.1 ± 0.6	3.9 ± 0.7	4 ± 0.8	4.3 ± 0.7	3.4 ± 0.5	3.5 ± 0.4
TAC, μ mol/l	0.801 ± 0.044	0.833 ± 0.048	0.806 ± 0.037	0.799 ± 0.028	0.802 ± 0.028	0.794 ± 0.027	0.800 ± 0.036	0.808 ± 0.032	0.812 ± 0.030
NO _x , μ mol/l	29.3 ± 8.2	37.2 ± 9.4	33 ± 7.4	29 ± 8	27.4 ± 6.5	29.6 ± 10.5	29.1 ± 16.3	21.6 ± 14.3	21.1 ± 11.2
MDA, nmol/ml	2.2 ± 0.2	2.4 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	2 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.3 ± 0.2

Values are means ± SE. T1 = Before intervention; T2 = 3 weeks after intervention; T3 = 6 weeks after intervention; LDL = low-density lipoprotein; HDL = high-density lipoprotein; MDA = malondialdehyde.

700, and for 37% it was >USD 700. Only 9% of the participants did not have overweight first- and second-degree relatives. Eighty-two percent of participants had at least one overweight first-degree relative and 9% had at least one overweight second-degree relative. Forty-four percent of subjects did not have first- or second-degree relatives with metabolic syndrome, and 46% had at least one first-degree relative with metabolic syndrome. Mean values for the number of diets completed and weight losses were 1 and 4.8 kg, respectively, but only 11% of participants maintained their weight loss. There were no significant differences in the general characteristics of the four groups.

Food intake of the groups, calorie intake and calories expended in activities before the run-in period are shown in table 2. The mean intake of milk and fruit in all of the groups was low. There were no differences before the run-in period with respect to food intake between the groups.

The effects of interventions on CVD risk factors using nested M-ANOVA for repeated measurements of a multifactor model are outlined in table 3. There were no significant differences among basal (before intervention) measurements in the four groups (not shown in table 3).

After 6 weeks during which L-arginine and selenium was added to the HDEL, the following results were obtained by repeated measurements of M-ANOVA in a crude model and after controlling for WC (table 3): (1) HDEL significantly reduced the hs-CRP level in the first

3 weeks and returned it to basal levels in the subsequent 3 weeks ($p = 0.022$) and (2) selenium significantly reduced fasting serum concentrations of insulin ($p = 0.05$) and HOMA-IR ($p = 0.04$).

With paired t test or Wilcoxon modeling, the following results were obtained (table 4): (1) HDEL reduced TG levels at 6 weeks ($p = 0.05$); (2) HDEL increased TAC in the first 3 weeks ($p = 0.011$); (3) HDEL + Arg + Se marginally reduced TAC in the first 3 weeks ($p = 0.077$); (4) HDEL increased the NO_x concentration at 3 weeks ($p = 0.028$) and 6 weeks ($p = 0.024$); (5) Se reduced the NO_x concentration in the first 3 weeks ($p = 0.028$), and (6) HDEL + Arg + Se reduced the NO_x concentration at 6 weeks ($p = 0.017$).

With an independent t test or Mann-Whitney U test model, we obtained the following results: (1) percentage of WC reduction at 6 weeks in the HDEL + Arg group was significantly ($p = 0.008$) more than in the HDEL group, and for the HDEL + Arg + Se group it was marginally more ($p = 0.067$); (2) in the first 3 weeks, the HDEL + Arg + Se group had a significantly reduced TAC compared with that in the HDEL group ($p = 0.016$); (3) in the first 3 weeks, the HDEL + Arg group had a marginally reduced TAC compared with that in the HDEL group ($p = 0.085$); (4) in the first 3 weeks, the HDEL + Se group had a significantly reduced NO_x concentration compared with that in the HDEL group, and (4) at 6 weeks, the HDEL + Arg + Se group had a significantly reduced NO_x concentration compared with that in the HDEL group.

HDEL + ARG + Se			Ptime	PSe	PArg	PArg + Se
T1	T2	T3				
91.9 ± 2.3	87.1 ± 2.5	85.9 ± 2.7	0.000	0.938	0.609	0.791
187.2 ± 7	183.2 ± 6.3	185.4 ± 5.6	0.928	0.488	0.931	0.928
110.5 ± 6.3	110 ± 5.1	111.3 ± 3.1	0.703	0.397	0.999	0.884
44.6 ± 1.6	44.2 ± 1.5	43.2 ± 1.9	0.742	0.388	0.938	0.396
160.5 ± 17.4	144.8 ± 18	154.2 ± 15.2	0.512	0.869	0.789	0.974
2.5 ± .5	2.1 ± .2	2.4 ± .4	0.023	0.533	0.621	0.481
18 ± 2.2	15 ± 1.5	16.7 ± 1.4	0.620	0.05	0.336	0.251
4.1 ± .5	3.5 ± 0.3	3.8 ± .3	0.748	0.040	0.461	0.312
0.805 ± 0.033	0.769 ± 0.036	0.782 ± 0.025	0.982	0.860	0.674	0.992
29.4 ± 14.2	29.3 ± 12.9	23.5 ± 13.5	0.74	0.47	0.89	0.85
2.3 ± 0.1	2.4 ± 0.2	2.5 ± 0.2	0.736	0.410	0.973	0.494

Table 4. Effect of interventions on risk factors for CVD in the four treatment groups by paired t test or Wilcoxon analyses

	Intervention											
	HDEL			HDEL + Arg			HDEL + Se			HDEL + Arg + Se		
	PT2-T1	PT3-T2	PT3-T1	PT2-T1	PT3-T2	PT3-T1	PT3-T1	PT3-T2	PT2-T1	PT3-T1	PT3-T2	PT2-T1
WC	0.000 ↓ (3.1 ± 0.5)	0.003 ↓ (1.5 ± 0.4)	0.000 ↓ (4.6 ± 0.5)	0.000 ↓ (5.4 ± 0.6)	0.010 ↓ (2.2 ± 0.8)	0.000 ↓ (7.6 ± 1)	0.000 ↓ (3.6 ± 0.5)	0.000 ↓ (1.8 ± 0.4)	0.000 ↓ (5.5 ± 0.6)	0.000 ↓ (5.1 ± 0.7)	0.046 ↓ (1.3 ± 0.6)	0.000 ↓ (6.4 ± 1)
Cholesterol										0.090 ↑ (3.5 ± 21)		
LDL	0.076 ↑ (14 ± 38)			0.053 ↑ (5 ± 11)								
HDL												
TG	0.009 ↓ (9 ± 13)		0.055 ↓ (12 ± 17)	0.001 ↓ (19 ± 19)	0.088 ↑ (14 ± 32)							0.084 ↓ (5 ± 19)
hs-CRP	0.018 ↓ (19 ± 73)	0.000 ↑ (59 ± 105)										
Insulin	0.039 ↑ (31 ± 49)						0.076 ↓ (10 ± 46)		0.093 ↓ (9 ± 30)			
HOMA-IR	0.002 ↑ (35 ± 41)	0.031 ↓ (29 ± 76)										
TAC	0.011 ↑ (4 ± 6)									0.077 ↓ (4 ± 14)		
NO _x	0.028 ↑ (15 ± 32)		0.024 ↑ (9 ± 14)					0.028 ↓ (4 ± 50)		0.055 ↓ (18 ± 76)		0.017 ↓ (35 ± 55)
MDA	0.068 ↑ (7 ± 17)											

Values in parentheses represent the percentage change in each parameter, shown as the mean ± SE. T1 = Before intervention; T2 = 3 weeks after intervention; T3 = 6 weeks after intervention; LDL= low-density lipoprotein; HDL = high-density lipoprotein; MDA= malondialdehyde.

Discussion

HDEL significantly reduced the hs-CRP level in the first 3 weeks and returned it to basal levels in the subsequent 3 weeks; adding L-arginine and/or selenium did not change the effect of HDEL. The effects of HDEL on hs-CRP levels were shown using nested M-ANOVA for repeated measurements in a multifactor model. This statistical model did not compare the two groups to each other but simultaneously compared the effect of all treatments by variance analysis and could be used to separate the effects of all treatments.

Inflammation has an essential role in atherosclerosis [29]. hs-CRP is an independent risk factor for CVD and a strong indicator of chronic inflammation [30]. Cytokines in adipose tissue stimulate CRP production, so there is a strong correlation between central obesity and the serum concentration of hs-CRP [31]. Clifton [32] showed that weight reduction reduced hs-CRP concentrations and that the macronutrient composition of diets

had no effect upon it. O'Brien et al. [33] and Selvin et al. [31] found similar results. However, Belza et al. [34] had different results compared with the results of the present study. In their study (which lasted 20 weeks), participants consumed 800-kcal diets for 8 weeks. At the end of 8 weeks, the weight of participants was reduced by 13% (13.7 kg), but a reduction in hs-CRP levels was not observed. Investigators then prescribed a maintenance diet for 4 weeks and followed it with a 1,000-kcal hypocaloric diet for a further 4 weeks. At the end of the second hypocaloric diet, participants showed an additional reduction in weight of 2.9 kg and also a 35% reduction in basal hs-CRP levels. These reductions were maintained during a second maintenance period which lasted 4 weeks [34]. Hence, if body fat mass decreases, hs-CRP will be reduced, but these reductions will occur when a new steady state is obtained. In the present study, one reason for this reduction in hs-CRP level in the first half of the study could have been body fat loss without an increase in plasma levels of free fatty acids. In the second half of the

study, loss of body fat was probably accompanied by enhancement of plasma levels of free fatty acids, which would have resulted in the production of cytokines and other acute-phase reactants in the liver. This cascade probably masked the beneficial effects of the loss of body fat upon reductions in hs-CRP levels in the second half of the study.

HDEL reduced TG levels, increased NO_x levels and increased TAC, but adding L-arginine and/or selenium eliminated the beneficial effect of HDEL. It is not surprising that L-arginine (the substrate for the oxidant NO) eliminated the beneficial effects of HDEL on TAC or that selenium (an antioxidant) stopped enhancement of NO_x levels, but why did selenium reduce TAC and L-arginine lower NO_x levels?

Because of the essential roles of antioxidants in health, measurement of TAC is very common [35]. One antioxidant is selenium, which has been used in a 'selenoprotein' form [36]. Various studies have shown that selenium consumption increases selenoprotein levels in a dose-dependent manner [37]. There are two classes of antioxidants: (1) nonenzymatic antioxidants (which have a low molecular weight) and (2) enzymatic antioxidants such as selenoproteins [38]. TAC measures only low-molecular-weight antioxidants, and selenoproteins do not have a role in TAC [39]. Hence, selenium could not increase TAC. It is likely that selenium interacts with other antioxidant nutrients in legumes [40]. Antioxidants in legumes that cannot interact with selenium can increase TAC. In selenium metabolism, glutathione (as an antioxidant) is used for reducing selenoamino acids, selenite and selenate [41]. Also, studies have shown that selenium has prooxidant effects and catalyses the oxidation of thiols and simultaneously produces superoxide [42–44].

Intake of large amounts of L-arginine suppresses the production of citrulline by glutamine, glutamate and proline in the intestine. Arginine immediately enters the circulation and some of it is converted to NO. However, citrulline is gradually converted to arginine in the kidneys and, instead of instantly increasing the NO concentration, increases it gradually [45]. This mechanism probably caused the increase in the fasting NO_x concentration in the HDEL group but not in the HDEL + Arg group.

Cheung et al. [46] showed that antioxidants (including selenium) tended to increase levels of very-low-density lipoproteins (TG-rich lipoproteins) in serum. Subjects who routinely used supplements of vitamin A and β-carotene showed an increase in serum levels of TG [47, 48]. Niacin in legumes is one agent that can reduce TG levels. Niacin affects the expression of some of the proteins in-

involved in lipid metabolism, and selenium supplements can interfere with it [46].

The beneficial effects of legumes on TG levels are derived from the fibers, niacin and certain amino acid compositions of the proteins of legumes [46, 49–51]. Addition of L-arginine could have caused disturbances in the composition of legume proteins, and the beneficial effects on TG levels mentioned above could have been eliminated.

The percentage reduction in WC in 6 weeks in the HDEL + Arg group was significantly more than in the HDEL group, and for the HDEL + Arg + Se group it was marginally more (*p* = 0.067). These findings have been discussed elsewhere [52]. In addition, interpretation of the effect of selenium upon reducing fasting concentrations of serum insulin and HOMA-IR has been evaluated (unpublished data).

Compared with HDEL, HDEL + Arg significantly reduced TAC in the first half of the study, and HDEL + Arg + Se marginally reduced it. As mentioned above, selenium cannot increase TAC. However, in some studies, L-arginine increased the production of oxidant radicals [53, 54]. Hence, the mean effect of L-arginine and selenium on TAC in the present study was reduction. In certain conditions, eNOS may cause an imbalance between the levels of oxidants and antioxidants. It has been shown that impaired eNOS can be an important source of endothelial O²⁻ in subjects with hypertension or type 2 diabetes mellitus [53, 55]. If eNOS is exposed to oxidative stress (e.g. to ONOO⁻) or if there is a deficiency in the BH₄ cofactor, eNOS produces O²⁻ instead of NO [56, 57]. In our participants, it is not clear whether impaired eNOS or a deficiency in the BH₄ cofactor caused TAC reduction. The answer to this question may be based on molecular studies. Consistent with other studies [58–60], selenium did not affect the serum MDA concentration in the present study.

In the two groups in which participants were supplemented with selenium (HDEL + Se and HDEL + Arg + Se groups), the serum concentration of NO_x was reduced. In most human studies [61–65], supplementation with L-arginine did not affect serum NO_x concentrations. Studies in which L-arginine was found to increase serum NO_x concentrations had one of the following criteria: (1) intravenous administration of L-arginine [66]; (2) oral supplementation of L-arginine in patients with peripheral atherosclerosis [67], or (3) measurement of the incremental area of NO_x concentration 1–10 min after L-arginine administration [7]. However, selenium supplementation can inhibit NOS [68], reduce iNOS gene expression [69] and thus inhibit NO production.

This was the first time that the simultaneous effects of a hypocaloric diet, L-arginine and selenium on CVD risk factors were studied. The advantage of our study was the statistical modeling. Even though each group had 17 cases, with nested M-ANOVA for repeated measurements of a multifactor model, simultaneous analyses were used on 68 cases.

The present study had several limitations. Firstly, estimation of body fat with advanced procedures was not possible. Secondly, serum concentrations of L-arginine and selenium were not measured. Thirdly, use of a basic hypocaloric diet affected the net effect of treatments. Fourthly, the side effects of legume consumption were not evaluated with questionnaires. Fifthly, we could not explain the causes of poor compliance. Lastly, we could not provide food to the participants.

We concluded that supplementation with L-arginine significantly reduced WC; selenium supplementation significantly lowered fasting concentrations of serum insulin and HOMA-IR; interactions between L-arginine and selenium significantly reduced fasting concentrations of NO_x, and HDEL significantly lowered TG levels

and WC and increased fasting concentrations of NO_x. Hence, except for WC, insulin levels and the HOMA-IR index, adding L-arginine and/or selenium not only could not improve the beneficial effects of HDEL on the other risk factors for CVD but also worsened them. Long-term studies are necessary to validate the results of the present study.

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Disclosure Statement

None of the authors had any personal or financial conflicts of interest.

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