Benefits of Semelil (ANGIPARS™) on oxidant-antioxidant balance in diabetic patients; A randomized, double-blind placebo controlled clinical trial

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Received 26 May 2009; Revised 31 Oct 2009; Accepted 4 Nov 2009

ABSTRACT

Background and the purpose of the study: Diabetes mellitus is one of the most common chronic diseases in the world with dreadful complications which not only is debilating for the patients but also puts a big burden on the health system.
One of the mechanisms by which the complications of diabetes occur is imbalance in the oxidant-antioxidant equilibrium in the body and therefore many studies have been performed to either correct this equilibrium or delay the occurrence of the complications.
Methods:In this randomized, double-blind placebo-controlled clinical trial, a total number of 61 subjects were divided into two groups and the antioxidant effects of a novel herbal drug, Semelil,was compared with placebo. Baseline laboratory tests including a complete blood count, fasting blood sugar,lipid profile,fasting plasma insulin, liver and renal function tests plus tumor necrotizing factor α (TNFα) and C-reactive protein (CRP) and homocystein were performed. Total antioxidant power assay, cellular lipid peroxidation assay, and nuclear damage (deoxyguanosine) and carbonly molecules were also measured to help determination of state of antioxidants- oxidants equilibrium in serum.
Result:Apart from deoxyguanosine, no significant change was found in TNFα or CRP levels in the studied group who received Semelil.
Conclusion: Mechanisms other than antioxidant effects are involved for Semelil in the treatment of diabetic foot ulcers.

Keywords: Diabetes Mellitus, Samelil, ANGIPARS™, Oxidant-antioxidant Balance

INTRODUCTION

Diabetes mellitus is one the most common metabolic diseases around the world. Diabetes mellitus type 2 or non insulin dependent diabetes is a multi factorial disease which results in impaired glucose homeostasis. In insulin dependent and independent diabetes oxidative stress is elevated significantly (1). Oxidative stress is caused by an imbalance between production of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. The relationship between uncontrolled blood glucose levels and oxidative stress is well established (2). Different factors have been related to higher production of free radicals in diabetes such as hyperglycemia which acts by activating oxidative stress related pathways. There is also a relationship between homocysteine levels and lipid peroxidation and oxidative stress in diabetes mellitus. Homocysteine has an oxidative-pathway-related role in vascular damage and also low levels of glutathione and superoxide dismutase have been observed in diabetic patients (3,4). The effects of oxidative stress pathway activation and inflammation can be measured by the increases in the systolic and diastolic blood pressure, increased C reactive protein and WBC and the change in lipid profile and other oxidative stress markers. Inflammatory cytokine such as TNFα play a role in insulin resistance and elevating vascular inflammatory response (5). Different mechanisms have been proposed as a way to reduce free radicals and oxidative stress (6). Reducing oxidative stress in direct and indirect ways have been the goal of many studies in order to reduce oxidative stress markers and lipid peroxidation in diabetic patients. Daily workouts and weight loss can help balance the oxidants-antioxidants equilibrium in the body (7). With a great interest in nonsynthetic and natural resources for antioxidants in recent studies, it is believed that such drugs could help to improve treatment of diabetic patients. Coumarins and flavonoids have been studied and it has been shown

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that they have antioxidant effects (8). In this study the antioxidant effects of a new herbal drug, Samelil (ANGIPARS™), which is derived from a plant named *Melilotus Officinalis* which contain compounds such as 7 Hydroxy coumarin and Flavonoids was investigated. ANGIPARS™ has been proven as a safe and effective drug in different clinical trials and useful in healing diabetic foot ulcers (9).

MATERIALS AND METHODS

Study Design

In this randomized, double-blind placebo-controlled clinical trial, a total number of 61 subjects were selected from patients with previously confirmed and documented type 2 diabetes according to the standard world health organization criteria, who were referred to Shariati Hospital Diabetes Clinic over a period of 9 month (Aug 2008-May 2009).

After the selection of the patients to enroll in the study, all participants were fully informed of the nature of the study and provided a written informed consent. The study protocol was approved by the ethics committee of Tehran university of Medical Sciences.

Inclusion and Exclusion Criteria

The following inclusion criteria were applied for selection of the patients eligible to take part in the study:

Male and females aged between 20-60, confirmed type 2 diabetes mellitus under therapy with oral anti-hypoglycemic agents, no recent infections or other inflammatory process during the last 4-6 weeks. The exclusion criteria consisted of patients with proliferative retinopathy, significant renal impairment (serum creatinine> 270 μmol/l), coronary artery disease, chronic liver disease, diabetic foot ulceration and gangrene, pulmonary infection, smoking, pregnancy, age less than 20 or over 60 years, periodontal disease, and supplementation with multivitamins or traditional herbs in the previous 4-6 weeks and not filling the informed consent.

Assessment of the patients

During the primary assessment, a detailed past medical history of the patients was taken which stressed on duration of diabetes mellitus and drug allergies. A comprehensive thorough physical examination of the patients was carried out by trained physicians searching for any previous ulcers or sites of infections or other complications of diabetes.

The participants were informed of the possible effects of taking any multivitamin supplements or traditional herbs during the study which may have on the results and were asked not to use them during the course of the study, and also were asked not to engage in any heavy exercise and not to change their routine life style and diet during the study.

Treatment Protocols

Following selection of the qualified patients to take part in the study, the subjects were randomly alienated into groups A (n=31) and B (n=30). Before administration of ANGIPARS™ or Placebo, one fasting blood sample (after fasting for over 12 hrs) of approximately 15 ml, was taken from every eligible participant in both groups A and B. Blood samples were drawn into trace mineral–free plastic tubes containing EDTA. The blood samples were centrifuged within 3 hrs of sampling at 3000 *g* for 20 min to obtain serum. Serum samples were frozen at –80 °C and were immediately sent to the Endocrinology and Metabolism Research Center (EMRC) and Pharmaceutical Sciences Research Center (PSRC) laboratories for further analyses. Baseline laboratory tests including a complete blood count, fasting blood sugar, HbA1C, insulin, lipid profile, liver and renal function, TNFα, CRP, and homocystein were performed on serum samples. Total antioxidant power assay, lipid peroxidation assay, deoxyguanosine and carbonyl containing molecules assays in serum were also performed.

ELISA was used to measure fasting plasma insulin using Monobind kits. Homocysteine measurements were performed by HPLC assay. ELISA (Cayman Chemical Co. kit) was used to determine TNFα levels. Homeostasis model assessment estimate of insulin resistance (HOMA-IR) was calculated using the standard formula (10). In the lipid peroxidation assay, deoxyguanosine and carbonyl containing molecules assays, kits from Cayman Chemical Co. were used. After baseline laboratory tests, patients of the group A received 100 mg of ANGIPARS™ capsules twice a day and patient of the group B were administered 100 mg placebo capsules (a nonabsorbable polymer) for 12 weeks (both produced by Pars Roos Co., Tehran). The patients were visited biweekly and evaluated for possible side effects. Assessment of likely adverse drug reactions were documented by physicians in every visit. After therapy for 12 weeks, another set of blood samples was taken in both group as previously stated and all hematological, biochemical, and oxidative stress parameters were evaluated again.

2.1 Total antioxidant power assay

Antioxidant power of plasma was determined by measurement of their abilities to reduce Fe^{3+} to Fe^{2+} established as named ferric reducing antioxidant potential (FRAP) (11). In this test, the medium is exposed to Fe^{3+} and the antioxidants present in medium start to produce Fe^{2+} as a criteria of their antioxidant activity. The reagent included 300 mmol/l acetate buffer, pH 3.6 and 16 ml $C_2H_4O_2$ per liter of buffer solution, 10 mmol/l TPTZ in 40 mmol/l HCl, 20 mmol/l $FeCl_3$ $6H_2O$. Working FRAP reagent was prepared as required by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3$ $6H_2O$ solution. Ten microliters of H_2O-diluted

sample was then added to 300 μL of the freshly prepared reagent warmed at 37 °C. The complex between Fe^{2+} and TPTZ gives a blue color with absorbance at 593 nm.

2.2 Lipid peroxidation assay

Malonedialdehyde (MDA) is the end product of the oxidation of polyunsaturated fatty acids and its concentration in the medium is an established measure of lipid peroxidation extent. In this test, the reaction of MDA with thiobarbituric acid (TBA) makes a complex which is determined spectrophotometrically and lipid peroxidation in samples are assessed in terms of produced thiobarbituric acid reactive substances (TBARS) (12). Briefly, the samples were diluted by buffered saline (1:5) and 800 ml of trichloroacetic acid (TCA, 28% w/v) was added to 400 μL of this mixture and centrifuged at 3000 × g for 30 min. Then, 600 ml of the supernatant was added to 150 μL of TBA (1% w/v). The mixture was incubated for 15 min in a boiling water bath then treated with, 4 ml of n-butanol, the solution was centrifuged, cooled and absorption of the supernatant was recorded at 532 nm by spectrophotometer (Japan).

2.3 Carbonyl group

Carbonyl content was determined by taking the spectra of the representative samples at 355-390 nm). The carbonyl content was calculated from peak absorption (370 nm) using an absorption coefficient (e) of 22,000 M^{-1} Cm^{-1}. The carbonyl containing molecules were expressed as nmol/mg protein. The protein content was determined by the Lowry method using BSA as standard (13).

2.4 Deoxyguanosine

8-Hydroxy-2-deoxyguanosine (8-OHdG) is a commonly used biomarker to assess oxidative lesions to DNA. ELISA tests provide a fast and simple method to measure this adduct. Determination of 8-OHdG in serum could allow an integrated measure of damage and repair (14).

Data Analyses

Statistical analysis of the results were performed using SPSS 14 software and data were presented as means ±standard deviation (SD). Unpaired 2-sample Student's *t* test for continuous data and chi-square tests for categorical data was used. Paired *t* test was applied to compare before and after intervention. P-values< 0.05 were considered statistically significant. After selection of the patients to enroll in the study all participants were fully informed of the nature of the study and provided a written informed consent. The study protocol was approved by the ethics committee of Tehran University of Medical Sciences.

RESULTS

Basic Characteristics

A total number of 61 patients were enrolled in this study. Of which 31 patients (50.8%) were in group A who received ANGIPARS™ and 30 patients (49.2%) were in group B who received placebo. Female participants in group A were 80% as compared to 76.7% in group B. As shown in table 1, the basic characteristic of the participants such as age, BMI and weight were similar at the beginning of the study in both groups A and B.

Laboratory Parameters

Hematologic parameters, hemoglobin, platelet and WBC and other biochemical parameters plus TNF-α, HS-CRP, insulin and HOMA-IR before and after 3 month of drug or placebo administration are shown in table 2. At the beginning of this study there was only a statistically significant difference between triglyceride levels in groups A and B (p= 0.04). After 3 month of treatment in group A who received ANGIPARS™ and placebo, there was a significant fall in WBC (p=0.000). The same pattern was observed in fasting plasma nsulin and HOMA-IR. There was a rise in AST (p= 0.006) in the control group B who received placebo and a statistically significant decrease in triglyceride (p= 0.044). Homocystein was surprisingly higher in group A as compared to group B.

Table 3 shows the oxidative stress parameters before and after the drug and placebo administrations. There was no statistically significant difference in oxidative stress parameters between two groups before the study. Following the course of the study, there was a statistically significant fall in deoxyguanosin in group A who received ANGIPARS™ (p= 0.05) but no change was observed in TBARS or FRAP levels.

DISCUSSION

Type 2 diabetes is characterized as a chronic, progressive disease with insulin resistance and insulin deficiency. The production of free radicals or defect in scavenging abilities of endogenous antioxidants cause a condition known as oxidative stress that result in macro- and micro vascular complications in diabetes (15).

Various antioxidants have been in the center of many studies and have or have not been proven useful in improvement of oxidative stress. For example, vitamin E, in supra-antioxidant doses have been reported useful in normalizing oxidative stress and vascular dysfunctions (16).

Most of the clinical trials which have been preformed previously were short in duration, had few participants, and used early surrogate markers, and in contrast to culture and animal studies, clinical trials have shown conflicting and confusing results. In a large study named "Heart Outcomes Prevention Evaluation", in which vitamin E was used, no microvascular benefit was observed in more than 3000 patients with diabetes (17).

In this study, it was found that ANGIPARS™ lowers levels of deoxyguanosin after administration for 3

Table 1: Baseline characteristics of subjects in mean ±SD Group A=Diabetic patients on drug.

	Group A	Group B
Variable		
Age	51.9 ±6.2	51.4±5.4
BMI(kg/m^2)	28.23±3.97	29.98±5.32
Baseline weight(Kg)	73.7±11.7	78.3±13.8
Length of diabetes	7.6±4.8	10.7±9.7

Group B=Control group BMI=Body Mass Index

Table 2: Mean Levels of biochemical parameters plus Homocystein* Insulin levels and HOMA-IR before and after treatment in drug (A) and placebo(B) groups.

	Group A			Group B		
Variable	Pre-Treatment	After 12 weeks	P value	Pre-Treatment	After 12 weeks	P value
WBC	7.62±1.07	6.13±1.23	0.00	8.11±0.84	6.33±1.44	0.00
Hgb(g/dl)	13.1±1.6	13.2±1.5	0.63	12.6±1.5	12.9±1.4	0.26
Platelet	266.96±59.29	268.39±57.16	0.86	243.48±51.13	258.82±56.35	0.24
Triglyceride(mg/dl)	178±76.7	126±45.4	0.00	148.3±72.6	123.5±53.4	0.09
HBA1C(%)	8.15±1.37	7.73±1.68	0.15	7.4±1.47	7.14±1.2	0.30
FBS(mg/dl)	156±41	156±47	0.94	155.7±47.8	146.6±46.5	0.34
Cholesterol(mg/dl)	181.4±41.3	179.8±49.2	0.82	157.7±46.6	162.2±35.4	0.52
Creatinine	0.9±0.1	0.9±0.1	0.08	0.9±0.1	1.0±0.1	0.22
ALT (IU/L)	21.3±15.2	20.7±12.2	0.84	17.5±6.9	18.1±6.4	0.71
AST (IU/L)	21.4±13.3	19.8±6.7	0.48	17.5±5.3	20.4±6.3	0.00
TNFα*(pg/ml)	88.66±1.48	83.1±1.5	0.28	87.04±1.28	77.75±1.38	0.16
CRP(mg/L)	2.26±1.74	3.31±3.59	0.07	3.57±3.21	3.24±3.55	0.42
Homocystein*(µmol/L)	8.85±1.35	10.05±1.44	0.00	10.25±1.64	11.29±1.55	0.13
Insulin(Miu/ml)	12±4.6	3.7±1.8	0.00	16.1±9.0	6.8±7.1	0.00
HOMA-IR	2.4±2.7	1.5±1.1	0.00	6.6±4.4	2.4±2.7	0.00

*Geographic mean ± SD:for parameters have no normal distribution curve was shown.
FBS=Fasting blood sugar

Table 3: Mean oxidative stress parameters before and after treatment in drug (A) and placebo(B) groups.

	Group A			Group B		
Variable	Pre-Treatment	After 12 weeks	P value	Pre-Treatment	After 12 weeks	P value
TBARS*(nmol/ml)	3.1±2.51	2.27±1.62	0.15	4.67±1.99	2.47±1.76	0.00
FRAP(nmol/ml)	838.4±227.46	897.3±242.01	0.13	763.8±171.08	870.34±190.52	0.00
DeoxyGuanosin(pg/ml)	545.38±70.36	495.18±93.39	0.05	574.52±81.54	512.04±86.43	0.21
Carbonyl Group(nml/ml)	0.038±0.00	0.043±0.00	0.15	0.04±0.01	0.031±0.01	0.33

* Geographic mean ± SD:for parameters have no normal distribution curve was shown.
TBARS= Thiobarbituric acid reactive substances, FRAP= fluorescence recovery after photobleaching

month ($p<0.05$) which is an oxidative stress marker in diabetic patients (18). ANGIPARS™ is a novel effective drug in treatment of diabetic foot ulcers which had no acute or chronic toxicity in preclinical and clinical studies (19-23).

Melilotus Officinalis has been proved to be helpful in reducing inflammation, regulation of the immune system and improvement of vascular blood flow which is thought to be due to the inhibitory effect of natural coumarins on oxidative DNA damage and formation (24,25). Similar results in lowering oxidative stress with coumarins in diabetic rats have been reported (26). No previously human or animal study has been published on antioxidative stress characteristics of ANGIPARS ™.

Although ANGIPARS™ did lower deoxyguanosine but no significant change was observed in other oxidative stress markers such as FRAP, TBARS or carbonyl containing molecules. A rise in FRAP or reduction in TBARS was observed in the control group who received placebo which could be explained by oxidative stress changes depending on many factors such as exercise, diet, or a reflex of placebo effect, etc. There was also a rise in homocystein level in group A as compared to the placebo group which again could be due to other factors that were mentioned above and all could affect the final oxidant-antioxidant equilibrium.

Apart from our specific oxidative stress markers, no significant change was observed in TNFα or CRP levels in the studied group who received ANGIPARS™ as compared to the control group.

Other factors affecting oxidative stress have been previously studied. Hyperhomocysteinaemia in patients with type 1 diabetes could reduce antioxidant defense in patients with diabetes. However homocystein itself is altered by many drugs (27, 10). Allopurinol has been reported to have the potential of reducing plasma ROS and also reducing plasma antioxidant enzymes in comparison to placebo group but many conflicting results have been reported (28, 29). Pentoxifylline has also reduced lipid peroxidation in type 2 diabetic patients, but not changed HbA1c significantly (30). ANGIPARS™ is a herbal extract which has passed all necessary steps of regulatory tests as a novel treatment for diabetic foot ulcers, with a possible mechanism of angiogenesis (31). In this study it was found that although ANGIPARS™ is a very effective drug in treatment of diabetic foot ulcers but the mechanism by which its effect is taken place is other than its antioxidant effects. Patients were monitored closely for any adverse drug reactions but no systemic or localized drug reactions or adverse effects were reported.

Limitations of this study are small sample size and short length of the study and not controlling different factors such as diet and exercise. This study is being continued for another 6 month to find out whether it gives better results.

ACKNOWLEDGEMENT

The authors would like to thank all the staff of the Endocrinology and Metabolic Research Center and Pharmaceutical Sciences Research Center and all the individuals who took part in this study. Authors also thank ParsRoos Company that provided ANGIPARS™ and placebo.

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