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Effect of *Nigella sativa* oil extract on inflammatory cytokine response and oxidative stress among people with type 2 diabetes mellitus: a randomized, double-blind, placebo controlled trial

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Summary. *Objectives:* Diabetes mellitus (DM) is the most common chronic disease and a challenging global health problem. Inflammation plays a key role in the pathogenesis of DM. The experimental studies have shown that *Nigella sativa* (*N. sativa*) oil extract is a potential anti-inflammatory and anti-oxidant substance. The present study was conducted to evaluate the anti-inflammatory and antioxidant effects of *N. sativa* among adults diagnosed with type 2 diabetes (T2D). *Methods:* A double-blind placebo controlled trial was designed. A total of 43 participants were assigned into either intervention (N=23) or control (N=20) conditions. The intervention group received 500 mg *N. sativa* capsules twice a day, while control group received identical amounts of placebo capsules twice a day for 8 weeks. Dietary intake, physical activity, anthropometric indices, and fasting blood samples were measured at baseline and one week post-intervention. *Results:* Findings revealed significant reductions in malondialdehyde (MDA) and nitric oxide (NO) within the intervention condition, although between-group analysis did not show any significant changes neither for MAD nor NO. Besides, within- and between-group analyses did not show any significant differences pre- and post-intervention for interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α), superoxide dismutase (SOD), and catalase (CAT). *Conclusions:* The current study revealed favorable effects for *N. sativa* oil extract in reducing MDA and NO among people with T2D. However, future research is needed to establish potential anti-inflammatory and anti-oxidative responses of *N. sativa* in diabetes.

Key words: Type 2 diabetes mellitus, inflammation, *Nigella sativa*, oxidative stress

Introduction

Diabetes mellitus (DM) is a global challenge, a chronic condition with active inflammatory state. Because DM leads to multiple complications, such as blindness, kidney failure, amputations, heart attacks, and strokes, it is considered as a devastating illness (1, 2).

The Etiology of diabetes is unknown, but it is generally believed that diet, psychological stress, obesity, physical inactivity, genetic propensity, immunological factors and oxidative stress have participated in occurrence of this disease (3-6). Moreover, T lymphocytes, B lymphocytes, macrophages, and monocytes may play a key role in the pathogenesis of DM by producing various inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-B (IL-β), and interleukin 6 (IL-6) (7, 8). TNF-α triggers cytokine cascade activation via NF-*κB* signaling pathway contributing to the inflammatory disease processes (9). Nitric oxide (NO), IL-1β, and TNF-α have been implicated

as immune effector molecules that mediate B-cell dysfunction associated with both type of diabetes (10-12). Also, oxidative stress has been implicated in the pathogenesis of type 2 diabetes (T2D) (7). However, severe oxidative stress possibly cause cell death by damaging cellular lipids, proteins, and DNA particularly leading to autoimmune diseases. Imbalance between free radicals and antioxidant defense is also a highlighted feature of many acute and chronic diseases. Under the oxidative stress conditions, an endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT), and glutathione may be failing to counter reactive oxygen species (ROS) (13). Biomarkers of oxidative stress have been widely used to assess the relation between oxidative damage to macromolecules (lipids, DNA, proteins) and disease progression (14). Elevated serum malondialdehyde (MDA), a biomarkers of lipid peroxidation, and decreased activities of antioxidants, including CAT, SOD and glutathione peroxidase have been reported in DM patients compared to healthy individuals (15).

Nigella sativa (N.sativa), commonly known as black cumin or black seed belongs to the plant family of Ranunculaceae, has long been used in traditional medicine for treating various conditions (15).

The most of the therapeutic properties of this plant are due to the presence of thymoquinone (TQ) which is the main component of the essential oil (7). Pharmacology studies showed that oil and TQ of black seeds have anti-inflammatory and anti-immunologic benefits (16). TQ compromises the maturation, cytokine release and survival of dendritic cells (17). Also, one animal study reported that TQ reduced the levels of pro-inflammatory cytokine and pro-oxidant molecules and increased the level of antioxidant mediators (18). The aim of present randomized clinical trial was to investigate the effect of N.sativa on the inflammatory cytokines and oxidative stress among patients with T2D.

Materials and methods

Study design and Subjects

A randomized double-blinded placebo-controlled clinical trial was designed. The study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences and the trial was registered in the Iranian Registry of Clinical Trials (IRCT201307221640N1).

Sample size was determined based on the primary information obtained from a study by Shah et al. in T2D (19). For a significance alpha value of 0.05 and 80% of power, the sample size was calculated as 40 in total. To compensate for attrition during the study, 50 participants were selected. The participants were randomly allocated into two groups using a block randomization procedure (of size 4), when adjusted for sex, age and BMI in each block. Participants were recruited from the Iranian Diabetes Association (IDS), Tehran, Iran, May to Nov 2014. Finally, 50 T2D patients who met the inclusion criteria were invited and provided with written informed consent.

Participants were considered having diabetes if fasting blood glucose (FBG) was≥ 126mg/dl or they were using oral glucose-lowering agents. Eligible participants were clinically diagnosed with T2D, had body mass index (BMI) of 25–35 kg/m^2, aged between 30–60 years, were non-smokers, not currently receiving insulin therapy without history of diseases including liver, renal and thyroid disorders.

The intervention participants received two 500 mg N.sativa capsules per day for 8 weeks while identical amounts of placebo capsules were given to controls (Barijessence co., Kashan, Iran). Daily doses of N.sativa and duration of intervention are based on previous studies (20, 21). For concealment purposes, N.sativa oil extract or placebo bottles were encoded by external researcher not involved in the study.

At baseline and at post-intervention, weight, height, demographic information (age, job, disease history, medication, marital status, etc), physical activity (IPAQ questionnaire), and psychological stress (State-Trait Anxiety Inventory Y form [STAI-Y] questionnaire) were assessed. Moreover, food intake was evaluated by 3-day dietary records (24-hour diet recall) at pre- and one weeks post-intervention. Pro-inflammatory cytokine (TNF-α and IL-1B), antioxidant biomarkers (SOD and CAT) and pro-oxidant biomarkers (NO and MDA) were also measured.

To establish compliance with treatment, unused capsules which previously participants were asked to

leave them in their bottles counted at the end of the trial. Patients were monitored via phone calls every 15 days for any least probable adverse events.

Blood sampling

After 10-12 hours of overnight fasting, 10 ml venous blood samples were drawn which were then centrifuged for 15 minutes at 1500 g to obtain serum. Serum samples were stored at -20°C until biochemical analysis is performed.

Biochemical assays

Cytokines

Serum levels of TNF-α and IL-1B were determined by commercially available cytokine ELISA kits (Diaclone Besancon, France) following instructions of the manufacturers at 450 nM wavelength in an ELISA plate reader apparatus (Awareness, Statfax-2100 model, USA).

Antioxidant defense system (SOD and Catalase)

SOD activity was measured by Spectrophotometric method using a Ransod kit (Randox Laboratory, UK). CAT activity was measured by the Aebi method (Aebi et al., 1984). All tests were performed using automatic analyzer (Abbott model Alcyon 300, USA).

Oxidative stress

Serum MDA and NO concentrations were assessed with colorimetric method by Cayman American Company and Active Motif Japan Company kits, respectively using the Selectra 2 auto-analyzer.

Statistical analysis

The data were analyzed using SPSS version 16 (SPSS Inc., Chicago, IL, USA). Two-way p-values of <0.05 were set as significant. The quantitative and qualitative data are presented as mean (SD) and median (25th and 75th percentiles), respectively. The normality of variables and homogeneity of variances were tested using the Kolmogorov–Smirnov and Leven test, respectively. Comparisons for demographic characteristics were tested by Chi-square. Baseline differences were assessed by independent t-test or Mann–Whitney test. Mean values at pre- and post-intervention within groups were compared using the paired t-test or Wilcoxon test. Analysis of covariance (ANCOVA) was used to examine the treatment effects between two groups for dependent measures after adjusting for the baseline variables and covariates (i.e., changes in BMI and state and trait anxiety scores throughout the study as well as menopausal status).

Result

Initially, 50 women with T2D were recruited, while 43 women completed the study. Eight percent (8%) of the N.sativa group and 20% of placebo group did not complete the 8-week treatment course. No severe adverse effects for the treatment were reported.

Baseline characteristics

Baseline characteristics of the patients are presented in Table 1. No baseline differences were observed between the two groups (p>0.05). At the end of the study, there was no statistically significant differences in BMI values between or within groups. Further, there were no baseline differences for type and dosage of medications between the two groups. Baseline STAI-Y scores and physical activity level showed no significant differences between groups.

Dietary Intake

Table 3 shows the results of energy and macronutrient intakes at baseline and post-intervention. No significant between-group and within-group differences were observed.

Biochemical data

As presented in the Table 2, SOD and CAT was significantly increased within the intervention group (P < 0.01). Also, MDA was significantly decreased within the intervention group (P < 0.05). ANCOVA did not show any significant differences in the serum levels of CAT and MDA (p> 0.05). SOD differed significantly between the two groups either at baseline or at the end (P> 0.05). Moreover, the study revealed no significant within- and between-group changes in levels of TNF-αIL-1B and NO following the intervention (p> 0.05).

Table 1. Baseline characteristics of study participants.

Variables	Placebo group (*N = 20*)	*N. sativa* group (*N = 23*)	*P*
Age (year)	56.00 (3.4)*	51.4 (9.2)*	0.275†
BMI (kg/m^2)	28.8 (8.1)*	28.4 (4.4)*	0.703†
education‡			0.88††
Under Diploma	15 (75)	18 (78.3)	
Upper Diploma	5 (25)	5 (21.7)	
Family history of diabetes‡	7(35)	6(26.08)	0.7
Duration of diabetes*	5.25 (0.96)	5.39 (1.4)	0.76
The number of women / men‡	10/10	13/10	0.43
Metformin tablets‡	19 (95)	20(87)	0.54
Glibenclamide tablets‡	18 (62)	19(82)	0.32
Antihypertensive drug‡	5 (18.8)	(6 (17.3	0.53
Use of lipid lowering drugs‡	8 (6.2)	(9 (4.3	0.43

*† Independent Student t test for age and BMI; Chi-square test; ††Mann-Whitney U test; * Mean (SD); ‡Frequency (percentage)*

Discussion

The main finding of the current study was significant reduction in MDA after 8 weeks of supplementation with N.sativa oil extract among diabetic patients. Although we observed increases in SOD and CAT in intervention group, but these observation did not reveal statistical between-group significance. Long-lasting hyperglycemia induces oxidative stress and inflammation, and weakens antioxidant defense system. Hence, anti-inflammatory or anti-oxidant agents may provide a useful approach for the prevention of complications in diabetes. Free radicals (Pro-oxidants) react with lipids and produce lipid peroxidation products such as MDA and NO (22, 23). SOD and CAT are antioxidant enzymes that result in repairing biologic damage from free radical and reduce inflammation. To our knowledge, this is the first clinical trial that performed to evaluate the effects of N.sativa oil extract on inflammatory cytokine response and oxidative stress status in people with T2D. Improving glycemia is the desired outcome when assessing effectiveness of interventions in diabetes. It should be noted that in the current study, fasting blood glucose was decreased significantly in the intervention condition compared to the placebo group (-23 ± 39.1 vs. 8.5±2.2, respectively; P=0.03) and the results will be well explained in another article by the authors.

Congruent with current findings, Kaleem et al.'s animal study show that N.sativa extracts can recover antioxidant status (24). They reported that 300 mg/kg NS supplement can decrease blood glucose, blood lipids, and lipid peroxidation products and increase antioxidant enzymes such as catalase and superoxide dismutase after 30 days.

Also, another study examined the effects of N.sativa on oxidative stress and damage to pancreatic beta cells in streptozotocin(STZ)-induced diabetic rats. Daily intraperitoneal administration of 2.0 ml/kg TQ for 4 weeks increased the activity of SOD, CAT, glutathione peroxidase and reduced serum levels of glucose, MDA and NO (25).

Based on available evidence, the main component of NS (thymoquinone) potentially increases expression and activity of antioxidant enzymes such as CAT, SOD, glutathione peroxidase and glutathione reductase and also decreases NO synthase expression and lipid peroxidation thereby reducing free radicals and oxidative stress in diabetes (26, 27).

Our findings did not reveal significant changes in TNF-α and IL-1 (inflammatory markers) following 8 weeks of supplementation with black seed extract. The

Table 2. The inflammatory, antioxidant and oxidative stress biomarkers at baseline and at post-intervention.

Variables	Placebo group (*N = 20*)	*N. sativa* group (*N = 23*)	*P*
TNF-α (pg/ml)			
Baseline	15.3±3.9	16.3±3.5	0.22†
Post-intervention	15. 8±3.5	15.1± 4.1	0.33*
Change	0.45±3.4	-1.3 ± 4.2	0.08*
IL-1β (pg/ml)			
Baseline	12.5±2.05	14.4±3.3	0.02†
Post-intervention	13.9±2.7	14.1±3.2	0.41*
Change	1.38±1.9	-0.37±3.4	0.28*
SOD (U/ml)			
Baseline	214±11.4	198±25.92	0.013†
Post-intervention	207±15.5	205±25.3	0.07*
Change	-7.1±16.7	7.5±16.9	0.017*
CAT (U/ml)			
Baseline	8.8±4.6	6.2±4.01	0.03†
Post-intervention	7.9±5.1	8.1±4.1	0.15*
Change	-0.86±4.2	1.8±27.6	0.098*
MDA (nmol/l)			
Baseline	5.5±1.7	6. 02±1.8	0.34†
Post-intervention	6.5±3.04	5.3±1.7	0.21*
Change	0.98±2.6	-0.7±1.3	0.02*
NO (nmol/l)			
Baseline	3.6±1.3	3.8±1.3	0.66†
Post-intervention	3.5±1.03	3.2±0.9	0.38*
Change	-0.16±1.6	-0.6±1.5	0.19*

*TNF- α: Tumor necrosis factor-alpha; IL-1β: Interlukin-1β; SOD: Superoxide dismutase; CAT: catalase; MDA: Malondialdehyde; NO; Nitric oxaid. Mean (SD) and Median (percentiles 25 and 75) are presented for normally and not normally distributed measures, respectively; pg, picograms; ml, milliliters; U, units; nmol, nanomoles; †Mann-Whitney U test; * Based on ANCOVA adjusted for baseline measures.*

finding is not in line with the results of some animal studies (28, 29). Although there are limited studies in this context, Umar et al. investigated the effects of 5 mg/kg TQ for 21 days on the inflammatory cytokines in rats (26). They found that TQ reduced inflammatory cytokines such as IL-1β, IL-6, and TNF-α and increased anti-inflammatory cytokines such as IL-10. Another study found that 20 mg/kg TQ can reduce IL-1β, IL-6, and TNF-α and PGE2 and increase IL-10 and IL-2 in diabetic rats (30). Furthermore, statistically significant reductions were demonstrated for inflammatory cytokines such as IL-1β, IL-4, and TNF-α following supplementation of albino rats with N.sativa (31). A recent study showed that Nigella sativa inhibits nitric oxide production, a mediator of pro-inflammatory cytokines (27).

Inhibitory effects of NS on TNF-α-medicated activation of NF-KB as well as reducing transportation of NF-KB (transcription factor aggravating the inflammation) from the cytosol to the nucleus has

Table 3. Energy and macronutrient intake in the two experimental groups at baseline and throughout the study.

	Placebo group (n = 19)	***N. sativa* group (n = 23)**	**p**
Energy (kcal)			
Baseline	1628 ±320	1889.82 (564.32)	0.215*
Post-intervention	1692.41 (523.30)	1894.82 (548.17)	0.909**
p-value***	0.890	0.769	
Protein (g)			
Baseline	56.35 (14.73)	58.86 (15.80)	0.439*
Post-intervention	59.00 (14.23)	60.21 (14.17)	0.389**
p-value***	0.619	0.811	
Fat (g)			
Baseline	55.06 (17.25)	62.60 (11.12)	0.272*
Post-intervention	56.85 (18.13)	60.80 (14.02)	0.690**
p-value***	0.174	0.185	

**Independent Student t test; ** Based on ANCOVA adjusted for baseline measures; ***Paired Student t test; Mean (SD) are presented for the measures*

been also reported (32). N.sativa prevents gene expression of p65 subunit of NF-KB, phosphorylation and degradation of the NF-KB inhibitor (I-KBα) (32). Studies also demonstrate protective effect of N.sativa on the inflammatory immune response through inhibition of NF-KB pathway and preventing phosphorylation of ERK / P38 (33, 34).

In this study, black seed extract had no effect on the inflammatory markers (TNFα and 1IL-β). Low doses of supplementation in the present intervention may account for insignificant findings. Future studies with higher doses of N.sativa extracts for a longer period of time are suggested for a better understanding of the effects of N.sativa on the inflammatory processes.

This study partially supports N.sativa as an anti-inflammatory and anti-oxidative herbal medicine among patients with diabetes and could be a beneficial adjunct therapy if results are confirmed by future studies.

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