Original Article

Effects of *Nigella sativa* Extracts on the Lipid Profile and Uncoupling Protein-1 Gene Expression in Brown Adipose Tissue of Mice

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Abstract

Background: Uncoupling protein-1 (UCP-1) is the index protein of the brown adipose tissue (BAT), used in the obesity studies. We evaluated the effects of thymoquinone (TQ), hydroalcoholic, and hexane extracts of *Nigella sativa,* on the *UCP-1* gene expression in BAT, and also on the recovery from oxidative stress, due to a high-fat diet. **Materials and Methods:** Fifty mice were divided into five groups: the first group was fed with a usual diet and the second, third, fourth, and fifth groups with a high-fat diet, hydroalcoholic extract, hexane extract, and TQ, respectively. After completing the course, the lipid profile, paraoxonase 1 (PON1), serum total antioxidant capacity (TAC), and malondialdehyde (MDA) were measured. *UCP-1* expression in BAT was evaluated at the gene and protein level. **Results:** The weight of mice, receiving TQ, hydroalcoholic, and hexane extracts, was decreased ($P < 0.05$), compared to the second group ($P < 0.05$). MDA was increased in the second group, compared to the first group ($P < 0.05$); however, TAC, liver catalase enzyme, and PON1 were decreased ($P < 0.05$). Furthermore, MDA of the third, fourth, and fifth groups had decreased, and the activity of PON1, liver catalase enzyme, and the amount of TAC was increased ($P < 0.05$). UCP-1 expression of the third and fourth groups was increased, compared to the second group ($P < 0.05$). **Conclusion:** The results suggest that TQ, hydroalcoholic, and hexane extracts of *N. sativa* have a protective and therapeutic role in the oxidative stress, caused by high-fat diets. The hydroalcoholic and hexane extracts can induce weight loss, by positively affecting UCP-1, at the gene and protein level.

Keywords: *Antioxidant capacity, malondialdehyde, Nigella sativa, obesity, uncoupling protein-1*

Introduction

Obesity is a chronic and multifactorial disease, in which social, psychological, behavioral, cellular, molecular, and metabolic factors are involved.[1] Obesity is characterized by the aggregation of fat in body tissues. According to the World Health Organization, obesity is defined by a body mass index (BMI) equal to or above 30 kg/m^2.[2] Obesity on a global scale is considered our biggest health problem, and it is not confined to industrial countries. In fact, studies showed that in developing countries, the prevalence of obesity is increasing, especially in women.[3] Obesity is one of the most important risk factors for developing cardiovascular and chronic kidney diseases, as well as diabetes; it is also an important risk factor for cancer in the nonsmoking population, with the growing rate of developing cancer. Cardiac fibrosis, causing metabolic disorders, such as heart failure and arrhythmia, is also linked to obesity.[4,5] In humans, the adipose tissue consists of the brown adipose tissue (BAT) and the white adipose tissue; the BAT comprised polyhedral fat cells with abundant mitochondria; uncoupling protein-1 (UCP-1) has a high expression level in these mitochondria and it plays an important role in the thermogenic activity of this tissue.[6] UCP-1 is present in the membrane of the mitochondria of adipose tissue; by transferring protons through the latitude of the membrane into the matrix of the mitochondria, it decreases the proton gradient; in this state, the energy from aerobic oxidation produces heat instead of adenosine triphosphate. This process controls the homeostasis of energy and keeps living creatures alive, during cold temperatures. Meanwhile, the

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BAT, as the natural antiobesity organ of the body, burns fat, produces heat, and therefore causes weight loss only with the expression of the *UCP-1*, without affecting the appetite.[7,8] For this reason, nowadays, the BAT is widely used in obesity-related researches.[7] Polyphenols present in herbal plants have beneficial prophylactic effects on cardiovascular diseases, diabetes, and cancer.[9] Herbal plants have fewer side effects, compared to chemical drugs. Many herbal plants, including *Nigella sativa,* have antiobesity effects, such as reduction of total cholesterol, triglyceride, and low-density lipoprotein (LDL). The major compound in *N. sativa* is thymoquinone (TQ), having anti-inflammatory, antioxidant, and antiobesity effects.[5,10-13] Because of the high prevalence of obesity and its side effects, as well as people's inclination to lose weight without using chemical drugs, utilization of herbal plants can be beneficial. The purpose of this study is to evaluate the effects of TQ, hydroalcoholic, and hexane extracts of *N. sativa* on the lipid profile, serum antioxidant capacity, and *UCP-1* gene expression in the BAT of mice.

Materials and Methods

N. sativa seeds were purchased from a grocery in Shahrekord, Iran, and were authenticated in Medical Plants Research Center of Shahrekord University of Medical Sciences, Iran.

The hydroalcoholic extract of *N. sativa* was prepared by maceration method. 300 g of the *N. sativa* was chopped in a suitable container and 500 ml ethanol 70% was added and kept at room temperature for 1 week. Then, it was filtered and the solvent was evaporated using a rotary evaporator. Finally, it was concentrated in 37°C in an oven and kept in a fridge until use.[14]

In this study, fifty 6-week-old mice (C-57) with an average weight of 20–25 g were used and they were kept in a constant light (12 h) and temperature (22°C ± 2°C) conditions. Mice were allowed free access to food and water.

Mice were randomly assigned into five groups (10 mice per group): the first group was fed with a regular diet and received 1 ml distilled water per day by oral gavage; the second group was fed with a high-fat diet and received 1 ml of sunflower oil per day by oral gavage; the third group was fed with a high-fat diet along with 200 mg/kg body weight dose of *N. sativa*'s hydroalcoholic extract; the fourth group was fed with a high-fat diet along with 300 mg/kg body weight dose of *N. sativa*'s hexane extract; and the fifth group received a high-fat diet along with 100 mg/kg body weight dose of TQ.

The duration of the study was 30 days. The hydroalcoholic extract was thoroughly dissolved in 1 ml of distilled water. The hexane extract and TQ were thoroughly dissolved in 1 ml of sunflower oil, and all mice were fed daily by oral gavage. The high-fat diet consisted of 57% regular mouse food with 43% high-fat food, including 15% saturated animal fat, 5% vegetable oil, 20% sucrose, 2.5% cholesterol, and 0.5% cholic acid.

After completing the course of the diet, treated mice were anesthetized with chloroform to measure the paraoxonase 1 (PON1) enzyme, malondialdehyde (MDA), and the lipid profile, using blood collected from the mice's heart; a section of the liver's tissue was excised to measure the activity of the hepatic catalase enzyme. BAT was excised from between the shoulder blades, at the back of the neck, in order to evaluate UCP-1, at the gene and protein level. After the tissue samples were washed with physiologic serum, they were stored in a − 70°C freezer, until further laboratory tests were performed.

The lipid profile of the serum was examined, using the commercial kits (Pars Azmun Company) and the AutoAnalyzer device (BT3000, Italy). The serum very low-density lipoprotein cholesterol (VLDL-C) and LDL cholesterol (LDL-C) levels were calculated, using the Friedewald formula.[15] The antioxidant capacity of the serum was measured by evaluating the serum's ability to reduce iron (III) to iron (II), using the Ferric reducing ability of plasma (FRAP) method.[16] The MDA concentration was determined by high-performance liquid chromatography, based on the Agarwal method.[17] The activity of the enzyme PON1 was measured with the spectrophotometer, and phenylacetate was used as the synthetic substrate of the enzyme.[18] The activity of the catalase enzyme was measured via the Abei method, and hydrogen peroxide was used as the substrate in homogenized hepatic tissue.[19] To measure the expression of the *UCP-1*, total RNA was extracted from the BAT, using a commercial kit (Kit BIOFLUX-Trizol Reagent). The quality and quantity of RNA were evaluated, using the Nanodrop device (Thermo Scientific Nanodrop 2000 spectrophotometer, USA). To synthesize cDNA, the First-Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used, according to the manufacturer's guidelines. The intended primers were designed, using the OLIGO 7 software (Molecular Biology Insights Inc., Colorado Springs, USA). The *UCP-1* gene expression was compared with the expression of housekeeping gene *β-Actin* and relative expression was reported. The primers used to detect *β-Actin* were: (Forward: 5'-CGGTCAGGTCATCACTATCGG-3', Reverse: 5'-TCTTTACGGATGTCAACTCACAC-3'); and the primers used to detect UCP-1 were: (Forward: 5'-TCAGGATTGGCCTCTACGACT-3', Reverse: 5'-GCATTCTGACCTTCACGACCT-3'). Amplification of the *UCP-1* and *β-Actin* was carried out, according to the following protocol in the Rotor-Gene 3000 apparatus (Corbett-Australia). Before amplification, the reaction mix was incubated for 10 min at 95°C and then real-time polymerase chain reaction was performed in a

3-step cycle procedure, consisting of 40 cycles of 15 s in 95°C, 20 s in 62°C, and 20 s in 72°C. UCP-1 protein was isolated from the BAT using radioimmunoprecipitation assay buffer and its concentration was determined using the Bradford method; subsequently, 40 μg of each sample was transferred into sodium dodecyl sulfate gel and electrophoresis was performed; it was then blotted onto the surface of polyvinylidene difluoride membrane. The membrane was blocked and was then incubated with primary specific antibodies (anti-β-actin [ab8227] or anti-UCP-1 [ab23841]); after washing, the membranes were incubated with the secondary antibody (anti-rabbit immunoglobulin G). Finally, 1 ml enhanced chemiluminescence solution was added to the membrane and scanned.[20]

Results were presented as means ± standard errors. Statistical analysis was performed by the Mann–Whitney test, using the SPSS software version 20 (SPSS Inc., Chicago, IL, USA). P <0·05 was considered statistically significant.

Results

According to the weekly weight chart, Group 2 had gained weight compared to Group 1 ($P < 0.05$), and Groups 3, 4, and 5 had lost weight compared to Group 2 ($P < 0.05$) [Figure 1].

Table 1 shows that after 30 days of high-fat diet, the serum thyroglobulin (TG), total cholesterol, and LDL-C levels were increased in Group 2 compared to Group 1 and the level of high-density lipoprotein cholesterol (HDL-C) was decreased ($P < 0.05$). The serum TG, total cholesterol, and LDL-C levels were significantly decreased in Groups 3 and 5 compared to Group 2 ($P < 0.05$). The total cholesterol and LDL-C level in Group 4 were significantly decreased, compared to Group 2 ($P < 0.05$); however, HDL-C level was significantly increased in Groups 3, 4, and 5 compared to Group 2 ($P < 0.05$). The serum VLDL level of Group 2 was significantly increased compared to Group 1 ($P < 0.05$); also, it was significantly decreased in Groups 3 and 5 compared to Group 2 ($P < 0.05$). The serum MDA level in Group 2 was significantly increased compared to Group 1 ($P < 0.05$); it was significantly decreased in Groups 3, 4, and 5 compared to Group 2 ($P < 0.05$); also, the serum total antioxidant capacity (TAC) level in Group 2 was significantly decreased compared to Group 1 ($P < 0.05$); TAC level was significantly increased in Groups 3, 4, and 5 compared to Group 2 ($P < 0.05$). The activity of serum PON1 and hepatic catalase enzymes in Group 2 was significantly decreased compared to Group 1 ($P < 0.05$) and was significantly increased in Groups 3, 4, and 5 compared to Group 2 ($P < 0.05$). Our data showed that the relative expression of *UCP-1* mRNA was significantly decreased in Group 2 compared to Group 1 ($P < 0.05$); whereas, it was significantly increased in the third and fourth groups compared to the second group ($P < 0.05$).

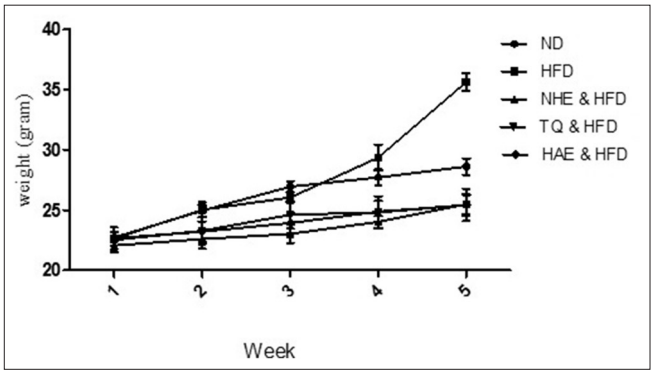

Figure 1: Weekly weight chart. There are 10 mice in each group. Group 1 normal diet (ND), Group 2 high-fat diet (HFD), Group 3 N-hexane extract and high-fat diet (NHE and HFD), Group 4 thymoquinone and high-fat diet (TQ and HFD), and Group 5 hydroalcoholic extract and high-fat diet (HAE and HFD)

Table 1: Data of the variables, examined in the 30-day diet

Variables	Group 1	Group 2	Group 3	Group 4	Group 5
Tg (mg/dl)	146.8±6.3	185.4±5.1[a]	160.3±2.8[b]	169±2	157.4±3.1[b]
Cholesterol (mg/dl)	107.7±2.3	330.4±22.7[a]	189.15±7.2[b]	194±6.23[b]	181.6±1.5[b]
HDL-C (mg/dl)	71.7±1.1	61.6±1.6[a]	72.6±0.97[b]	67.8±0.96[b]	77.2±1.2[b]
LDL-C (mg/dl)	6.66±1.85	208.74±21.4[a]	84.4±6.8[b]	92.4±6.4[b]	78.95±2.8[b]
VLDL-C (mg/dl)	29.37±1.27	37±1[a]	32±0.55[b]	33.8±0.4	31.5±0.6[b]
MDA (μM)	25.08±1.37	70.7±2.4[a]	29.2±1.85[b]	25.65±0.97[b]	21.77±2[b]
TAC (μM)	556±27.1	360.9±29.2[a]	545.9±37.5[b]	516.2±12.25[b]	533.6±27.8[b]
Hepatic catalase (unit per mg of protein)	35.72±2.7	21.1±1.2[a]	38.04±1.32[b]	37.33±1.5[b]	36.46±2.34[b]
Paraoxonase 1 (unit/ml)	46.3±2.67	23.13±0.8	46.7±1.6[b]	45.81±0.89[b]	45.81±0.76[b]
UCP-1 (fold change)	1	0.52±0.084[a]	2.15±0.22[b]	2.7±0.26[b]	0.64±0.17

[a]P<0.05 is considered statistically significant compared to Group 1, [b]P<0.05 is considered statistically significant compared to Group 2. The numbers are shown as mean±SE. There are 10 mice in each group. Group 1 is normal control, Group 2 is high-fat control, and Groups 3, 4, and 5 are test groups fed with hydroalcoholic extract (200 mg/kg), hexane extract (300 mg/kg), and thymoquinone (100 mg/kg), respectively. UCP-1: Un coupler protein-1, MDA: Malondialdehyde, TAC: Total antioxidant capacity, HDL-C: High-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, Tg: Triglyceride, SE: Standard error, VLDL-C: Very low-density lipoprotein cholesterol

Effects of the 30-day diet on the uncoupling protein-1 protein expression of brown adipose tissue

As shown in Figure 2, in Group 2, UCP-1 protein expression was decreased compared to Group 1; on the other hand, in Groups 3 and 4, by loading the same amount of protein, stronger bands were observed, compared to the bands of Group 2; this is an indicator of the stronger expression of the UCP-1 protein in these groups.

There are ten mice in each group: 1 – normal diet, 2 – high-fat diet, 3 – group fed with high-fat diet and hexane extract (300 mg/kg), 4 – group fed with high-fat diet and TQ (100 mg/kg), 5 – group fed with high-fat diet and hydroalcoholic extract (200 mg/kg).

Discussion

Obesity can decrease the activities of body's protective antioxidants, and can change the activity of enzymes, such as catalase and superoxide dismutase .[21,22] In a study by Ismail M. *et al*., carried out on rats fed with high-cholesterol diets, they found that the LDL and total cholesterol levels of rats treated with *N. sativa* had significantly decreased. In addition, the expression of genes involved in the catalase and superoxide dismutase activity had increased;[23] increased catalase gene expression was concomitant with an increase in the activity of hepatic catalase, reported in the present study as well. The most abundant compound in *N. sativa* is TQ that possesses antioxidant, anti-inflammatory, and antiobesity properties; it was also revealed that *N. sativa* decreases LDL, total cholesterol, and TG levels.[10-13]

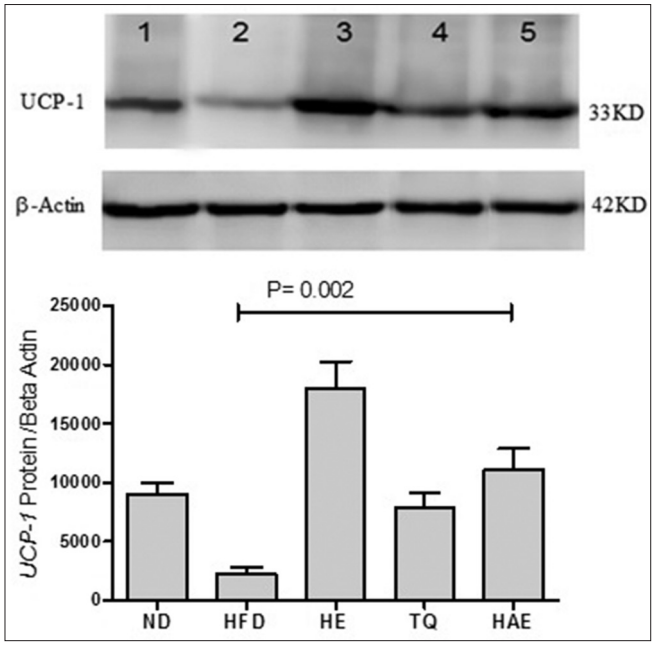

Figure 2: Western blot analysis of UCP-1 and β-Actin. 1 – Normal Diet (ND), 2 – High-fat diet (HFD), 3 – Hexane extract (HE), 4 – Thymoquinone (TQ), 5 – Hydroalcoholic extract (HAE)

In 2013, Ahmad S. *et al*. showed that treatment with TQ decreased the lipid peroxidation marker, MDA in the serum, and increased the TAC of the plasma to a normal level.[24] According to a study on the lipid profile performed by Farzaneh *et al*., they reported that *N. sativa* lowered total cholesterol, triglyceride, LDL, and BMI and increased HDL.[11] In the current study, the serum levels of TG, LDL, and cholesterol were significantly increased in the high-fat control group compared to the normal controls ($P < 0.05$). However, in the groups receiving TQ, hydroalcoholic, and hexane extracts, the cholesterol and LDL levels were significantly decreased after 30 days, in comparison to the high-fat control group ($P < 0.05$); also in the groups receiving TQ and hydroalcoholic extract, serum TG level was significantly decreased compared to the group fed with the high-fat diet ($P < 0.05$). The serum HDL level was significantly decreased in the high-fat control group compared to the normal control group ($P < 0.05$); on the other hand, the HDL of groups receiving TQ, hydroalcoholic, and hexane extracts was significantly increased compared to the high-fat diet group ($P < 0.05$). Most likely, these changes are due to an increase in the catabolism or burning of fats, entering the body via the high-fat diet.

UCP-1 is an uncoupling protein that decreases the proton gradient generated in oxidative phosphorylation in the mitochondria of BAT. Therefore, the compounds that increase this protein may be used to increase the metabolic energy expenditure and weight loss.

In this study, the *N. sativa* extracts have been able to increase the *UCP-1* expression-induced by high-fat diet due to polyphenolic compounds. Therefore, these compounds may be used as weight-reducing agents.

The UCP-1 expression alterations observed in this study can partly explain the lipid alterations demonstrated in the groups receiving extract. Weight increase and a decrease in TAC, the catalase enzyme activity, and the antioxidants of the serum observed in the second group compared to the first group can be due to the free radicals produced by the high-fat regimen that has dominated the antioxidant capacity of the serum. According to a study by Noeman *et al.* performed on rats receiving a high-fat diet, the group with the high-fat diet compared to the control group had higher MDA and lower PON1 levels.[25] Furthermore, in the current study, the PON1 levels of the high-fat control group were higher than the levels in the normal control group; yet in the groups receiving TQ, hydroalcoholic, and hexane extracts, we have shown a renewed increase and restoration in PON1 levels, compared to the high-fat control group, suggesting that in the high-fat control group, the levels of endogen lipids and fatty acids have increased and the liver tends to produce more triglyceride, resulting in a reduction in the synthesis of HDL. On the other hand, HDL is required for transferring PON1 from the liver to the bloodstream,

whereas decrease in the secretion of HDL is concomitant with decrease in the PON1 serum level; however, in groups receiving TQ, hydroalcoholic, and hexane extracts, following the increase in the production of HDL, we also observed an increase in the PON1 serum level. The serum MDA level in high-fat control group compared to the normal control group increases, which is due to increase in fatty acids and decrease in serum antioxidant capacity. In Groups 3, 4, and 5, the serum MDA level was decreased compared to Group 2, which is due to increase in serum antioxidant capacity and decrease of oxidative stress from the high-fat regimen, following the use of the antioxidants in the extracts. The BAT has an important role in weight and metabolism regulation and converts the stored energy of TG to heat, through UCP-1. In a study by Shen *et al.*, on the antidiabetic effects of cinnamon on mice, it was found that the intake of aqueous extract of 30 mg/kg body weight cinnamon for 22 days decreased the blood glucose level. The antidiabetic effects were exerted through two mechanisms: one increasing the *UCP-1* gene expression of BAT and another one increasing the glucose transporter type-4 in muscular and BATs.[10] In a study, Rossato *et al.* found that the UCP-1 gene expression in white adipose tissue induces the production of heat.[26] In a study carried out by Lee *et al.* on high-fat diet rats, a decrease in the UCP-1 gene expression was found in the high-fat diet group.[27] In the current study, the gene and protein expression of UCP-1 were also significantly decreased in the high-fat control group compared to the control group ($P < 0.05$); however, the expression of UCP-1 was significantly increased in the groups receiving alcoholic and hexane extracts ($P < 0.05$), which is consistent with previous studies. Our data showed that the aforementioned doses of TQ, hydroalcoholic, and hexane extracts of *N. sativa* amend the antioxidant level, by increasing the activity of catalase and PON1 enzymes, increasing the levels of HDL-C and TAC, and decreasing the levels of MDA, LDL, cholesterol, and TG.

Conclusion

Our data suggested that weight loss through UCP-1 is less dependent on TQ, and it is likely that other compounds present in both extracts can decrease weight by increasing UCP-1 expression although there is another possibility that some compounds in the extracts have a synergistic effect with TQ, leading to the further reduction of UCP-1 expression. In spite of the significant change in the UCP-1 gene expression, in the group receiving TQ, the weight loss can be due to the role of other genes, such as *UCP-2*, *BMP7,* and *BMP8b*. Further studies are needed to confirm the role of these genes.

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Conflicts of interest

There are no conflicts of interest.

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