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Effect of Black Cumin on the Expression of Genes Related to Lipid Metabolism in Adipose Tissue: A Mouse Model Study

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ABSTRACT

Obesity, as a complex multifactorial disease, can be considered as a suitable goal for treatment by traditional medicine. This study aimed to investigate the effect of black cumin on the expression of some important genes in the metabolism of lipids. For this purpose, 14 male Balb/c mice were divided into control and treatment groups. Mice in the control group were fed standard food, and mice in the treatment group were fed with a mix containing 50% standard food and 50% powdered cumin for 35 days. After anesthetizing the mice, their adipose tissue was collected. Total RNA was extracted, and after the qualification by agarose gel electrophoresis, cDNA was synthesized. The expression changes of *Leptin*, *AdipoQ*, and *PPARG* genes were measured by real-time RT-PCR. In addition, adipose tissue and cell size were analyzed using the Oil Red O staining method and quantitation using Image J software. Finally, GraphPad prisim 9.0 was applied to statistically evaluate the cell size difference and gene expression changes. The results of this study showed a significant decrease in *Leptin* gene expression levels (FC= 0.42, Sig.= 0.00, P< 0.05) and also a significant increase in the expression of *AdipoQ* and *PPARG* (FC= 1.8, Sig.= 0.00, P< 0.05) genes in adipose tissue in the treated group compared with the control group. Also, a considerable decrease in adipose tissue cell size in the treatment group was observed (210/520, Sig.=0.00, P<0.05). The results of this study are supported by similar studies and confirmed that the compounds in black cumin can be useful in preventing abnormal fat storage and problems such as metabolic syndromes by modulating the cellular metabolism of lipids.

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Introduction

Nowadays, obesity has reached a world epidemic and has become one of the health problems. Obesity is caused by an energy imbalance between calories received and calories consumed, which causes abnormal fat accumulation in the body. The use of herbs in the prevention and management of obesity in humans has been confirmed by several studies. Medicinal plants or certain chemical compounds in them contain many active ingredients that may act as inducing elements to promote functional genes that play an important role in metabolism. In this study, we focused on studying the effect of black cumin (*Carum carvi*) on the expression of important genes involved in metabolic pathways related to obesity (Haque and Ansari, 2018). Black cumin seeds are an excellent source of secondary metabolites such as polyphenols, flavonoids, and alkaloids (Johri, 2011). Its flavonoids inhibit glucose reabsorption and lower blood sugar, and lipid phytosterols prevent the absorption of bad cholesterol in the intestine and blood circulation. The biologically active compounds in black cumin seeds have antimicrobial, antioxidant, anti-inflammatory, and anti-cancer activities, which are caused by some of its ingredients, such as carvacrol (polyphenols) and unsaturated fatty acids

(Saremi *et al*., 2024). Adipose tissue is known as the place where energy is stored in the body. They store energy as triglycerides when food is plentiful in the body and release it as fatty acids when needed. Adipose tissue is structurally composed of two groups of cells. 1. White adipose 2. Brown adipose. When the white part of adipose tissue is affected by stimuli such as obesity and the increase of fat cells, it is stimulated. It secretes many biologically active molecules called adipokines (Kadowaki *et al*., 2006).

The first adipokine discovered was a protein called *leptin*. It was first discovered in 1994 by a researcher named Jeffrey Friedman. *Leptin* is secreted from adipose tissue into the bloodstream. The amount of *leptin* in the blood is directly proportional to the amount of adipose tissue and relates the state of energy storage to the brain. It plays a role in regulating appetite, endocrine function, and energy homeostasis. *Leptin* binds to its receptors at the cell surface body mass index, metabolic hormones, and sex are the factors that have the greatest impact on circulating *leptin* concentrations (Allison and Myers, 2014). *Adiponectin* is expressed exclusively in white adipose tissue. By connecting to its receptors *adipoQ* R1/R2, it initiates the transmission of tissue-dependent signals, which affect fat and glucose metabolism, insulin sensitivity, and possibly energy balance. Blood levels of adiponectin reduce obesity, insulin resistance, and type 2 diabetes (Guerre-Millo, 2008).

Peroxisome proliferator activating receptors are a group of intranuclear hormone receptors that contain three different isotypes, including alpha, delta, or beta and gamma, that function at the transcriptional level. Like other nuclear receptors, they contain a second ligand binding and a DNA binding domain that bind to *PPAR*-responsive elements (*PPREs*) in the promoter of target genes. Some *PPARs* are required to bind to this sequence and form a heterodimer complex with *RXR*. These receptors are activated by their ligands. The family of transcription factors that activate peroxisome proliferative receptors (*PPARs*) play a key role in lipid metabolism and many diseases, the most important of which is obesity. The *PPARγ* nucleus receptor was discovered by Tontonoz et al. In the early 1990s, it was located on chromosome 3P25.2 in humans and is known as the major regulator of fat (Schoonjans *et al*., 1996). The main objective of this study is the investigation of the effect of a diet enriched with black cumin on the expression of the gene involved in lipid metabolism balance and phenotypic changes of adipocytes.

Material and Methods

Methods of preparation of caraway

The study was conducted on adult male BALB/c mice, weighing approximately 24± 2 grams, in a controlled environment with a temperature of 25°C and a 12 hours light/dark cycle. Ethical clearance was obtained from the local Animal Research Ethics Committee, adhering to Shahrekord University guidelines. Fourteen mice were divided into two groups: Group I, the control group, was fed standard pellet feed, while Group II received a specialized diet of 50% standard feed and 50% black cumin powder. In detail, the standard mouse food and black cumin were powdered in an equal weight ratio and again mixed with the appropriate amount of water, and food tablets with a new composition were made and the mice were fed with it.

Adipose tissue biopsy and oil red staining

Mice were anesthetized using chloroform, and their adipose tissue was promptly extracted under sterile conditions. For cell size quantification, adipose tissues were first fixed with 10% formalin for two weeks at room temperature. Subsequently, they were washed with phosphate-buffered saline (PBS) and after preparing the sections with a microtome (Hisure, China), staining was performed for 1h with 0.5% Oil Red O in 60% isopropanol. Following a thorough wash with distilled water, the stained tissues were examined under a light microscope (Euromex, Netherlands).

RT-PCR assay

Following the manufacturer's protocol, the tissue was directly subjected to RNA extraction using RNXplus buffer (Sinaclon Co., Iran). Subsequently, cDNA synthesis was performed using approximately 50 ng of RNA, oligo (dT) primers, and random hexamers according to the manufacturer's instructions with a cDNA

synthesis kit (SmoBio Co., Taiwan). The specific primers for the *ActB (*as reference gene), *Leptin*, *Adipoq*, and *PPARG* genes listed in Table 1 were designed using Gene Runner software (version 6.5.52). Real-time RT-PCR was performed using an ABI StepOne device (Applied Biosystem Co., USA) and SYBER green 2x master mix (Biofact Trading Company, Taiwan). Each reaction mixture, in a 10µl final volume, containing 5µl of 2X master mix, 3.5 µl of DEPPC water, and 0.5 µl each of forward and reverse primers and template cDNA. All reactions were executed in duplicates for every sample and gene, in 40 cycles. Also, to calculate the melting curve, the reaction was subjected to 95ºC for 60 seconds and melting curve was calculated between 65 ºC and 95 ºC with 0.3 ºC/step ramping rate for 1 minute.

Table 1. List of primers and their properties that were used in this study.

Primer name	Oligomers (5'→3')	Target length	Target gene	Accession no.
mActF	5'- GGACTCCTATGTGGGTGACG-3'	119bp	*ActB*	NM_007393.5
mActR	5'- AGGTGTGGTGCCAGATCTTC-3'			
FAQ1	5'-ACTTGTGCAGGTTGGATGG-3'	139bp	*AdipoQ*	NM_009605.5
RAQ1	5'-CTGTCTCACCCTTAGGACC-3'			
FLEP	5'-CACACACGCAGTCGGTATCC-3'	133bp	*Leptin*	FJ374142.1
RLEP	5'-CAGGTCCTCACCAGCCTGCC-3'			
FPPRG	5'-TACCCTTTACTGAAATTACC-3'	131bp	*PPARG*	NM_001308352.1
RPPRG	5'- TGTGGTAAAGGGCTTGATGTC-3'			

Gene expression and statistical analyses

Gene expression changes were calculated using the $2^{-\Delta\Delta ct}$ method. To check the significance of the differences in the results of the two groups, GraphPad Prism (version 9.0.0) software was applied. In all of the analyses, $P<0.05$ was considered.

Results

Total RNA was extracted using RNXplus solution and qualified in gel electrophoresis (Fig. 1). The presence of 28s and 18s rRNA bands showed an optimal quality.

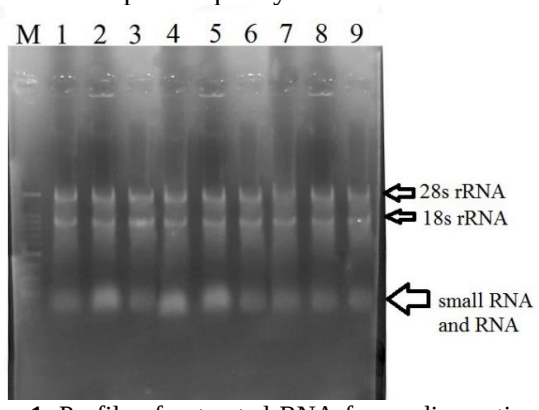

Fig. 1. Profile of extracted RNA from adipose tissue in the agarose gel electrophoresis: M= 100 pairs of DNA marker; Lane 1 to 9 RNA samples were extracted, of which 5 microliters were examined in 1% agarose gel.

After cDNA synthesis and Realtime RT-PCR, the fold change of the gene expression was calculated and subjected to statistical analysis. The results of the gene expression analysis for *Leptin*, *AdipoQ*, and *PPARG* are presented in Figure 2 (A-C). A decrease of about 60% in *Leptin* gene expression was observed ($P\leq0.05$, Sig. = 0.001, Fold change = 0.425) compared to the control group. Also, we reported an elevated expression for *AdipoQ* gene ($P\leq0.05$, Sig.= 0.000, FC= 1.8709) and *PPARG* gene ($P\leq0.05$, Sig.= 0.000, FC= 1.8075) compared to the control group.

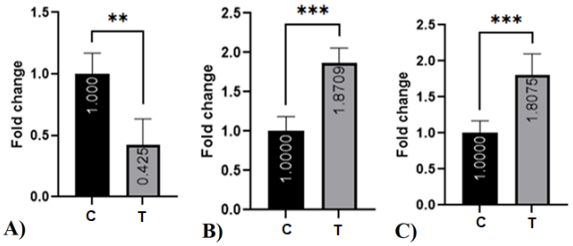

Fig. 2. Gene expression changes between the control and black cumin-treated groups: A) *Leptin* gene expression, which decreased ($P\leq 0.05$, Sig.= 0.001, Fold change= 0.425) compared to the control group; B) Changes in the expression of the *AdipoQ* gene, which increased ($P\leq 0.05$, Sig.= 0.000, FC= 1.8709) compared to the control group; C) Increase of *PPARG* gene expression ($P\leq 0.05$, Sig. = 0.000, FC = 1.8075) compared to the control group; C= control and T= treatment.

Quantitative examination of fat cells

Microscopic photos in similar resolution from any samples were prepared (Fig. 3) and by using Image J software, the size of adipocytes in adipose tissue of the control and treatment samples was measured as pixels units. Finally, the changes in the cell sizes were evaluated statistically (Fig. 4).

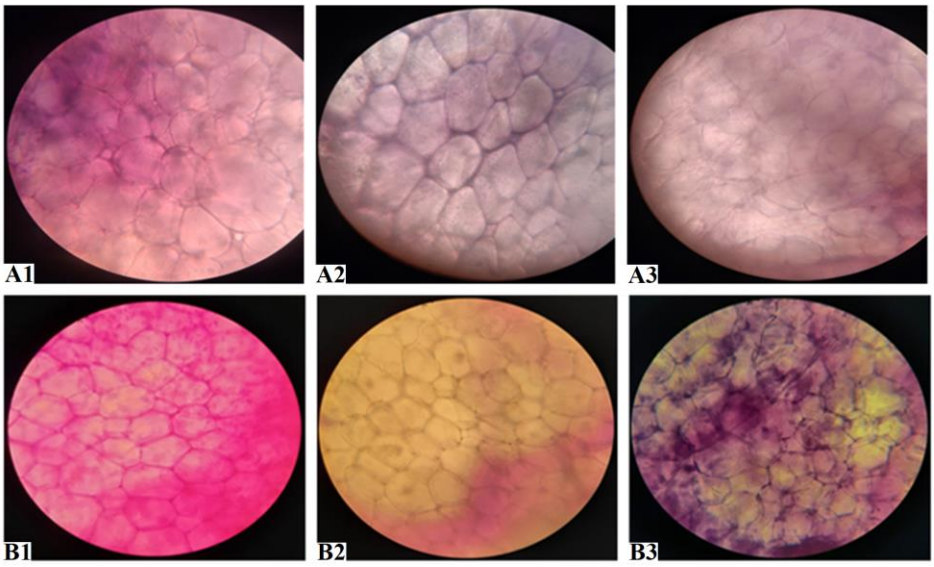

Fig. 3. Microscopic images of adipose tissue: A1-A3) Adipose tissue of control sample with light microscope stained with oil red O; B1-B3) Adipose tissue of the sample treated with black cumin.

Discussion

This study investigated the effects of black cumin treatment on the expression of *Leptin*, *AdipoQ*, and *PPARG* genes, which are involved in lipid metabolism, in adipose tissue. The $2^{-\Delta\Delta ct}$ method was employed to quantify gene expression changes, and statistical analysis was performed using GraphPad Prisim 9.0. Our results showed a significant decrease in *Leptin* transcript levels (FC=0.42, Sig.=0.00, P<0.05) and also a significant increase in the expression of *AdipoQ* and *PPARG* genes transcript (FC=1.8, Sig.=0.00, P<0.05) in adipose tissue in black cumin fed group compared with the control group. Also, the decrease in adipose tissue cell size in the treatment group was significant compared with control (210/520, Sig.=0.00, P<0.05). The importance of the present study and its selection as the main topic of this research can be justified when we know that obesity has reached epidemic proportions around the world and has become one of the most common health problems. Obesity and fat-related problems, in general, play a role in a variety of disorders and are associated with high mortality worldwide (Ibars *et al*., 2017).

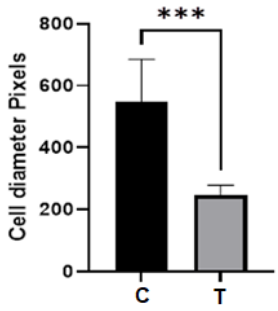

Fig. 4. Column diagram comparing adipose tissue of mice treated with black cumin compared to control sample tissue cells (Sig.= 0.00, p< 0.05); C= control and T= treatment.

An ideal treatment for weight loss is diet change, lifestyle modification, and regular exercise (Sun *et al*., 2016). In this study, we focused on using herbs because most medications were unsuitable due to their ineffectiveness or side effects. Several clinical and animal studies have been conducted using various herbal medicines. These studies have shown significant improvements in weight control, and importantly, no adverse side effects have been observed (Haque and Ansari, 2018). In addition to their anti-obesity function, herbs have many health benefits, and their use

can be an effective strategy for managing obesity and related disorders (Kaur *et al*., 2016). Creating negative calories is one of the features of a diet containing some plants (Shang *et al*., 2021). The main constituents of oil in black cumin seeds were caron and limonene, in addition to β-myrsen, transhydrocaron, and transcarole, α-pinene, sabinene, n-octanal, β-osimene, γ-terpinene, Linalool, cis and trans-limonene oxide, cis-dihydrocaron, cis-carole, peryldehyde, trans-anethole, and trans-beta-caryofylene. This diverse range of compounds contributes to the unique properties and potential therapeutic benefits of black cumin seed [13].

Adipose tissue, which has been studied in this study, is considered a storage tissue and is important for regulating the level of triglycerides in the body. Adipose tissue disorders can be diagnosed through the biomarker of the adiponectin-to-leptin ratio. *Leptin* and *adiponectin* are indicators of fat mass and triglyceride metabolism/insulin sensitivity, respectively. Elevated levels of this ratio are associated with improved triglyceride profiles (Samimi *et al*., 2021; Frühbeck *et al*., 2019). In response to this sensing mechanism, adipocytes secrete *leptin*, a hormone that carries out various physiological functions. Once released into the bloodstream, *leptin* binds to its receptors in the hypothalamus and has three main effects: First, *leptin* suppresses appetite and reduces food intake. Second, *Leptin a*cts on the sympathetic nervous system, stimulating a series of responses that ultimately lead to reduced fat production and increased fat breakdown. This cascade of events includes heightened activity of *AMP*-activated protein kinase (*AMPK*) in the liver, resulting in decreased lipogenesis and enhanced fatty acid oxidation. Furthermore, sympathetic activation increases B-adrenergic activity in adipocytes, promoting lipolysis and subsequent expression of uncoupling protein 1 (*UCP-1*), thereby coordinating fatty acid release from triglycerides and their oxidation. These effects are mediated by increased production of *cAMP* (Saltiel, 2012). Increased adipose tissue inhibits the production of *adiponectin*, which leads to insulin resistance, while calorie restriction in the body has an insulin-sensitive effect. Takmura et al. reported that *adiponectin* levels in obese patients were low and increased with weight loss and increased insulin sensitivity (Mohammadpour *et al*., 2020; Unamuno *et al*., 2018). *PPARG*, which is expressed in adipose tissue, increases fatty acid stores in adipose tissue and regulates the expression of hormones secreted by adipocytes, which affects glucose homeostasis and can convert calorie-storing white fat cells into fat-burning brown fat cells. Compounds that activate this protein can help people burn fat faster. The possible cellular and molecular mechanism of this pathway is such that beta-adrenergic receptors stimulate *P38MAPK*-activated protein kinase A in adipocytes via protein kinase A (Inagaki *et al*., 2016). Activated *P38MAPK* phosphorylates *PGC1α* and transcription activating factor 2 (*ATF2*) and controls *UCP1* gene expression through its interactions with *PPARG*. *PPARG* is the main enhancer of the *UCP1* gene. *ATF2* activation by *P38MAPK* is used as a sensor that increases the expression of *PGC1α* gene in brown adipose tissue (Wang *et al*., 2016). Scientists have been able to identify the elements responsible for active *PPAR* in the promoter region of the *adiponectin-expressing* gene. In addition, *PPARG* activators such as thiazolidinediones, widely used to improve insulin sensitivity and glucose tolerance in type 2 diabetes, increase the expression and secretion of the adiponectin protein (Lim *et al*., 2008; Dupont *et al*., 2008). *PPARG* activation increases the expression and transport of *GLUT-4* to the cell surface, thus glucose uptake into adipocytes. In addition, *PPARG* increases the plasma concentration of *adiponectin*, which in turn increases the oxidation of fatty acids in the liver and skeletal muscle. In general, *adiponectin* improves insulin sensitivity in skeletal muscle and liver and reduces glucose production in the liver, thereby reducing free fatty acids, triglycerides, and glucose (Monsalve *et al*., 2013; Watson *et al*., 2004).

Adiponectin levels increased significantly, while *leptin* levels decreased. As we know, the balance between adiponectin and leptin levels is very important in regulation of the blood glucose levels. In our study, with the consumption of black cumin by mice, the *leptin* gene transcript level has decreased. This decrease is in line with the appearance of the adipose tissue study that was done in our study. The size of fat cells decreases significantly so that the average

number of larger cells in terms of size that confirms more fat storage in the tissue of the treatment group is much less than the control group, indicating that the fat storage level is reduced due to increased fuel. We saw it in the body, which is done through the liver and tissues such as muscle, but the other point is that the level of *leptin* by reducing it has led cells to lose their fat stores.

Conclusion

Active compounds in black cumin may decrease fat storage by impacting energy expenditure and fat oxidation, resulting in increased energy consumption. These compounds not only reduce fat absorption in the intestinal tract but also play a role in preventing the differentiation and growth of fat cells. They trigger metabolic cycles and control factors that are crucial in fat metabolism, which in turn influences gene expression. Black cumin has been shown to increase insulin sensitivity in mice, resulting in lowered blood glucose levels. This effect is likely due to the observed changes in fat cell size in the model animal, indicating that black cumin may have long-lasting or even permanent effects in preventing the abnormal increase in adipose tissue volume.

Acknowledgment

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Ethical approval

This study was approved by the ethical committee of Shahrekord University with code: IR.SKU.REC.1399.015

Conflicts of Interest

The authors declare no conflict of interest.

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