Effects of Thymoquinone on Adipocyte Differentiation in Human Adipose-Derived Stem Cells

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Abstract
Inhibition of adipocyte differentiation would be a key strategy to control obesity. Human adipose tissue-derived stem cells (ADSCs) are a promising tool for adipocyte differentiation research. Thymoquinone (TQ) as a potent antioxidant molecule may inhibit adipocyte differentiation. Herein, we aim to investigate the inhibitory effect of TQ on lipid differentiation in ADSCs. Quantification of cell surface markers was used by Flow-Cytometry and the effect of TQ on cell viability was assessed using the AlamarBlue test. ADSCs were subjected to induction of differentiation in the presence of non-cytotoxic concentrations of TQ (6.25, 12.5 and 25 μg/mL). Lipid accumulation was assessed using the Oil-Red O staining technique. Moreover, the expression of PPARγ (Peroxisome proliferator-activated receptor-γ) and FAS (Fatty Acid Synthetase) proteins was evaluated using Western blotting. Flow-cytometry demonstrated the expression of CD44, CD90, and CD73 as mesenchymal stem cell markers on the cell surface. At concentrations ≤100 μg/mL of TQ, no significant difference in cell viability was observed compared to the control. Lipid accumulation in ADSCs significantly decreased at 25 μg/mL (P < 0.001) and 12.5 μg/mL (P < 0.01) of TQ. The findings of the qualitative examination of Lipid Droplets also confirmed these results. Western-blot showed that TQ at 12.5 (p < 0.05) and 25 μg/mL (p < 0.01) reduced FAS/β-actin ratio compared to the positive group. TQ also decreased the expression of PPARγ at 6.25 μg/mL but not at higher concentrations. In conclusion, TQ may reduce differentiation of fat stem cells into fat cells through inhibition of the expression of PPARγ and FAS proteins and might be a potential anti-obesity compound.

Keywords Obesity · Fat stem cells · Thymoquinone · Black cumin · FAS · PPAR-γ

Abbreviations

| Abbreviation | Full Form |
|--------------|-----------|
| PPARγ | Peroxisome proliferator receptor gamma |
| GAPDH | Glyceraldehyde phosphate dehydrogenase |
| FAS | Fatty Acid Synthetase |
| TQ | Thymoquinone |
| ADSCs | Adipose tissue-derived stem cells |
| WAT | White adipose tissue |
| BAT | Brown adipose tissue |
| SVF | Stromal vascular fraction |
| PBS | Phosphate buffered saline |
| DMEM | Dulbecco’s Modified Eagle’s Medium |
| DMC | Differentiation medium cocktail |
| ECL | Enhanced Chemiluminescent |
| LDL | Low-density lipoprotein |
| HIF1α | Hypoxia-inducible factor 1α |
| GSIS | Stimulated insulin secretion |

Introduction
The liver and adipose tissue, a type of connective tissue containing lipid-rich cells called adipocytes, are important organs regulating lipid metabolism in the whole body. Obesity is a complex disorder characterized by excessive lipid accumulation in adipose and liver tissues and may lead to metabolic syndrome [1]. Recently, obesity has become one of the major health concerns worldwide [2] due to its
noxious effects on the quality of life, career productivity, and social problems. WHO has prioritized reducing the obesity-related burden on societies and diminishing its prevalence [3].

Obesity is predominantly associated with a dramatic increase in adipocyte count (hyperplasia) in adults. On the other hand, adipocyte enlargement (hypertrophy) is a common adaptive mechanism to accommodate excessive energy in the form of triglyceride (TG) in adults [1]. Adipocyte enlargement also serves to preserve adipose tissue buffering capacity to protect other tissues from lipotoxicity [4]. Two types of adipose tissue, “white (WAT)” and “brown (BAT)”, are present in the human body. WAT is the main site of energy storage and an endocrine tissue that produces cytokines and adipokines. In obesity conditions, WAT is associated with inflammation and oxidative stress leading to insulin resistance and ultimately, metabolic syndrome [5, 6]. WAT hyperplasia results in hormonal imbalances and elevated secretion of inflammatory cytokines and adipokines, causing alteration in the normal energy homeostasis which in turn leads to a wide spectrum of diseases. Excessive releases of adipokines can interrupt insulin signaling and cause insulin resistance and eventually type 2 diabetes mellitus (T2DM) [6]. Several molecular mechanisms are responsible for these events. The peroxisome proliferator-activated receptor-γ (PPARγ) has been considered a major regulator in the adipocyte differentiation process. Activated PPARγ induces the expression of CCAAT-enhancer binding protein α (C/EBPα), which triggers the expression of several genes associated with adipocyte differentiation to mature adipocytes [7]. On the other hand, fatty acid binding protein 4 (FABP4), adiponectin, and fatty acid synthase (FAS) are key players in the formation of mature adipocytes [6]. Adipose-derived stem cells (ADSCs) are considered the predominant cell type present in the stromal vascular fraction (SVF) of adipose tissue. ADSCs are a group of mesenchymal stem cells with the ability of self-renewal and differentiation into chondrocytes, adipocytes, myocytes, osteoblasts, and even neurocytes [8]. ADSCs have the advantages of being multipotency, possessing high expansion capacity, being passaged numerous, being cryopreserved for a long time [9], and reflecting donor- and depot-specific characteristics [5]. Moreover, ADSCs’ harvesting procedure is far less painful than that of bone marrow stem cells [8]. These advantages have prompted the use of ADSCs as a promising tool for stem cell differentiation research [3].

Various approaches including dietary regimen, exercise, behavioral modification, and pharmacotherapy are commonly used for lowering body weight. However, these strategies are often ineffective in the long term because they require a constant struggle to succeed [10]. Moreover, although conventional anti-obesity medications are to some extent effective, the presence of undesirable side effects such as insomnia, constipation, headache, and cardiovascular stroke may limit their acceptability [7]. Therefore, finding and developing anti-obesity drugs with minimum adverse effects would be promising. Today, the use of medicinal plants and traditional medicine systems plays a pivotal role in finding new therapeutic molecules. Natural molecules such as anthocyanins, catechins, and some polyunsaturated fatty acids (PUFA) have shown desirable anti-obesity properties [10]. *Nigella sativa* L. known as black cumin is a medicinal plant of the Ranunculaceae family that has been used in Traditional Persian Medicine (TPM) to attenuate liver problems [11, 12] which are important components of metabolic syndrome. *N. sativa* seed is an important ingredient in several multi-herbal oral and transdermal formulations used to manage obesity and steatohepatitis [12, 13]. *N. sativa* seed has been shown to increase both insulin secretion and insulin sensitivity [14, 15]. Thymoquinone (TQ), a natural quinone (C10H12O2) abundantly present in *N. sativa* seeds has been reported to possess anti-inflammatory, antioxidant, antidiabetic, and anti-hyperlipidemic properties [16, 17].

However, to our knowledge, no data on the effect of TQ on cell differentiation and lipid accumulation in ADSCs is available. Therefore, in this study, the effects of TQ on adipocyte differentiation and lipid accumulation in human ADSCs based on the expression of adipocyte-specific markers FAS and PPARγ were investigated.

### Materials and Methods

#### Materials

We purchased thymoquinone (≥99%; CAS No. 490–91-5; CAT No. 274666) from Sigma–Aldrich (Germany), collagen type1, fetal bovine serum (FBS), penicillin-streptomycin (Pen-Strep) from Gibco (USA), AlamarBlue, Dulbecco’s Modified Eagle’s Medium (DMEM), Protease inhibitor cocktail, and indomethacin from Sigma (Germany), mouse anti-human CD45-FITC, mouse anti-human CD34-FITC, mouse anti-human CD44-FITC, mouse anti-human CD90-FITC antibodies, mouse anti-human CD11b-PE, mouse anti-human CD73-PE, β-actin (8H10D10) Mouse antibody, PPARγ (81B8) Rabbit mAb and FAS (C20G5) Rabbit mAb from Cell signaling (USA), Oil Red O from Merck (Germany), dexamethasone from Iran hormone (Iran), insulin from Elixir (Iran) and ECL Western blot detection reagent from Bio-Rad (USA).

#### Cell Culture and Differentiation

Fat samples obtained from liposuction surgery patients aged 38–45 years and body mass index (BMI) ≥ 30 kg/m² were
transferred to the laboratory in sterile conditions. After washing several times with phosphate-buffered saline (PBS) containing 1% antibiotic, the fat samples were digested by collagenase type1 (0.5 mg/mL) at 37 °C for 45–60 min. The cell suspension was centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the pellet was re-suspended in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS, penicillin /streptomycin (Gibco, USA) and incubated at 37 °C with 5% CO₂. The medium was refreshed twice per week to obtain up to 80% confluence [18].

Quantification of Cell Surface Markers by Flow Cytometry

Upon third passages, ADSCs were trypsinized and suspended in PBS (6^10 cells/mL) and subsequently, were incubated with mouse anti-human CD45-FITC, mouse anti-human CD34-FITC, mouse anti-human CD44-FITC, mouse anti-human CD90-PE and mouse anti-human CD11b-PE antibodies and incubated for 30 min at 4 °C in the dark. PBS was added to the stained cells and centrifuged for 5 min at 1200 rpm. Finally, cells were re-suspended in PBS and analyzed by flow cytometry (Becton Dickinson).

Cytotoxicity Assay

ADSCs (5 x 10^3 cells/well) were treated with TQ (6.25, 12.5, 25, 50, 100, 150 and 200 µg/mL), and incubated with 5% CO₂ at 37 °C for 24 h. Afterward, 10 µl of alamarBlue® was added to each well. The intensity of fluorescence was measured at 540 nm excitation and 560 nm emission by an ELISA plate reader (Biotech, USA). Each test was repeated three times.

Oil Red O Staining

Adipogenesis of human ADSCs was assessed by Oil red O staining. ADSCs from the third passage were cultured in 24-well plates and left to reach 70–80% density. The cells were then treated with a differentiation medium cocktail (DMC) containing 1 µM dexamethasone, 10 µM insulin, 100 µM indomethacin (Sigma, Germany) in DMEM medium (Francis et al. 2010), and a maintenance medium cocktail containing 10 µM insulin in DMEM medium in the presence or absence of TQ (6.25, 12.5 and 25 µg/mL).

The DMC was replaced every 2 days till day 21. Then, the cells were fixed with 10% formaldehyde and stained with Oil Red O. Stained lipids were extracted by isopropanol and the absorbance was measured at 540 nm [19].

Western Blot Analysis

After 21 days of adipocyte differentiation, the samples were subjected to western blotting analysis to evaluate the expression level of PPARγ and, FAS proteins. Then cells were harvested and lysed in cell lysis buffer. Equal amount of protein from each sample was loaded onto 10–15% SDS-polyacrylamide gel and subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad, USA). After blocking in 5% skim milk dissolved in TBST (TBS plus 0.1% Tween-20) at room temperature for 2 h, the membranes were incubated with primary antibodies including PPARγ (81B8) Rabbit mAb (1:1000) and FAS (C20G5) Rabbit mAb (1:1000)) diluted in TBST (Tris-buffered saline and tween 20). After washing with TBST, the membrane was probed with secondary antibodies (1:3000 in TBST) for 1 h at 37 °C. β-actin (8H10D10) Mouse antibody was used as control. The protein bands were detected by Enhanced Chemiluminescent (ECL) Flourchem and analyzed with Image J software. Bands were visualized by a chemiluminescent system. Protein expression is normalized against β-actin level.

Statistical Analysis

All data were presented as Mean ± SEM. Statistical analysis was performed using Graph Pad Prism 6.0. One-way analysis of variance (ANOVA) was performed for comparing different groups. P < 0.05 was considered to indicate a statistically significant difference.

Results

Characterization of Adipose Derived Stem Cells

The stem cells isolated from adipose tissue had adhered to the surface of the cultured plate during the first days of culture. The ADSCs rapidly proliferated in vitro and formed fibroblast-like spindles after 5 days of culture (Fig. 1a-c). Flow cytometry analysis demonstrated the expression of CD44 and CD90 and CD73 markers as mesenchymal stem cells (MSCs) markers on the surface of ADSCs. The results also showed the absence of CD45 and CD34, CD11b markers on the surface of ADSCs (Fig. 1d).

Cytotoxicity Assay

At concentrations of 100 (p < 0.05), 150, and 200 µg/mL (p < 0.001), TQ decreased the viability of mesenchymal stem cells, but at concentrations below 100 µg/mL, no significant difference was observed compared to the control group (Fig. 2).
Lipid Accumulation

ADSCs were treated with TQ to investigate its effects on lipid accumulation. The adipogenesis process was evaluated using Oil red O staining after 21 days. As presented in Fig. 3, lipid droplet accumulation was detectable as red vacuoles. The amount of Oil-Red O stained lipid significantly increased in the differentiation group (positive control) compared to the control group (untreated with differentiation medium). Lipid accumulation process significantly decreased at the concentrations of 25 μg/mL \((P < 0.001)\) and 12.5 μg/mL \((P < 0.01)\) of TQ. At the concentration of 6.25 μg/mL no significant decrease in lipid accumulation was observed. The results of the qualitative examination of lipid droplets also showed that the highest amount of fat vacuoles are formed at the concentration of 6.25 μg/mL of TQ (Fig. 3).

Fig. 1 ADSCs under inverted microscope (10x magnification) and Flow cytometric analysis of ADSCs (a) ADSCs 3 days after isolation. b ADSCs 5 days after isolation. c ADSCs 10 days after isolation. d Flow cytometry results showing surface markers of ADSC cells.

ADSCs expressed CD90 and CD44 and CD73 while CD45 and CD34 and CD11b expression was negative which authorizes the characteristics of ADSCs.
Protein Level of PPAR-γ and FAS

Hitherto, the results showed that treatment of ADSCs with TQ decreased lipid accumulation by arresting adipocyte differentiation. Accordingly, it was hypothesized that the protein expression of key regulators responsible for adipocyte differentiation, especially FAS and PPARγ would be inhibited by TQ. To verify this, we performed a Western blot analysis to determine whether TQ suppressed adipocyte differentiation by down-regulating the expression of adipogenic factors, FAS and PPARγ. The results of Western blot analysis showed that TQ at 12.5 ($p < 0.05$) and 25 μg/mL ($p < 0.01$) reduced the FAS/β-actin ratio compared to the positive group. However, at 12.5 ($p < 0.05$) and 25 μg/mL, TQ had no significant effect on the amount of PPARγ protein compared to the positive group while it exhibited a decrease in PPARγ protein at 6.25 μg/mL (Fig. 4).

Discussion

During the past ~50 years, the prevalence of obesity has increased worldwide, reaching pandemic levels. Obesity can increase the risk of diseases such as T2DM, fatty liver disease, hypertension, cardiovascular problems, dementia, etc., thereby leading to disturbances in the quality of life and life expectancy [20]. Adipocyte differentiation plays a key role in the growth of adipose tissue mass and obesity. Accordingly, it is assumed that the inhibition of adipocyte differentiation might be a possible strategy to control obesity and related problems [3]. In the present study, the inhibitory effect of TQ on adipocyte differentiation in ADSCs was evaluated. A brief molecular mechanism for the inhibition of adipocyte differentiation by TQ is shown in Fig. 5.

*Nigella sativa* seed has been used for centuries to combat various diseases such as diabetes, dyslipidemia, and cardiovascular ailments, particularly in the Middle East, Southeast Asia, and the Mediterranean [21]. Pharmacological studies have revealed its hypoglycemic, anti-hypertensive, anti-oxidative, anti-hyperlipidemia, and anti-inflammatory properties. Moreover, *N. sativa* has been shown to moderately reduce body weight, BMI, and waist circumference [21–23].

Thymoquinone, the main component of *N. sativa* seeds can improve serum lipid profile by decreasing total lipids, TG, and low-density lipoprotein (LDL) levels possibly through diminishing hepatic HMG-CoA reductase activity, enhancing arylesterase activity, regulating genes affecting cholesterol metabolism, and alleviating oxidative stress [24, 25]. The important characteristic of TQ is the presence of the lipophilic quinine constituent in its structure which efficiently facilitates the entrance of the compound into cellular and subcellular structures, as well as affecting the intracellular transcription factors and master regulators [26].

TQ could ameliorate metabolic dysfunction caused by HFD-induced obesity through the activation of genes involved in MPK/PGC1α/SIRT1pathway [27]. TQ also improved glucose tolerance and insulin sensitivity and ameliorated inflammation, lipid dysregulation, and diabetic weight gain in the diet-induced obesity murine model of T2DM possibly via activating SIRT-1 and AMPK pathways [14]. As per the reported studies, we hypostatized that TQ
could have beneficial effects in the treatment of obesity. Therefore, we investigated the effects of TQ on adipocyte differentiation and lipid accumulation in an in vitro model of human ADSCs.

Flow cytometry analysis demonstrated the expression of CD44, CD73 and CD90 on the surface of ADSCs as undifferentiated MSCs markers, which confirms the mesenchymal nature of the isolated cells.

In Cytotoxicity Assay, alamarBlue®, a redox indicator, was used to quantify cell viability and proliferation [28]. TQ at concentrations of 6.25, 12.5, 25, and 50 µg/mL, exhibited no significant difference in cell viability compared to the untreated control. Numerous studies showed TQ possesses cytotoxic effects in several cancer cell lines such as breast, liver, cervix, brain, and colon cancer cell lines in higher concentrations via increasing PPAR-γ activity (at doses higher than 20 µg/mL), inhibition of COX-2 expression, and down-regulating the expression of pro-apoptotic genes including Bcl-2, Bcl-xL, and survivin [29, 30]. However, TQ not only has no toxicity in normal cells in low to moderate concentrations but also may protect normal cells against various cytotoxic agents via its antioxidant effect through activation of catalase, superoxide dismutase, glutathione reductase, glutathione-S-transferase and glutathione peroxidase thus preventing lipid peroxidation. Also, TQ could promote the expression of HO-1 gene and enhance phosphorylation of Akt and AMPK-α which in turn leads to phosphorylation of Keap1 serine/threonine residues [26]. It has been reported that inflammation and expression of inflammatory mediators in the WAT and (pre)adipocytes trigger expansion of the WAT by increasing adipogenesis [31]. COX-2 is a key player in regulating adipose inflammation including macrophage recruitment and subsequent immune response and insulin resistance [32]. It is assumed that inhibition of COX-2 in (pre)adipocytes might be a potential target in suppressing WAT expansion and obesity inflammation [31]. TQ has been reported to exert anti-inflammatory and cytotoxic activities through several signaling pathways, predominantly via COX inhibition [33, 34]. COX-2 inhibition can also partly contribute to the anti-inflammatory effect of TQ in preadipocytes and differentiated ADSCs and further its anti-obesity effect.

Oil Red O is a lipophilic dye capable of staining triglycerides and lipids in tissue and cells. The Oil Red O staining is used as a quantitative method to evaluate the alterations in the degree of adipocyte differentiation and the capacity of cultured preadipocytes to differentiate under different experimental conditions [3, 35]. In the Oil-Red O staining test, we observed that lipid accumulation within cells was
significantly decreased at the concentrations of 25 and 12.5 μg/mL of TQ. This is consistent with Shen et al. report in which TQ (1–4 μM) could decrease the lipid content and adipocyte expansion in preadipocytes, 3T3-L1 murine fibroblasts [36]. Moreover, TQ downregulated hypoxia-inducible factor 1α (HIF1α) expression in WAT of high-fat diet (HFD)-fed mice. It is known that the expression of HIF1α and secretion of pro-inflammatory adipokines are triggered by adipocyte hypertrophy-induced hypoxia to mediate obesity-associated insulin resistance [37].

Fatty acid synthase (FAS) is an enzyme that catalyzes the synthesis of long-chain fatty acids in the cytoplasm by condensation of acetyl-CoA and malonyl-CoA, in the presence of NADPH. FAS triggers the formation of mature adipocytes and its inhibition can attenuate preadipocyte differentiation (Fig. 5) [38]. Western blotting analysis revealed that TQ treatment (12.5 and 25 μg/mL) reduced the FAS/β-actin ratio compared to the positive group. Gray et al. also demonstrated that chronic exposure (72 h) to TQ (0.5 μM) decreased the glucose overdose-induced elevation in FAS in rat insulinoma cell, INS-1 832/13 (a useful model for studying the regulation of insulin secretion). Moreover, acute and chronic treatment with TQ and N. sativa extract increased glucose-stimulated insulin secretion (GSIS) which was in accordance with their ability to enhance the ATP/ADP ratio. TQ also attenuated the glucose overload-induced GSIS impairment in β-cells by suppressing the accumulation of malonyl-CoA, increasing acetyl-CoA carboxylase, and fatty acid-binding proteins [39].

PPARγ is a ligand-dependent transcription factor that regulates adipocyte differentiation, the expression of genes responsible for lipid metabolism and glucose homeostasis [40]. In the fasting state, PPARα has been shown to accelerate the formation of fatty acid in adipose tissue of the liver by regulating the expression of apolipoprotein, which leads to an increase in plasma levels of high-density lipoprotein cholesterol and a reduction in levels of low-density lipoprotein cholesterol [41]. Activated PPARγ decreases free fatty acid content in all organs except adipose tissue and circulating blood, thus causing an increase in the capacity of adipose tissue to store TG [42]. In our study, western blotting revealed that TQ exhibited a decrease in PPARγ protein at the low concentration of 6.25 μg/mL. This indicates that low concentrations of TQ can suppress PPARγ expression leading to a decrease in the capacity of adipose tissue to store TG. Higher concentrations of TQ (12.5 and 25 μg/mL) did not significantly affect PPARγ protein expression compared to the positive group which is in harmony with the results of a study conducted by Woo et al. [43]. To our knowledge, this was the first report on the effects of low concentrations of TQ on PPARγ protein expression in ADSCs.

Herein, we provided scientific evidence for the effects of TQ as the main component of N. sativa seed, which is widely used to manage obesity and metabolic syndrome.

Conclusion

Our findings demonstrated the expression of CD44 and CD90 markers as MSCs markers on the surface of ADSCs by Flow cytometric analysis. At concentrations below 100 μg/mL of TQ, there was no significant difference in cell viability of ADSCs compared to the control. Lipid accumulation in ADSCs was significantly reduced at 25 μg/mL (P < 0.001) and 12.5 μg/mL (P < 0.01) of TQ. The qualitative examination of Lipid Droplets was in harmony with these results. Western blot analysis demonstrated that TQ at 12.5 (p < 0.05) and 25 μg/mL (p < 0.01) could decrease the FAS/β-actin ratio compared to the positive group. Moreover, TQ decreased PPARγ protein expression only at the low concentration of 6.25 μg/mL. Overall, the results of the present study revealed that TQ is capable of inhibiting adipocyte differentiation by reducing FAS and to some extent PPARγ proteins expression. Our findings suggest that TQ may serve as a promising candidate for the treatment or prevention of obesity and metabolic disorders in humans.

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Author Contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Z.T.N., S.A.E., B.J., and M.S. The first draft of the manuscript was written by M.S. B.J. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of interest The authors declare no competing interests.

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