Beneficial Effect of 3% Thymoquinone on Stem Cell-Mediated Improvement in Immune System and Anti-Inflammatory Function

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RESEARCH

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ABSTRACT

Background/Aim: Black seed oil containing Thymoquinone (TQ) has therapeutic benefits as a functional food to reverse the harmful effects of a high-fat diet (HFD) in obesity, diabetes, and metabolic syndrome. We studied TQ and its effects on mesenchymal stem cells (MSCs) during differentiation, proliferation and the consequences of TQ and Vitamin D3 on their additive effects on immune-mediated inflammation.

Methods: In in-vitro studies we treated MSCs with TQ, Commercial Black seed Oil (CBSO), and their respective combinations with Vitamin D3, compared to control for seven days. We examined the effects of TQ on MSC proliferation, levels of inflammatory biomarkers, changes in lipid droplet number and size, adipocyte differentiation, mitochondrial biogenesis, and stem cell function. Using C57BL/6J male mice fed a high-fat diet (HFD, 23 weeks) and then divided into three groups: Lean controls, HFD with no intervention, and HFD treated with TQ, for an additional eight weeks, we examined hepatic inflammation and ANG-1 levels.

Results: TQ increased the levels of Mfn-2 and PGC-1α. The addition of Vitamin D3 enhanced these effects. Also, TQ increased ANG-1 and decreased the inflammatory cytokine TNFα. ANG-1 action on MSCs directly affected MSC differentiation but not proliferation while improving immune function with a reduction in TNFα levels. HFD Mice treated with TQ all showed a decrease in lipid droplet size and number.

Conclusion: TQ is a functional food that increases stem cell (MSC) proliferation and significantly reduces the inflammatory state, both alone and with Vitamin D3. TQ had a powerful effect on MSC differentiation and expansion with a reduction in inflammation. TQ offers a potential

therapy for the chronic immune-mediated inflammatory state caused by HFD induced obesity.

Keywords: Functional foods, immune-mediated inflammatory disease, biomarkers, heme oxygenase, cytokines, mitochondrial function and biogenesis, thermogenesis, obesity, high fat diet, fatty liver, nafld, chronic inflammation.

INTRODUCTION

Black seed oil (*Nigella Sativa*) is a plant that is indigenous to Southeast Asia. Multiple reports document the health benefits of TQ on various chronic inflammatory conditions with immune dysfunction, such as obesity, type 2 diabetes, hypertension, rheumatoid arthritis, cancer, neurodegenerative diseases, and digestive tract diseases [1]. Black seed oil is a functional food that goes beyond nutrition; it has shown benefits in both treating and preventing disease (2]. This herbal medicine is known as black cumin, although it is not chemically related to cumin. The main active component is Thymoquinone, for which multiple studies support its antioxidant, anti-inflammatory, and pro-apoptotic properties (3]. Despite several cultures highly regarding black cumin due to its medicinal properties, no long-term studies have examined its safety and optimal dosing regimens for humans. Notably, most of the medicinal properties of black cumin lie in the essential oils of its seeds (4]. N. Sativa seeds are unsaturated fatty acids comprised of linoleic acid (50-60 %, omega-6), oleic acid (~20%, omega-9), and palmitic acid (~14 %, omega-9). Linoleic acid and oleic acid are monounsaturated acids, the latter being the most common fatty acid in nature. Palmitic acid is a saturated long-chain fatty acid. The body can manufacture omega-9, but not omega-3 or omega-6 oils (5]. High fat diets (HFD) have been associated with weight gain, and chronic inflammation that results in heart disease and cancer; HFD can also contribute to increasing the inflammatory state in many chronic diseases like obesity, diabetes and rheumatoid arthritis [5, 15]. HFD can increase LDL and VLDL and total cholesterol, which further contributes to heart disease risk [33].

Cold-pressed black cumin seed oil containing 3% Thymoquinone (TQ) has therapeutic benefits as a functional food that can reverse the harmful effects of a high-fat diet (HFD) in obesity, diabetes, and metabolic syndrome [6]. We now study the beneficial effects of TQ as a functional food in metabolic syndrome, diabetes, and the downstream systemic complications of HFD induced- obesity. Functional foods are foods that are capable of treating and preventing disease in addition to providing nutritional value. TQ as a functional food has been known for decades, but the mechanism of action was unclear. The purpose of this study was two-fold. First, to establish a mechanism by which TQ works and second, to establish the right amount of free fatty acids in the formulation, since there are many commercially available formulations with different free fatty acid content. Mechanistically, we studied TQ and its effects on mesenchymal stem cells (MSCs) during differentiation, proliferation and the consequences of TQ and Vitamin D3 on their additive effects on immune-mediated inflammation.

Thymoquinone makes up approximately 30-48% of the black seed oil. We study the beneficial effects of TQ as a functional food in metabolic syndrome, diabetes, and the downstream systemic complications of HFD induced obesity. A commercial formulation of cold-pressed Nigella sativa oil, ThymoQuin (TQ) with low concentration of free fatty acids (1.8%) and contains about 3 % Thymoquinone.

TQ has been studied recently in the cytokine storm of COVID-19 and shown to have excellent reductions of cytokines with clinical improvement clinical improvement [7-9]. TQ has been shown to reduce the inflammation in asthma [7] and has been beneficial in hypertension, diabetes, bronchitis, headaches, influenza, dizziness, and arthritis [8, 9]. However, the effect of TQ on Mesenchymal stem cells (MSCs) with or without Vitamin D may have a crucial role in modulating immune function in chronic inflammatory diseases [10], and it is well-established that

Vitamin D deficiency increases the risk of immune dysfunction.

MSC are multipotent adult stem cells that can differentiate into multiple tissues, including fat, bone, cartilage, and muscle. In addition, MSCs can modulate inflammatory responses [12, 13]. Chronic or dysregulated inflammation can lead to immune-mediated inflammatory disease [14, 15]. MSCs modulate the inflammatory process and can sense different inflammatory signals [16], either promoting or attenuating the inflammatory response [15, 17]. This is dependent upon the MSC microenvironment, which can switch MSCs between pro-inflammatory and anti-inflammatory phenotypes. Depending on the inflammatory response stage, MSCs can exert pro- or anti-inflammatory effects [14, 18]. Therefore, regulation of MSC differentiation may offer a portal to understand the immunoregulatory plasticity of MSC's via vascular maturation [19].

Plasticity of MSCs allows them to undergo conversion from adipose tissue to endothelial cells and vice versa. We hypothesized that TQ has a direct effect on MSC and regulate adipogenesis and immune system. To address this, we explored the effect of TQ on the immunoregulatory plasticity of MSCs via the upregulation of Angiopoietin-1 (ANG-1).

METHODS

Human bone marrow-derived adipocyte mesenchymal stem cells

Human bone marrow-derived mesenchymal stem cells were from All Cells (Emeryville, CA). After thawing, cells were resuspended in α-minimal essential medium (α-MEM, Invitrogen, Carlsbad CA) as described in [21]. Upon confluence, MSCs were recovered by adding 0.25% trypsin/EDTA (Invitrogen, Carlsbad, CA). MSCs (Passage 2–3) were plated as described by Shen [6].

On day 7, cultures were switched to treatment medium: MSC Basal Medium supplemented with an MSC growth kit (ATCC). In addition, cells were treated for 4 days with ThymoQuin (TQ, Black seed oil, from TriNutra Ltd. which contains 3% Thymoquinone, 1.3% p-Cymene and 1.8% Free Fatty Acid), Commercial Black Seed Oil (CBSO), Vitamin D3, TQ, and Vitamin D3 in their respective concentrations CBSO + Vitamin D3, all compared to control. After 4 days, the supernatants were recovered. The medium was replaced as previously described [21].

Oil Red O staining

We used red oil staining to examine the effect of TQ on generating small healthy adipocytes, using 4 groups of cultured MSC-derived adipocytes as follows: group 1: control grown in regular MSC media (n=4); group 2: treated with CBSO (n=4); group 3: treated with Vitamin D3 (n=4); group 4: treated with TQ; group 5: treated with TQ + Vitamin D3 (n=4). Oil Red O staining was conducted using 0.5% Oil Red O solution (Sigma-Aldrich, St. Louis, MO). Briefly, adipocytes were fixed as previously described [21]. We used bright-field microscopy to visualize staining (Olympus Microscopes, Center Valley, PA, USA) [30]. We analyzed lipid droplet size with Image-Pro (advance Imaging Concept, Inc., Princeton, NJ, USA) [6].

Animal Protocols

All animal experiments followed an institutionally approved protocol in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight-week-old C57BL/6J mice were divided into five groups of 4 animals each as follows: group (1) lean controls; group (2) fed HFD; group (3) HFD and treated for eight weeks with cold-pressed Thymoquinone oil (TQ); group (4) HFD and treated for eight weeks with Vitamin D3; and group (5) HFD and treated for eight weeks with cold-pressed Thymoquinone oil (TQ) and Vitamin D3. The mice were fed their respective diets for 23 weeks before being fed the indicated treatments for the last eight weeks. Control mice (group 1) were fed ad libitum a normal diet containing 11% fat., 62% carbohydrate and 27% protein with a total calorie count of 12.6KJ/g. The remaining animals (groups 2,3,4,5) were fed a high-fat diet containing 58% fat (from lard), 25.6% carbohydrate, and 16.4% protein for a total calorie count of 23.4KJ/g. (Bio-SERV, Frenchtown,

NJ) for 23 weeks. After 23 weeks of HFD diet, TQ was added to HFD for 8-weeks. TQ oil was mixed into the HFD food and made into pellets using a mixer. The mice were sacrificed at the end of the experiment, and liver tissue was dissected for western blot analysis. Liver samples were frozen in liquid nitrogen, pulverized in a mortar and pestle, centrifuged, and the supernatants saved for western blot as in (22).

Western-blot analysis

MSCs cultured in T75 flasks were washed with PBS and trypsinized (0.05% trypsin w/v with 0.02% EDTA). Cells were scraped and lysed as previously described in T-PER lysis buffer containing protease and phosphatase inhibitors (Complete TM Mini and PhosSTOP TM, Roche Diagnostics, Indianapolis, IN, USA). Livers were dissected and lysed in radioimmunopreci pitation assay (RIPA) lysis buffer (RIPA, Sigma-Aldrich, St. Louis, MO) as previously described (21).

The following primary antibodies was used to measure Mfn-1, SDF-1, ANG-1, TNFα, and β-Actin (Cell Signaling Technology, Danvers, MA, USA) and PGC1-α (Santa Cruz Biotechnology) and immunoreactivity signals were determined as previously described (21, 31).

Statistical analysis

Data are presented as mean ± standard error (SE) across the experiments. A p-value <0.05 was considered significant. The null hypothesis was tested for comparison between treatment groups by single-factor analysis of variance (ANOVA) for multiple groups or unpaired t-test for two groups.

RESULTS

TQ decreased lipid droplet size and adipogenesis in stem cells

The effect of TQ on adipogenesis was examined by utilizing MSC-derived adipocytes. We counted cells with lipid droplets in the cytoplasm and stained positive with Oil Red O (ORO). Figure 1A shows representative images for the different treatment groups and corresponding quantitative analysis. Treatment with Commercial Black Seed Oil (Commercial TQ) resulted in significant ORO staining and increased average lipid droplet size compared to control. ThymoQuin alone (TQ) preparation was highly effective in reducing average lipid droplet size, even more than Vitamin D3 alone. The combined treatment with TQ and Vitamin D3 showed the most significant reduction in average lipid droplet size (*p<0.05). Remarkably, commercial TQ increased TNFα levels, whereas ThymoQuin (TQ) markedly reduced the expression of this inflammatory cytokine (Figure 1B). Therefore, since our findings clearly showed a pro-adipogenic and pro-inflammatory effect of commercial TQ, we focused on examining the impact of our preparation of ThymoQuin (TQ) in the next series of studies. The results in Figure 2 show that ThymoQuin (TQ) markedly reduced the size of large lipid droplets and effectively increased the small lipid droplet area. Both these effects were enhanced when TQ was combined with Vitamin D3.

TQ increased mitochondrial biogenesis and energy metabolism, and reduced inflammation in stem cells

MSCs were grown in the presence of TQ, and the expression levels of markers of mitochondrial biogenesis and energy metabolism were evaluated by Western blot. In the presence of TQ, there was a marked increase in protein expression levels of Mfn-2 and UCP-1 (Figure 3A-B), comparable to the effects exerted by Vitamin D3 alone. Again, the combination of TQ and Vitamin D3 produced a more significant response than with either of these agents alone (Figure 3A-B). These findings suggest increased mitochondrial activity via increased amounts of mitochondria. In addition, we observed a similar pattern of response to TQ when protein levels of PGC1-α were examined: marked increase in the presence of TQ alone, which was enhanced in the presence of Vitamin D3 (Figure 3C; n=4, *p < 0.05). Notably, treatment with TQ alone resulted, like Vitamin D3, in a substantial reduction of TNFα protein levels, an effect that was significantly enhanced when TQ and Vitamin D3 were combined (Figure 3D). This is the opposite from what we observed with CBSO, which

increased TNFα expression (Figure 1B) and showed a strong anti-inflammatory effect of TQ.

TQ's effect on ANG-1 and SDF-1 levels in mouse hepatic tissue

As detailed under 'Animal Protocols', eight-week-old C57BL/6J mice were divided into three groups (n=4 each): group 1, lean control; group 2, HFD; group 3, HFD and treated for eight weeks with cold-pressed Thymoquinone oil (TQ). We assessed angiogenesis in mouse liver tissue using the biomarkers ANG-1 and SDF-1. The lowest levels of ANG-1 and SDF-1 were observed in the HFD group, whereas the livers from mice fed HFD and treated with TQ showed high levels of ANG-1 and SDF-1 (Figure 4A-B). This suggests that TQ could induce angiogenesis in vivo in hepatic tissue.

Finally, we showed that increasing doses of ANG-1 (Figure 5) can directly affect the size of lipid droplets, increasing the area of small lipid droplets and reducing large ones, both in a dose-dependent fashion. This supports the notion that ANG-1 contributes to the observed effect of TQ on lipid droplet size.

DISCUSSION

In this study, we provide evidence supporting the beneficial effects of TQ as a functional food to regulate adiposity. Functional foods are foods that supply more than nutrition, foods that are capable of treating and preventing disease. This has been known about TQ for decades, but the mechanism of action was unclear. The action of TQ as a functional food seems to occur, at least in part, by a direct influence on MSC differentiation, with the ability to reprogram the plasticity of these stem cells to generate brown-like adipocytes, which translates into decreased proliferation of adipose tissue and an increased number of small lipid molecules. Our data suggest that TQ limits adipogenesis, increases energy expenditure, and improves mitochondrial signalling and biogenesis. We also provide evidence indicating a strong anti-inflammatory effect of TQ at the level of MSCs and by reducing hepatic inflammation and regulating energy expenditure in the liver.

Increased MSC differentiation into adipose tissue from a HFD initiates inflammation, heralded by ROS, macrophage infiltration, and subsequent organ damage, primarily due to mitochondrial dysfunction and destruction [23]. Thus, modulating vascular maturation offers an excellent avenue to target immune-mediated inflammation of obesity [19].

Notably, TQ was effective in regulating adipose tissue plasticity before adipocyte generation, by increasing the expression of ANG-1. Importantly, our findings show that obesity causes a reduction in ANG-1. This reduction was reversed by treatment with TQ, in support of the notion that TQ affects MSC plasticity by altering MSC differentiation. TQ increases mitochondrial numbers and function, increases thermogenesis, and promotes adipocyte conversion to a healthy "beige" phenotype. ANG-1 is a potent suppressor of adipogenesis and is secreted by vascular pericytes surrounding the endothelium [19, 24]. We have previously shown the "crosstalk" between blood vessels and the surrounding adipose tissue [25]. The TQ-mediated increase in ANG-1 promotes this crosstalk, enabling adipose tissue remodeling to a healthier phenotype. MSCs can differentiate into adipose tissue; however, unregulated differentiation from a HFD leads to the accumulation of ROS and macrophages, promoting intracellular damage. The increased ANG-1 limits this mesenchymal stem cell (MSC) differentiation into adipocytes and promoting maturation of the vasculature, while attenuating adipose tissue plasticity and adipose tissue remodeling [19, 26]. In this study, TQ supplementation promoted the development of the vasculature surrounding the MSCs and adipose tissue, evidenced by the increased levels of ANG-1. The present study provides evidence that TQ can affect the immune system by upregulation of ANG-1.

Stromal cell-derived factor-1 (SDF-1) promotes the activation of signaling pathways necessary for stem cell migration and proliferation [27]. The significance of SDF-1 in maintaining MSC proliferation was thought to be essential

for limiting MSC plasticity. In the present study, we did not find a statistically significant effect of TQ on SDF-1 levels.

Clearly, unregulated adipocyte expansion causes aberrations in immune function from increased ROS; ROS and inflammatory cytokines reduce circulating endothelial progenitor cells [12, 28, 29]. Oxidative stress impairs MSC function, leading to unregulated adipogenesis and MSC-derived adipocyte differentiation [30-33]. Therefore, we can infer that TQ might hold a substantial role as a functional food in battling the negative consequences of unregulated ROS, since dietary TQ attenuated the detrimental effects of a HFD.

TQ upregulation of HO-1 might be responsible for the decreased production of TNFα, consistent with previous reports [34, 35]. Importantly, we discovered that TQ also increases the mitochondrial enzyme Mfn-2, required to improve mitochondrial function. The increase in the uncoupling protein UCP-1 and the HO-1 nuclear coactivator PGC-1α decreases lipid droplets and increases energy expenditure, further reducing inflammation. Our findings indicate that TQ acts upstream of PGC-1α and Mfn-2, resulting in increased thermogenic genes, mitochondrial signaling and biogenesis, and lipid metabolism. In association with an increase in UCP-1 and PGC-1α, this increase could reduce lipid droplet formation and limit ROS production, as TQ could contribute to the rise in energy expenditure. PGC-1α regulates adipocyte differentiation. TQ upregulates PGC-1α expression suggesting that TQ can regulate adipocyte progression through PGC-1α signaling. These novel findings highlight the operation of a TQ-PGC-1α interplay and suggest a significant role in adipocyte differentiation and proliferation.

The epidemic of obesity continues unabated, and we must direct therapy at decreasing adipocyte production and expansion and prevent the resultant chronic inflammatory state. Reducing inflammation is critical to prevent cardiovascular complications of obesity, diabetes, and metabolic syndrome [36, 37]. Dysfunctional adipocytes are responsible for the inflammation that results in damage to the endothelium [38].

Our novel findings support the operation of a TQ-HO-1-PGC-1α triad that can regulate adipogenesis at the level of the MSC. We propose to regulate this using Thymoquinone, with high levels and low fatty acid content, as a functional food to reverse disease and prevent inflammation (see Figure 6). Nigella sativa seed oil, with its active component TQ, is the very potent herb to effectively reduce the chronic inflammatory state of obesity and reduce the incidence of obesity-related cardiovascular complications. The addition of Vitamin D3 offers the potential for synergy with TQ. PGC-1α is the nuclear co-activator of heme oxygenase (HO-1), showing the effect is also the upregulation of the heme oxygenase system and its effect on the redox state [31]. Upregulation of the heme oxygenase system is key to the reversal of the chronic inflammatory state [39].

In summary, there are many black seed oil cold press preparations that are currently on the market. TQ is an excellent functional food that can help to address obesity and the resultant chronic inflammatory state it produces. TQ must have a low fatty acid content (2% or less) to be successful as a functional food. Making certain the public understands how to interpret the free fatty acid content of different TQ preparations will move this discussion forward with the public understanding how TQ acts as a functional food in obesity, metabolic syndrome and diabetes.

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PEER REVIEW

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Figure 1A

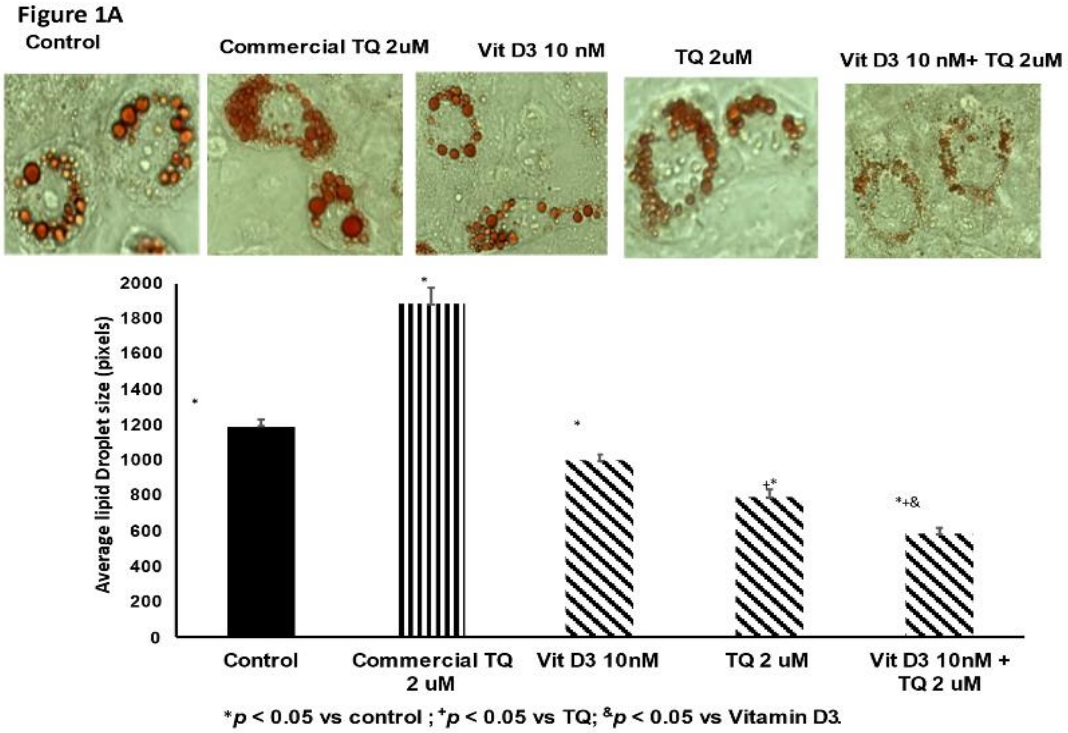

Figure 1B

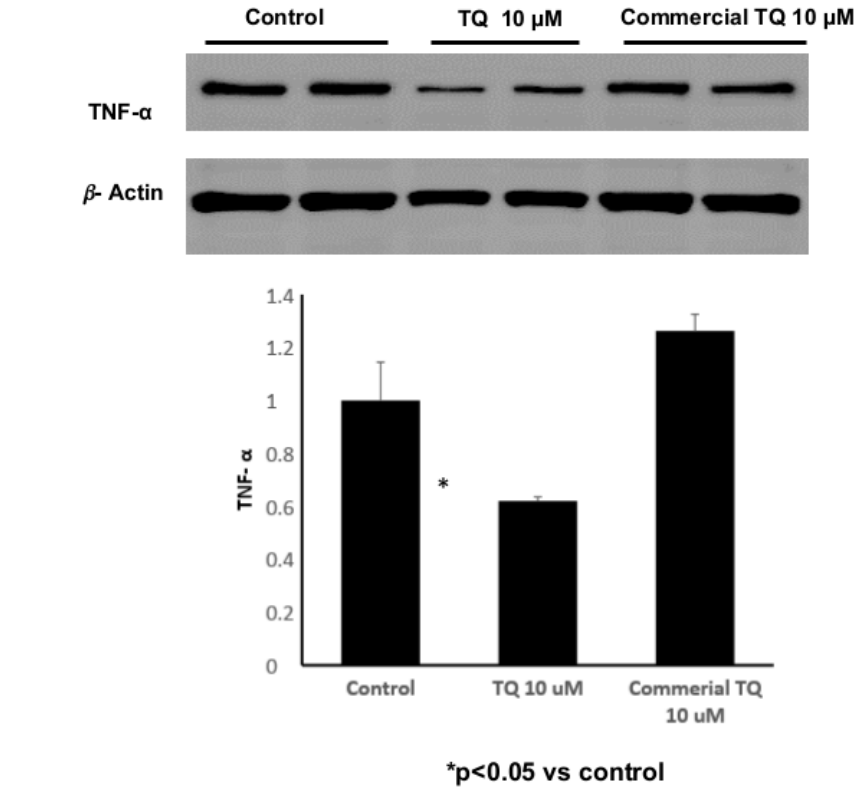

Figure 1. **A** Shown are representative images of adipocytes derived from MSCs and subjected to the indicated treatments for 7 days. Lipid droplet size was measured in all groups; corresponding quantifications are shown in the bar graph (n= 4, *p< 0.05 compared to control). **B** Western blot showing the effects of TQ and CBSO on TNFα levels. n=4 *p< 0.05 compared to control.

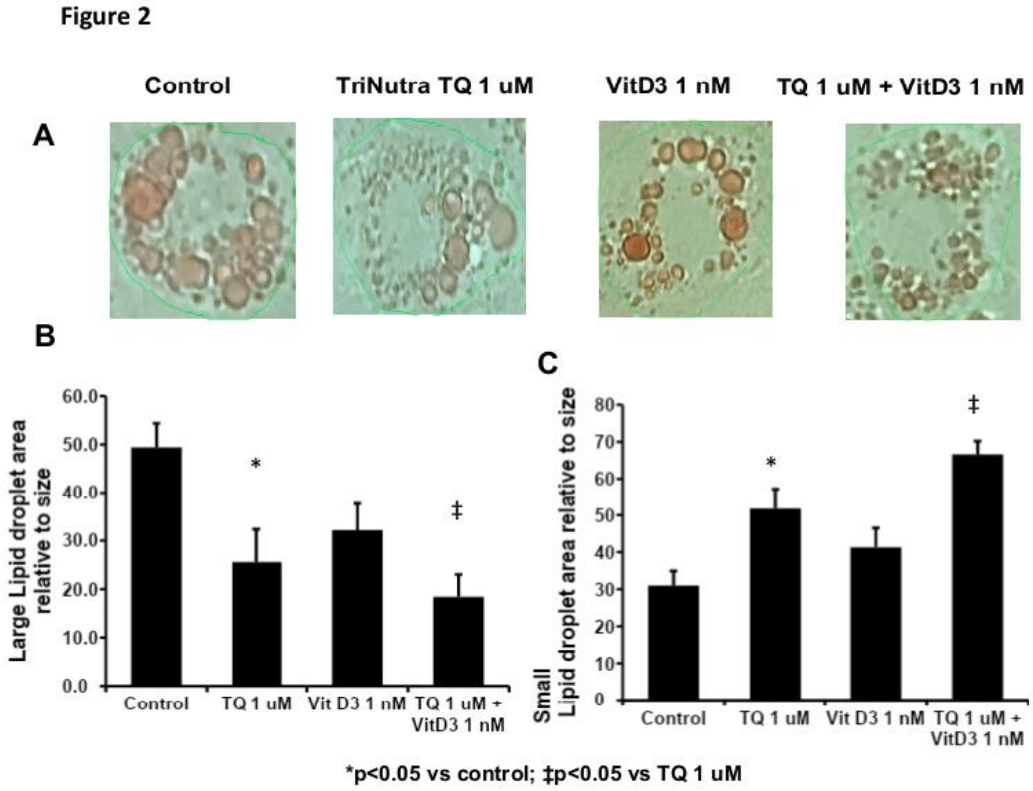

Figure 2. A Shown are representative images of adipocytes derived from MSCs and subjected to the indicated treatments for 7 days. Large and small lipid droplet sizes were measured in all groups; corresponding quantifications are shown in the bar graphs in panels **B** and **C** (n= 4, *p< 0.05 compared to control).

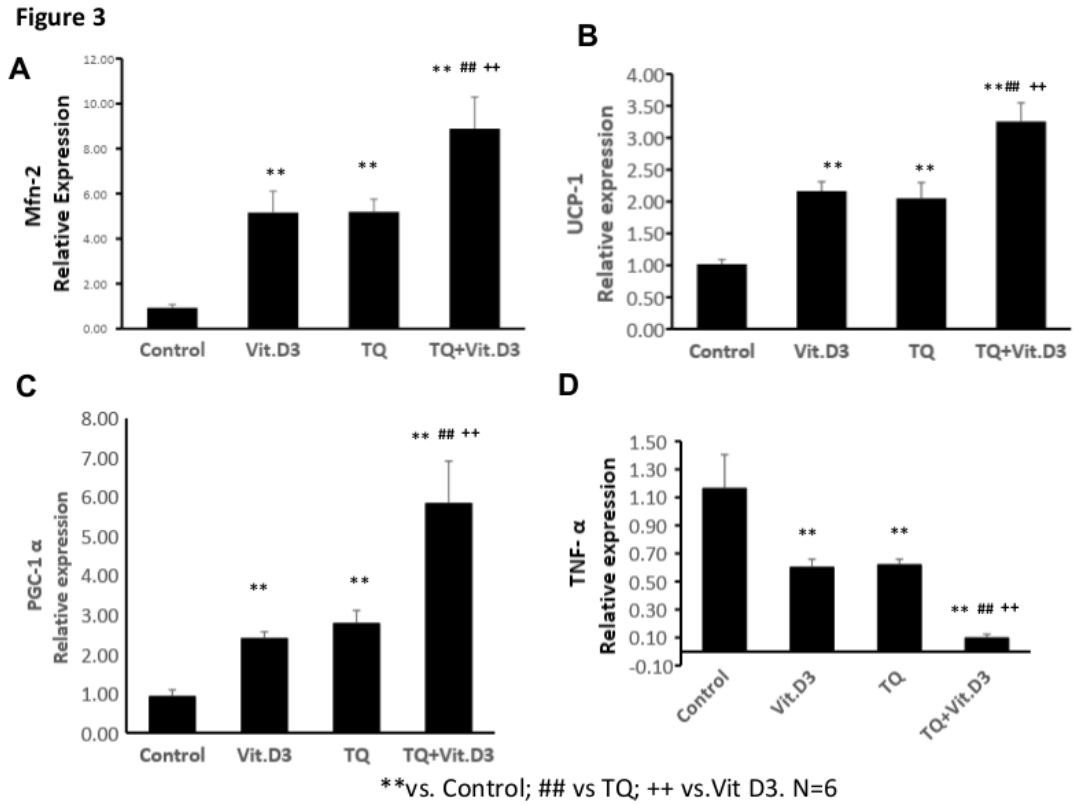

Figure 3. Densitometric quantification of the effect of TQ on the protein expression levels of MSC production of key regulators of immune mediated inflammation and mitochondrial biogenesis. Shown are average normalized densitometric values for Mitofusion-2 (Mfn-2, panel **A**), UCP-1 (panel **B**) and peroxisome proliferator-activated receptor gamma coactivator (PGC-1α, panel **C**). n=4 *p< 0.05 compared to control.

Figure 4

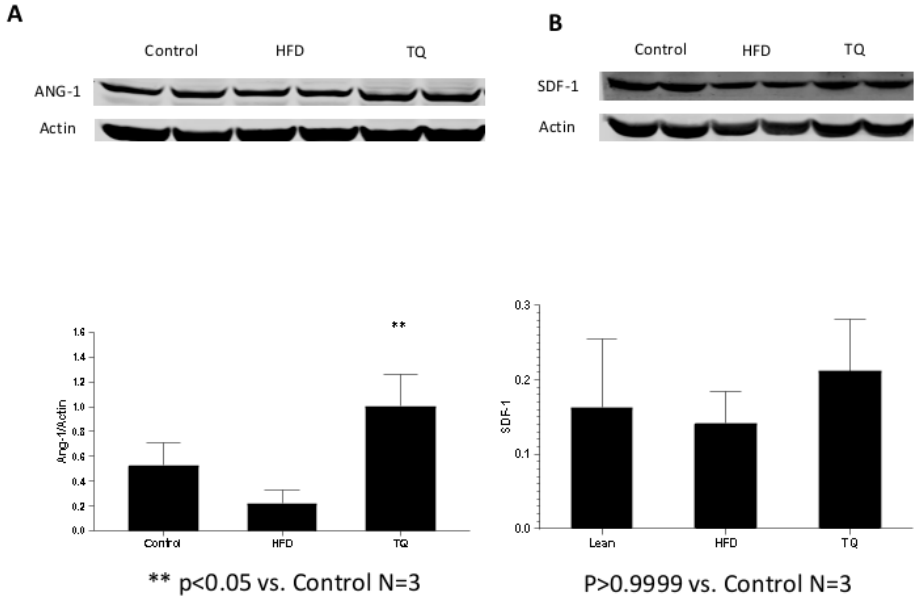

Figure 4. Eight-week-old C57BL/6J mice were divided into four groups of 4 animals each as follows: group (1) lean controls; group (2) fed HFD; group (3) HFD and treated for 8 weeks with cold-pressed ThymoQuin oil (TQ). Mice were fed their respective diets for 23 weeks before being fed the indicated treatments for the last 8 weeks. At the end of the experimental period, the mice were sacrificed, and liver tissue dissected for western blot analysis of angiopoietin-1 (ANG-1, panel A) and proliferation marker Stromal Derived-Factor-1 (SDF-1, panel B). Corresponding normalized densitometric quantifications are shown (n=3, *p< 0.05 vs. control).

Figure 5

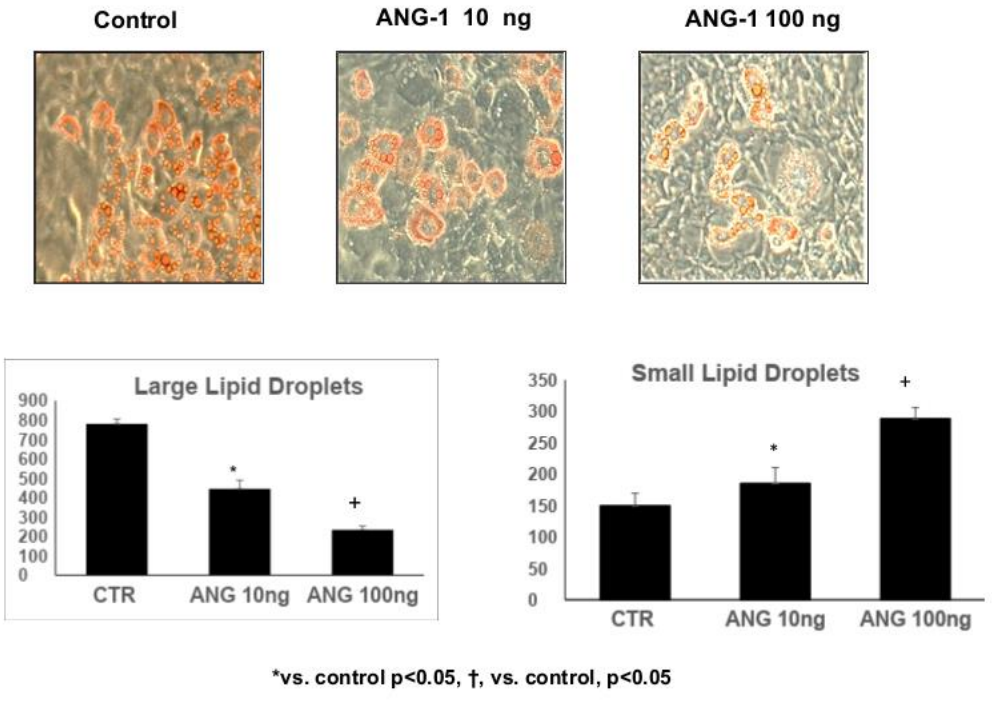

Figure 5. Shown are representative images of adipocytes derived from MSCs and subjected to the indicated doses of ANG-1 for 7 days. Lipid droplet size (large and small) was measured in all groups; corresponding quantifications are shown in the bar graphs (n= 3, *p< 0.05 compared to control).

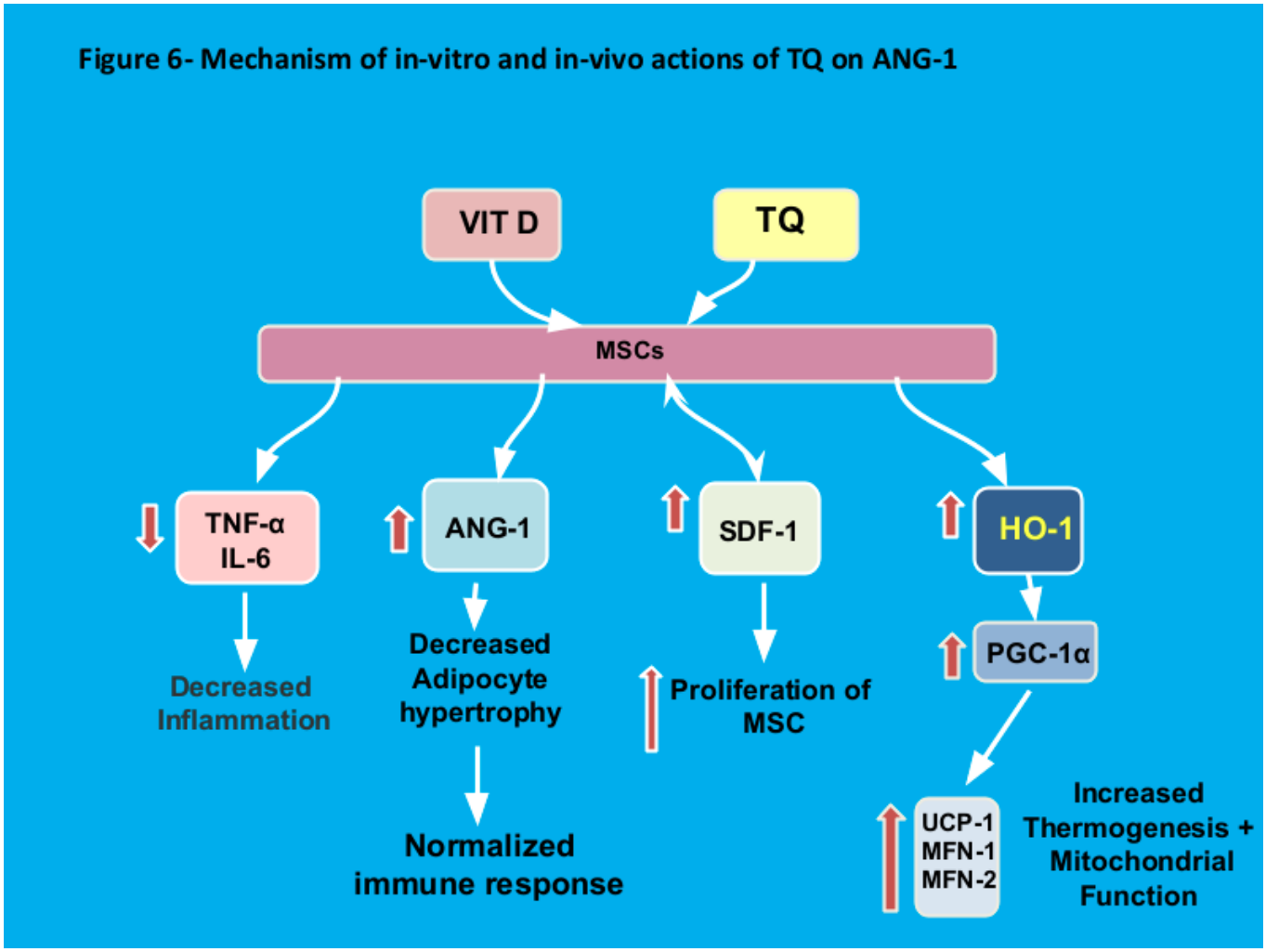

Figure 6. Diagram showing the effect of TQ on bone marrow derived MSCs, reducing adipogenesis and inflammation, and increasing thermogenesis and mitochondrial function, both *in vitro* and *in vivo.*