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RETRACTED: Cold-Pressed *Nigella Sativa* Oil Standardized to 3% Thymoquinone Potentiates Omega-3 Protection against Obesity-Induced Oxidative Stress, Inflammation, and Markers of Insulin Resistance Accompanied with Conversion of White to Beige Fat in Mice

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Received: 8 April 2020; Accepted: 1 June 2020; Published: 4 June 2020; Retracted: 20 June 2025



Abstract: Excessive lipid accumulation in white adipose tissue (WAT) results in adipocyte hypertrophy and chronic low-grade inflammation, which is the major cause of obesity-associated insulin resistance and consequent metabolic disease. The development of beige adipocytes in WAT (browning of WAT) increases energy expenditure and has been considered as a novel strategy to counteract obesity. Thymoquinone (TQ) is the main bioactive quinone derived from the plant *Nigella Sativa* and has antioxidative and anti-inflammatory capacities. Fish oil omega 3 (ω3) enhances both insulin sensitivity and glucose homeostasis in obesity, but the involved mechanisms remain unclear. The aim of this study is to explore the effects of TQ and ω3 PUFAs (polyunsaturated fatty acids) on obesity-associated inflammation, markers of insulin resistance, and the metabolic effects of adipose tissue browning. 3T3-L1 cells were cultured to investigate the effects of TQ and ω3 on the browning of WAT. C57BL/6J mice were fed a high-fat diet (HFD), supplemented with 0.75% TQ, and 2% ω3 in combination for eight weeks. In 3T3-L1 cells, TQ and ω3 reduced lipid droplet size and increased hallmarks of beige adipocytes such as uncoupling protein-1 (UCP1), PR domain containing 16 (PRDM16), fibroblast growth factor 21 (FGF21), Sirtuin 1 (Sirt1), Mitofusion 2 (Mfn2), and heme oxygenase 1 (HO-1) protein expression, as well as increased the phosphorylation of Protein Kinase B (AKT) and insulin receptors. In the adipose tissue of HFD mice, TQ and ω3 treatment attenuated levels of inflammatory adipokines, Nephroblastoma Overexpressed (NOV/CCN3) and Twist related protein 2 (TWIST2), and diminished adipocyte hypoxia by decreasing HIF1α expression and hallmarks of beige adipocytes such as UCP1, PRDM16, FGF21, and mitochondrial biogenesis markers Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), Sirt1, and Mfn2. Increased 5′ adenosine monophosphate-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC)

This paper has been retracted.
A retraction notice was published on 20 June 2025 in *Antioxidants* **2025**, *14*, 758; doi:10.3390/antiox14070758

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phosphorylation and HO-1 expression were observed in adipose with TQ and ω3 treatment, which led to increased pAKT and pIRS1 Ser^{307} expression. In addition to the adipose, TQ and ω3 also increased inflammation and markers of insulin sensitivity in the liver, as demonstrated by increased phosphorylated insulin receptor (pIR tyr^{972}), insulin receptor beta (IRβ), UCP1, and pIRS1 Ser^{307} and reduced NOV/CCN3 expression. Our data demonstrate the enhanced browning of WAT from TQ treatment in combination with ω3, which may play an important role in decreasing obesity-associated insulin resistance and in reducing the chronic inflammatory state of obesity.

Keywords: obesity; inflammation; thymoquinone; omega 3; beige adipocyte; insulin resistance

1. Introduction

Overweight individuals and obesity are growing worldwide public health concerns and pose a significant healthcare burden that warrants further investigation [1]. The major cause of obesity results from the imbalance of excess caloric intake and insufficient energy expenditure that may contribute to adipose tissue accumulation, expansion, and hypertrophy [2]. Increased adiposity is accompanied by the pathological impact of adipocyte hypoxia, leading to the activation of hypoxia-inducible factor 1α (HIF1α) and secretion of proinflammatory adipokines/cytokines such as leptin, interleukin (IL)-6, tumor necrosis factor (TNF) α, and nephroblastoma overexpressed (NOV/CCN3) [3–5]. These adipokines/cytokines play a pivotal role in the development of obesity-associated metabolic dysfunction such as insulin resistance, type 2 diabetes, hypertension, cardiovascular diseases, and certain types of cancer [6,7]. Thus, understanding the molecular and cellular mechanisms regulating adipogenesis and adipose inflammation is crucial for the management of obesity.

Three distinct kinds of adipose tissue have been found in mammals: white adipose tissue (WAT), brown adipose tissue (BAT), and beige adipose tissue [8]. Although WAT exists in subcutaneous depots, a much stronger link has been made between metabolic disorders, insulin resistance, and expansion of WAT deposits in the viscera [9,10]. Additionally, in humans, the expression pattern of browning genes is greater in visceral white adipose tissue (vWAT) than in subcutaneous white adipose tissue (sWAT,) thus further addressing the importance of vWAT browning to combat obesity in humans [10]. The main function of WAT is to store triglycerides and secrete adipokines, while BAT and beige adipose tissue are abundant in mitochondria and generate heat for thermogenesis via uncoupling protein 1 (UCP1) [11]. UCP1 is located in the inner mitochondrial membrane and possesses thermogenic potential by uncoupling the respiratory chain, allowing for rapid glucose and fatty acid oxidation with low ATP production [12]. A previous study indicated that the downregulation of UCP-1 accelerated the development of obesity in response to a high-fat diet (HFD) [13], while ectopic overexpression of UCP1 was related to WAT-reduced obesity and improved insulin sensitivity [14]. Besides UCP1, browning of WAT regulates genes including PR domain containing 16 (PRDM16), fibroblast growth factor 21 (FGF21), and peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α). Additionally, PGC1α activity is tightly regulated by energy sensors, 5′ adenosine monophosphate-activated protein kinase (AMPK), and silent mating type information regulation homolog (Sirt1) to control energy expenditure in the mitochondria [15]. Sirt1 downregulation results in insulin resistance. Thus, promoting WAT browning to beige adipose tissue offers a promising approach for the management of obesity and metabolic disorders.

Due to the adverse cardiovascular effects of anti-obesity medications, there is an emerging interest in bioactive dietary compounds that alleviate obesity-associated metabolic dysfunctions [16]. Polyphenols such as resveratrol [17], berberine [18], thymoquinone [19], and curcumin [20] have been reported to increase antioxidant genes and heme oxygenase 1 (HO-1) levels, which exert beneficial effects via increased insulin sensitivity and lipid metabolism in mice fed a high-fat diet (HFD). Thymoquinone (TQ) is the major bioactive constituent of black cumin and has been widely utilized for its anti-inflammatory,

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antioxidant, and anti-hyperglycemic effects [21,22]. Moreover, TQ has been shown to reduce hepatic glucose production and protect β-cells from oxidative stress in a streptozotocin-induced type 2 diabetes model [23]. TQ administration has improved mitochondrial biogenesis and oxygen consumption as well as decreased hepatic lipid accumulation in HFD mice [19]. Coadministration of TQ and turmeric at low doses further improved metabolic dysfunction of fructose-fed mice [24]. Fish oil is derived from the tissues of oily fish. It is rich in omega-3 polyunsaturated fatty acids (ω3) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and has been widely used as a natural hypolipidemic to reduce cardiovascular disease risk as well as prevention of obesity [25]. ω3 treatment attenuates the accumulation of body fat by reducing hypertrophy and hyperplasia of fat cells [26]. This metabolic switch in WAT lowers triglycerides and induces mitochondrial biogenesis [27]. Fish oil mainly reduces triglycerides and inflammation, but the results have been variable [28–30].

Prescription-strength omega-3 fatty acids typically contain both EPA and DHA. A newer version of the drug icosapent ethyl (ethyl eicosapentaenoic acid) has been much more successful than other formulations of omega-3-fatty acids. It is an Federal Drug Administration (FDA)-approved purified form of the EPA ethyl ester and is used to treat hypertriglyceridemia [31,32]. The data are not yet complete on the reduction of inflammation or ischemia, but they show promise.

We previously reported that 3% TQ treatment reduced obesity-related hepatic steatosis and insulin resistance in HFD mice [19]. However, strategies need to be developed to improve obesity-related metabolic syndromes with lower doses of drugs and decreased adverse effects. Thus, the objective of the present study was to investigate the potential effects of thymoquinone (TQ) in combination with omega-3 (ω3) on the metabolic profiles, inflammation, conversion of white to beige adipocytes and markers of insulin sensitivity in vivo and in vitro, and to explore the involved underlying mechanisms.

2. Methods

The formulation of thymoquinone, obtained from TriNutra, Ness Ziona Israel, is as follows: TQ 3.0%, p-cymene, 1.24%, carvacrol 0.08%, free faty acids (FFA) 1,29%, oleic acid 21.53%, palmitic acid 11.31%, linoleic acid 57.44%, other fatty acids 1.98%, and TPGS, 0.8%. The formulation of omega-3 oil, obtained from GC Rieber, Bergen, Norway, is as follows: eicosapentaenoic acid (EPA) 400 mg/g (min) as omega-3-ethyl esters/triglycerides (EE/TG) and docasahexaenoic acid (DHA) 300 mg/g (min) as EE/TG. TQ 3% and ω3 2% oil were mixed into the HFD food and made into pellets using a mixer.

2.1. Cell Culture and Differentiation

3T3-L1 murine fibroblasts were obtained from the American Type Culture Collection (Rockville, MD, USA). After thawing, cells were resuspended and seeded in DMEM, supplemented with 10% heat-inactivated bovine calf serum (BCS) at 37 °C in a humidified 5% CO_2 atmosphere for two days until confluent (Experimental protocol as Figure 1A). Adipogenesis was achieved as previously described [33]. In detail, the medium was replaced with adipogenic medium (Dulbecco's modified Eagle's medium (DMEM) with high glucose (Invitrogen) supplemented with 10% (*v*/*v*) FBS, 10 µg/mL insulin (Sigma–Aldrich, St. Louis, MO, USA), 0.5 mM dexamethasone (Sigma–Aldrich), and 0.1 mM indomethacin (Sigma–Aldrich), and the cells were cultured for an additional seven days during which time the medium was replaced every two to three days.

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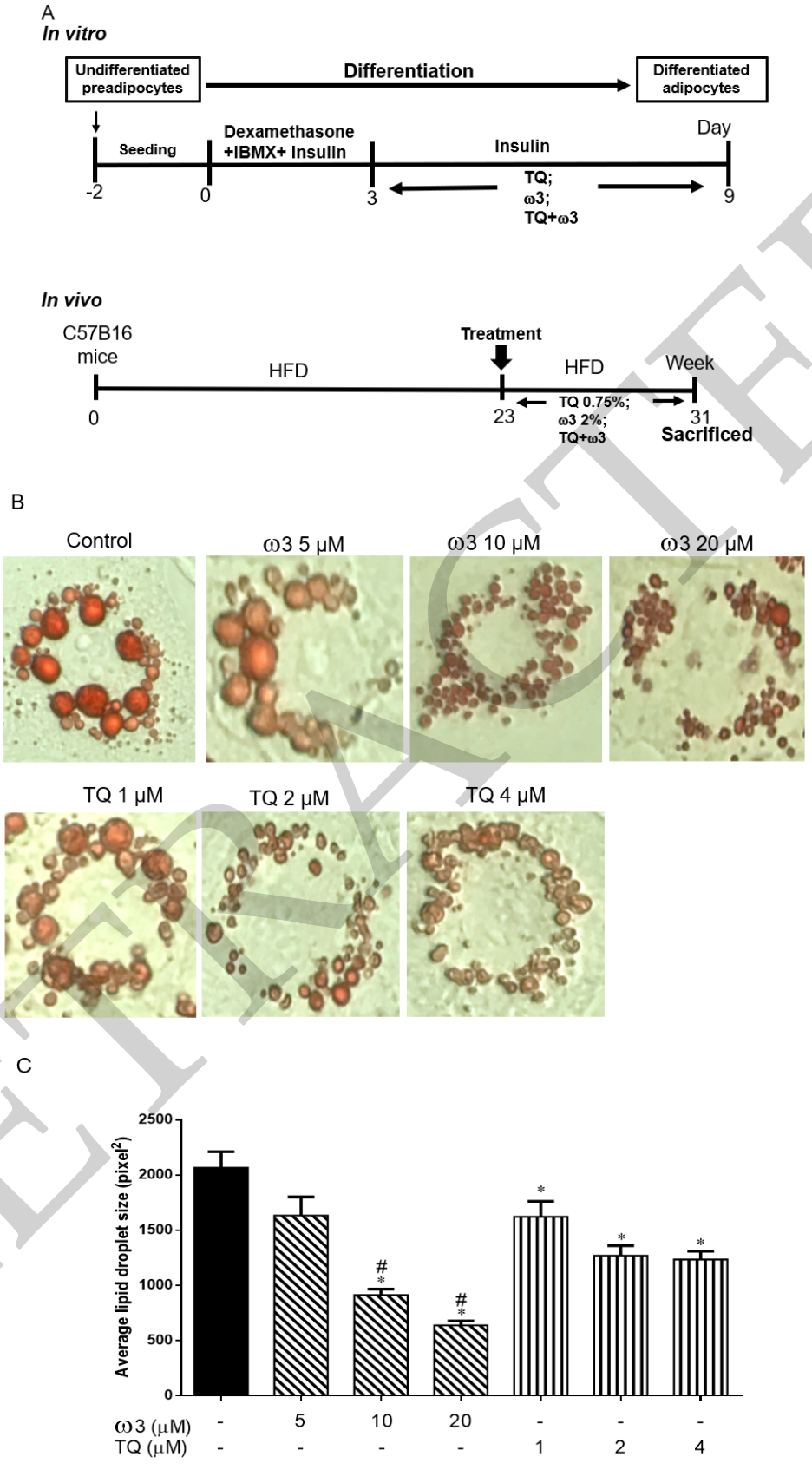


Figure 1. *Cont.*

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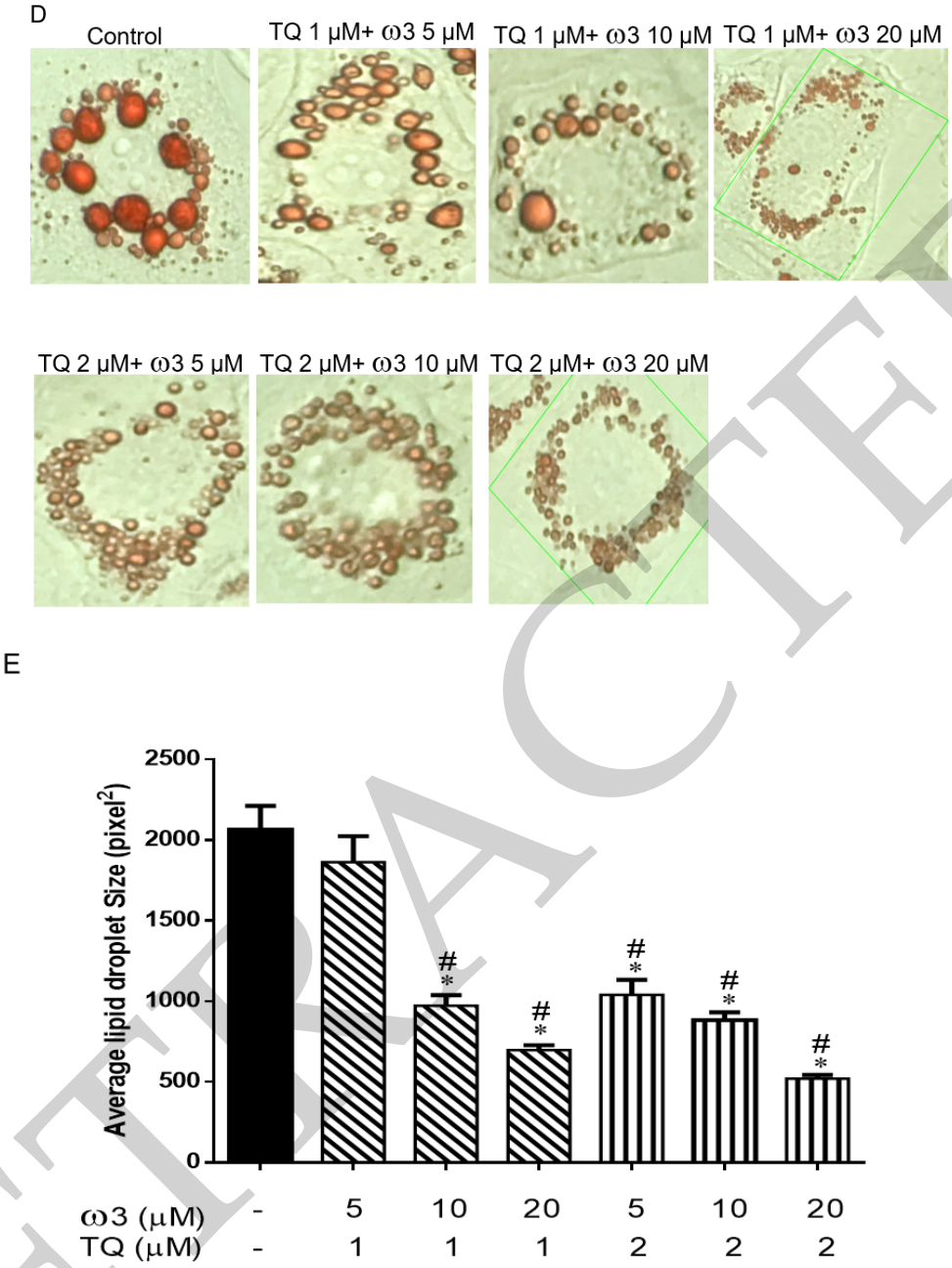


Figure 1. Scheme of the experimental protocol and dose-dependent effects of thymoquinone (TQ) and omega 3 (ω3) treatment on lipid droplets in 3T3-L1 adipocytes. Scheme of the in vitro and in vivo protocol (**A**). Representative and graph of the cells treated with TQ (1–4 μM), ω3 (5–20 μM) (**B**,**C**), and a combination of TQ (1–2 μM) and ω3 (5–20 μM) (**D**,**E**) for six days ($n = 4$); * $p < 0.05$ vs Control; # $p < 0.05$ vs ω3 5 μM. HFD—high-fat diet; IBMX—3-isobutyl-1-methylxanthine.

2.2. Oil Red O Staining

Differentiated adipocytes were washed with phosphate-buffered saline (PBS) and fixed in 3.7% formaldehyde for 10 min. Cells were stained with oil red O solution for 30 min at 25 °C. Staining was visualized using bright-field microscopy (Olympus Microscopes, CenterValley, PA, USA). Lipid droplet size was analyzed with Image Pro (advance Imaging Concept, Inc., Princeton, NJ, USA).

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2.3. Animal Protocols

Eight-week-old C57B16 male mice were fed high-fat diets Western diets, containing 42% kcal from fat, 42.7% carbohydrate, and 15.2% protein with total calories of 4.5 kcal/g and 0.2% cholesterol (Cat no TD.88137, Harlan, Teklad Lab Animal Diets, Indianapolis, IN, USA) for 23 weeks while lean mice were fed chow diets. Mice were divided into five groups: (1) Lean; (2) HFD; (3) Thymoquinone (TQ): HFD mice treated with black seed-cold press oil formulation containing thymoquinone (TQ) 0.75% mixed in the diet for eight weeks; (4) Omega 3 (ω3): HFD mice treated with Omega-3 (ω3) 2% mixed in their diet for eight weeks; (5) TQ+ω3: HFD mice were treated with TQ 0.75% and ω3 2% mixed in their diet for eight weeks. At the end of the experiment, all mice were sacrificed, and visceral adipose tissue and liver organs were dissected for further analysis. The handling of all animal experiments strictly followed the NYMC IACUC institutionally approved protocol in accordance with NIH guidelines.

2.4. Western Blot Analysis

Frozen adipose and liver tissues were homogenized and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease and phosphatase inhibitors (Complete TM Mini and PhosSTOP TM, Roche Diagnostics, Indianapolis, IN, USA). Protein samples were separated using 10% sodium dodecyl sulfate-polyacrylamide (SDS) gels and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After blocking, the membranes were then incubated at 4 °C overnight with the following primary antibodies: anti-UCP-1, anti-PRDM16, anti-FGF21, anti-Sirt1, anti-PGC1α, anti-pAKT, anti-AKT, anti-pACC, anti-ACC, anti-pIRS1 Ser^{307}, anti-NOV/CCN3, anti-TWIST2, anti-HIF1α, anti-β-actin (Cell Signaling Technology, Danvers, MA, USA), anti-pIR tyr^{972} (Millipore, Bedford, MA, USA), anti-HO-1 (Enzo Life Sciences, Farmingdale, NY, USA). Membrane incubations were carried out using a secondary infrared fluorescent dye conjugated antibody absorbing at both 800 nm and 700 nm. The blots were visualized using an Odyssey Infrared Imaging Scanner (Li-Cor Science, Lincoln, NE, USA)) and quantified by densitometric analysis after normalization with β-actin. Results were expressed as optical density (O.D.) as previously described [19].

2.5. Statistical Analysis

Statistical significance between experimental groups was determined by ANOVA with Tukey–Kramer post-hoc analysis for comparison between multiple groups (GraphPad Prism version 7, GraphPad Software, San Diego, CA, USA). The data are presented as means ± standard error of the mean (SEM), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of TQ and ω3 on Lipid Droplet Size and Adipogenesis in 3T3 L1 Cells

Compared to the control group, treatment with various concentrations of ω3 (5–20 μM) significantly decreased the average lipid droplet size in a dose-dependent manner (Figure 1B,C). TQ 1–4 μM treatment significantly reduced the lipid droplet size, but no marked difference was observed between the TQ 2 and 4 μM groups. Furthermore, TQ in combination with the ω3 treatment potentiated further lipid droplet size reduction in a dose-dependent manner (Figure 1D,E). Collectively, we then chose the concentration of TQ 2 μM and ω3 20 μM for further investigation of browning and mitochondria biogenesis in 3T3-L1 cells.

3.2. Effect of TQ and ω3 on Browning of White to Beige Adipocytes in 3T3 L1 Cells

Western blot analysis showed uncoupling protein 1 (UCP1) was significantly upregulated in 3T3-L1cells treated with TQ, ω3, and TQ in combination with ω3 as compared to the Control group (Figure 2A,B). PRDM16 and FGF21 are key transcriptional regulators in promoting brown fat adipogenesis and inducing beige adipocyte recruitment in WAT [34]. We further examined the

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effect of TQ and ω3 treatment on PRMD16 and FGF21 expression in 3T3-L1 cells. As shown in Figure 2A,C,D, PRDM16 and FGF21 protein expressions were significantly increased in TQ, ω3, and TQ in combination with the ω3 treatment groups when compared to the Control group ($p < 0.05$). Notably, TQ in combination with the ω3 treatment potentiated the protein expression of PRDM16 and was significantly increased as compared to the TQ- and ω3-only groups (Figure 2A,C).

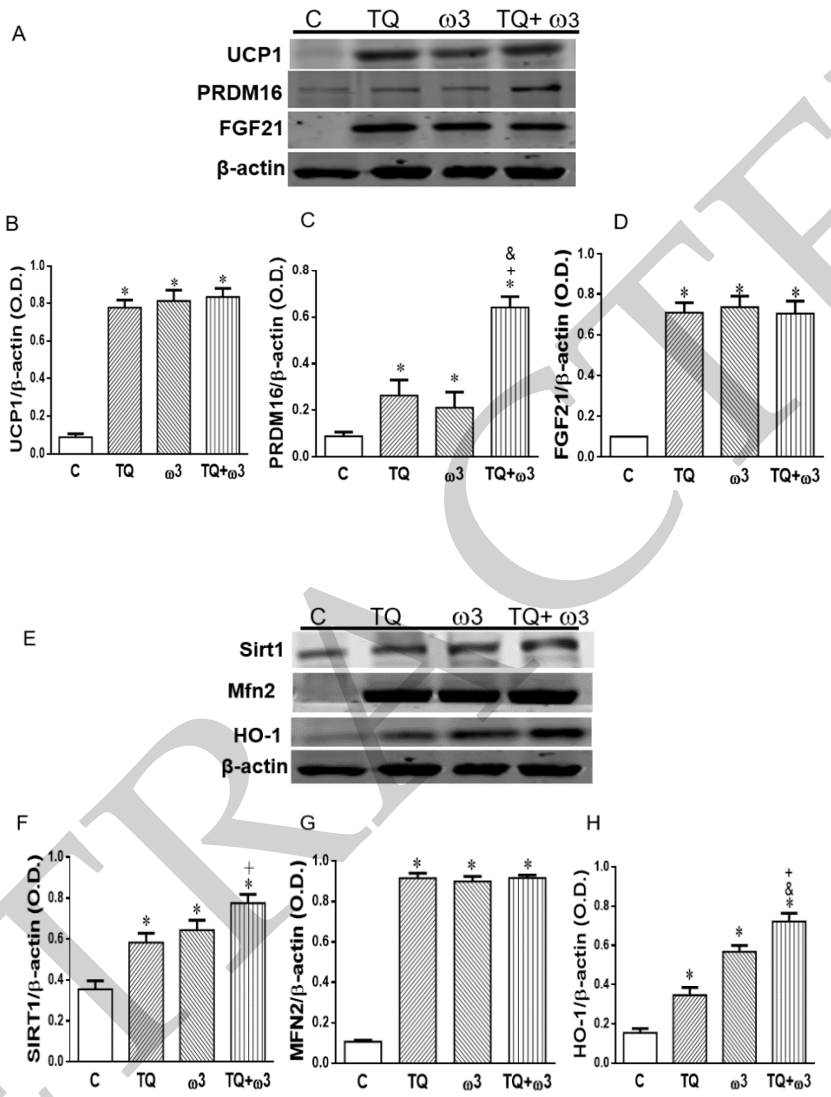


Figure 2. Effects of thymoquinone (TQ) and omega-3 (ω3) treatment on the protein expression of beige adipocytes and key regulators of mitochondrial biogenesis in 3T3-L1 adipocytes. Representative and graph of uncoupling protein 1 (UCP-1), PR domain containing 16 (PRDM16), and fibroblast growth factor 21 (FGF21) (**A**–**D**); Sirtuin 1 (Sirt1) Mitofusion 2 (Mfn2), and heme oxygenase-1 (HO-1) protein expression (**E**–**H**). 3T3-L1 adipocytes were treated with TQ 2 μM, ω3 20 μM, and a combination of TQ and ω3 for six days ($n = 4$). * $p < 0.05$ vs Lean; $^{+}$ $p < 0.05$ vs TQ; $^{\&}$ $p < 0.05$ vs ω3.

We further assessed the effects of TQ and ω3 on mitochondrial biogenesis marker Sirt1 and mitochondrial fusion-associated protein Mfn2 expression in 3T3L1 cells. Figure 2E–G show TQ and ω3 dramatically increased Sirt1 and Mfn2 expression. Moreover, the combination of TQ and ω3 significantly potentiated the antioxidant enzyme HO-1 induction (Figure 2H). Collectively, these results indicated that TQ and ω3 reduced lipid droplet size through the upregulation of mitochondria biogenesis/fission and conversion of white to thermogenic beige adipocytes.

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3.3. Effect of TQ and ω3 on the Insulin Receptor and AKT Phosphorylation in 3T3 L1 Cells

The insulin-AKT signaling pathway stimulates glucose uptake in adipocytes and skeletal muscle by promoting glucose transporter 4 (GLUT-4) translocation and cell membrane fusion [35]. Figure 3A,B show increased phosphorylation of IR tyr^{972} following TQ treatment as compared to the Control group. Furthermore, TQ in combination with the ω3 treatment exhibited an additional potentiation effect on pIR tyr^{972} expression that was significantly higher than that of the Control, TQ, and ω3 groups. Consistently, phosphorylation levels of AKT (pAKT) significantly increased in the TQ and ω3 groups compared to the Control group, and TQ in combination with ω3 treatment further potentiated pAKT (Figure 3A,C).

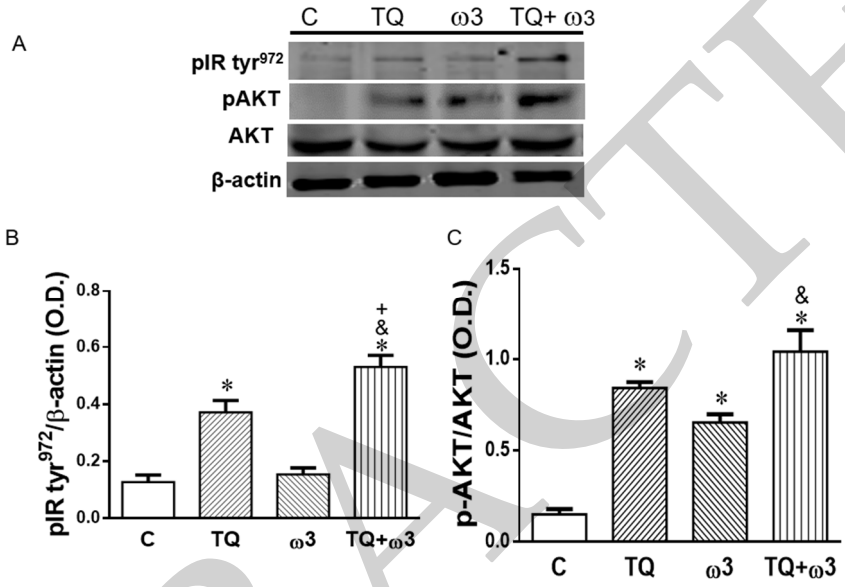


Figure 3. Effects of thymoquinone (TQ) and omega 3 (ω3) treatment on the protein expression of insulin signaling in 3T3-L1 adipocytes. Representative and graph of p-AKT and phosphorylated insulin receptor (pIR tyr^{972}), protein expression (**A**–**C**); 3T3-L1 adipocytes were treated with TQ 2 μM, ω3 20 μM, and a combination of TQ and ω3 for six days (n = 4). * $p < 0.05$ vs Lean; $^{+}$ $p < 0.05$ vs TQ; $^{\&}$ $p < 0.05$ vs ω3.

3.4. Effect of TQ and ω3 on Adipose Tissue Hypoxia-Induced Inflammation in HFD Mice

Adipocyte hypertrophy results in hypoxia and triggers the expression of HIF1α and secretion of proinflammatory adipokines to mediate obesity-associated insulin resistance [36]. Nephroblastoma overexpressed (NOV/CCN3) is a novel adipokine secreted by adipose tissue that specifically regulates the inflammatory and fibrotic responses, regulating insulin resistance in mice an HFD [5]. Aberrant expression of NOV/CCN3 and TWIST is evident in obesity-induced inflammation [3,5,37]. NOV/CCN3 expression was significantly increased in the HFD group as compared to the Lean group ($p < 0.05$) (Figure 4A,B). The increase of NOV/CCN3 was significantly reversed by TQ, ω3, and the TQ + ω3 combination treatment in HFD mice. In addition, TWIST2 was significantly augmented by HFD as compared to the Lean group (Figure 4A,C). TQ and omega3 treatment alone did not change the TWIST2 protein expression as compared to the HFD group. Interestingly, a combination of TQ + ω3 exhibited a significant reduction of TWIST2 expression in HFD mice, suggesting the additional effect of TQ + ω3 in reducing adipocyte inflammation.

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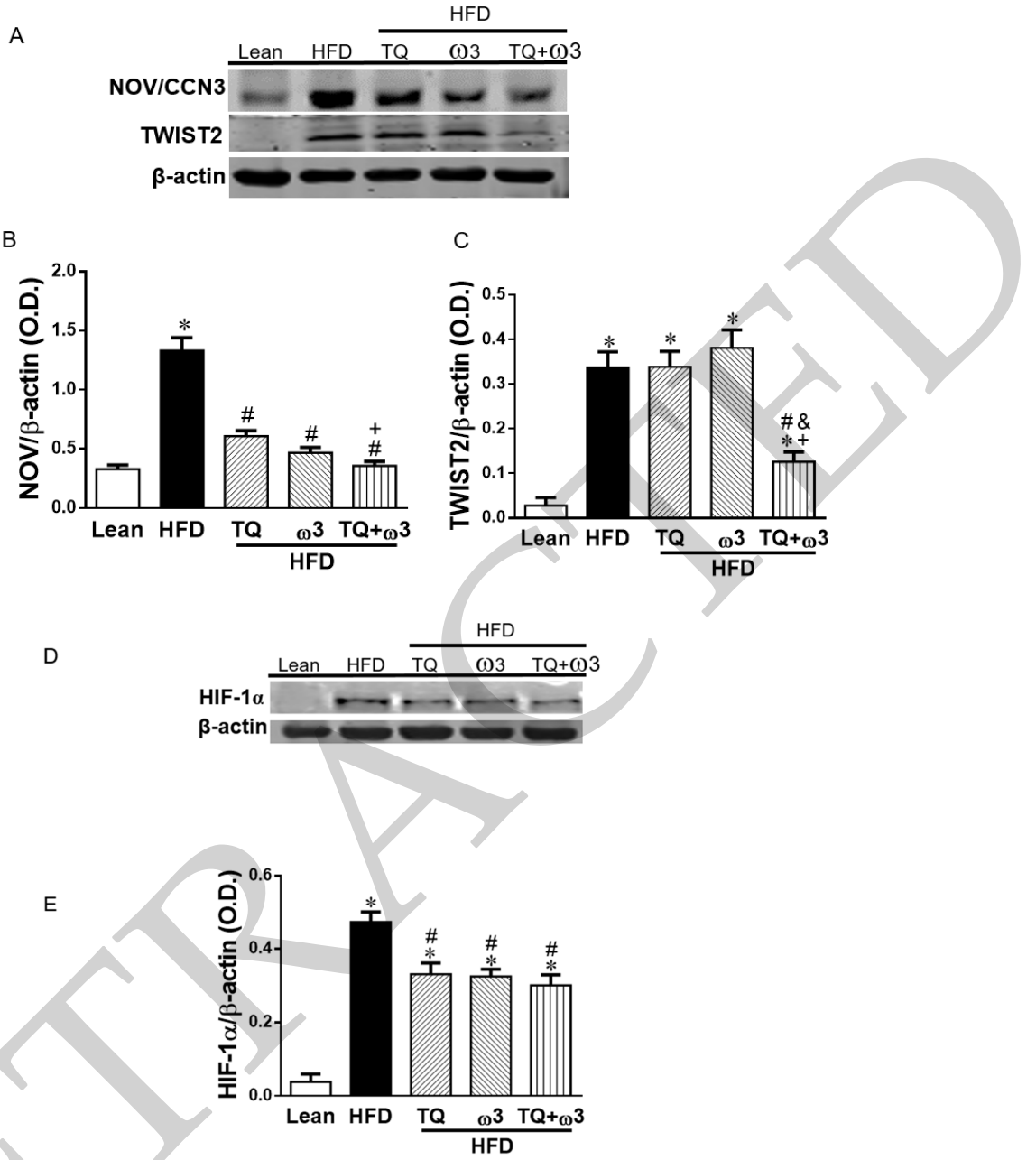


Figure 4. Effects of thymoquinone (TQ) and omega-3 (ω3) treatment on the inflammation and hypoxia in visceral adipose tissue of mice fed a high-fat diet (HFD). Representative Western blots (**A**) and densitometry analysis of Nephroblastoma Overexpressed (NOV/CCN3) (**B**) and Twist related protein 2 (TWIST2) (**C**); Representative densitometry analysis of hypoxia-inducible factor 1α (HIF1α) (**D**,**E**). *n* = five animals per group. * $p < 0.05$ vs Lean; # $p < 0.05$ vs HFD, + $p < 0.05$ vs TQ, and & $p < 0.05$ vs ω3.

3.5. Effect of TQ and ω3 on Adipocyte Hypertrophy

The key step in the development from lean to obese status is the increase in adipocyte size. When the adipocyte size enlarges to reach the diffusional limit of oxygen from the vasculature, the low oxygen tension in adipose tissue induces the activation of hypoxia-inducible factor 1α (HIF-1α), which plays an important role in triggering inflammation and obesity-related metabolic disorders [38]. Figure 4D,E indicate that HIF-1α was significantly induced in the HFD group compared to the Lean group, and treatment with TQ or ω3 or TQ in combination with ω3 showed a significant decrease in HIF-1α protein expression.

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3.6. Effect of TQ and ω3 on the Browning of WAT in HFD Mice

We next evaluated the effects of TQ and ω3 treatment on the browning of adipose tissue in HFD mice. Figure 5A–D show that HFD mice exhibited significantly lower levels of brown adipose tissue-related markers: UCP1, PRDM16, and FFGF21 than those in the Lean group ($p < 0.05$) (Figure 5A–D). TQ and ω3 treatment produced significant increases in protein expression of UCP1, PRDM16, and FGF21 compared to the HFD group. Moreover, the TQ + ω3 groups showed markedly higher protein expression of PRDM16 and FGF21 compared to the ω3 group.

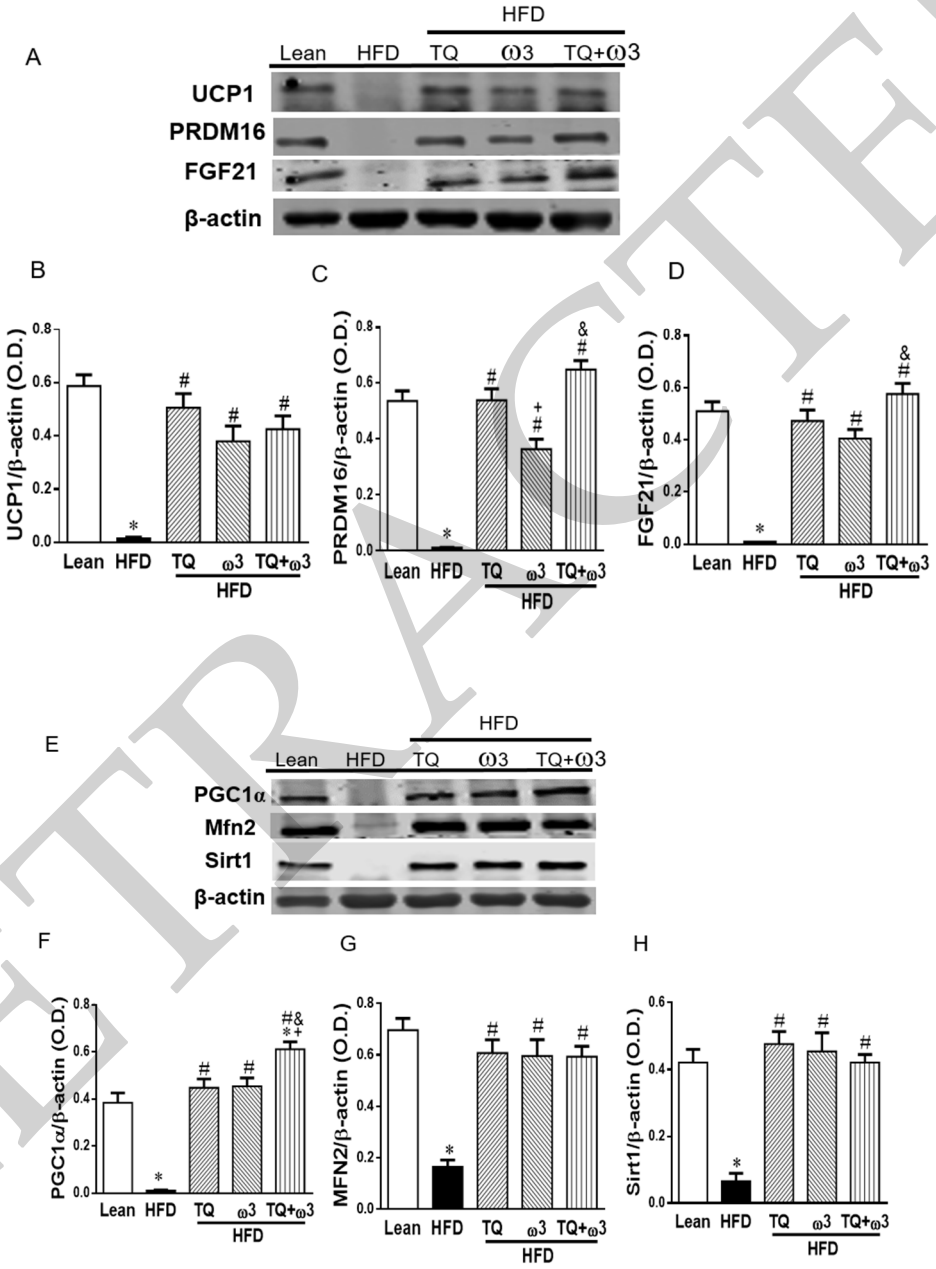


Figure 5. Effects of thymoquinone (TQ) and omega-3 (ω3) treatment on the key regulators of beige adipocyte and mitochondrial biogenesis in visceral adipose tissue of mice fed a high-fat diet (HFD). Representative Western blots (**A**) and densitometry analysis of uncoupling protein 1 (UCP1) (**B**), PR domain containing 16 (PRDM16) (**C**), and fibroblast growth factor 21 (FGF21) (**D**); Representative Western blots (**E**) and densitometry analysis of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) (**F**), Mitofusion 2 (Mfn2) (**G**), and Sirtuin 1 (Sirt1) (**H**). n = five animals per group. * $p < 0.05$ vs Lean; $^{\#}$ $p < 0.05$ vs HFD, $^{+}$ $p < 0.05$ vs TQ, and $^{\&}$ $p < 0.05$ vs ω3.

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Enhanced mitochondrial function is closely related to beige fat function and the energy dissipating capacity. As shown in Figure 5E–H, adipose tissue of HFD mice exhibited significantly lower protein expressions of PGC1α, Mfn2, and Sirt1, which are associated with mitochondrial biogenesis and integrity. The TQ, ω3, and TQ+ω3 groups exhibited drastic increases in the protein expression of PGC1α, Mfn2, and Sirt1.

3.7. Effect of TQ and ω3 on the Phosphorylation of ACC, AMPK, AKT, and HO-in of Adipose Tissue of HFD Mice

The HFD group demonstrated decreased phosphorylation of acetyl-CoA carboxylase (pACC) and adenosine monophosphate activated protein kinase (pAMPK) in the adipose tissue when compared to the Lean group (Figure 6A–C). Only the TQ treatment reversed the reduced expression of pACC (Figure 6A,B). However, TQ or ω3 or TQ in combination with ω3 treatment significantly increased pAMPK expression in the adipose tissue of HFD-fed mice (Figure 6A,C). Adipose tissue of HFD-fed mice exhibited lower levels of pAKT protein expression than those of lean mice. TQ and TQ + ω3 treatment, but not the ω3 groups, reversed the downregulation of pAKT (Figure 6D,E). In addition, the decreased HO-1 expression in HFD mice was markedly elevated by TQ, ω3, and TQ + ω3 treatment (Figure 6F,G). These results revealed TQ exerts a better capacity in improving insulin signaling in the adipose tissue of HFD mice.

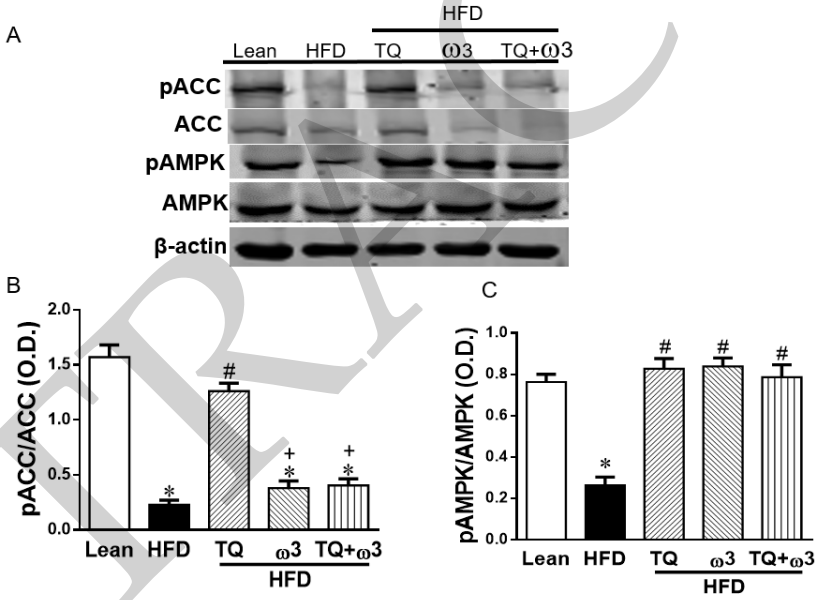


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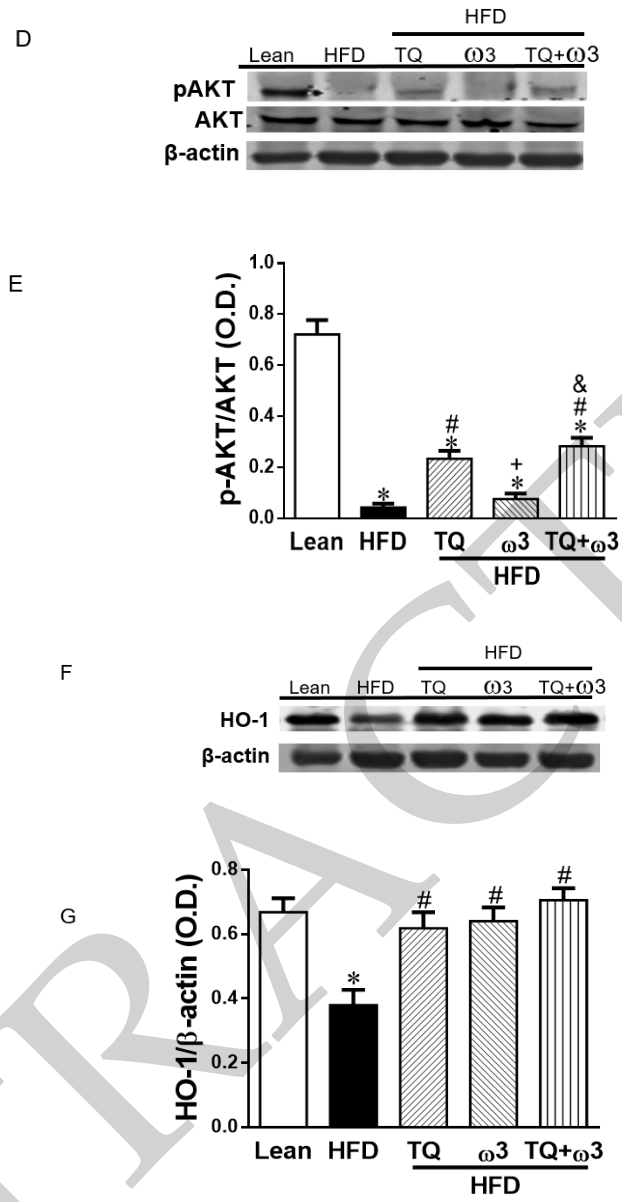


Figure 6. Effects of thymoquinone (TQ) and omega-3 (ω3) treatment on 5′ adenosine monophosphate -activated protein kinase (AMPK) and insulin signaling in visceral adipose tissue of mice fed a high-fat diet (HFD). Representative Western blots (**A**) and densitometry analysis of phosphorylated acetyl-CoA carboxylase/acetyl-CoA carboxylase (pACC/ACC) (**B**), pAMPK/AMPK (**C**); Representative Western blots (**D**) and densitometry analysis of p-AKT/AKT (**E**); Representative Western blots and densitometry analysis of HO-1 (**F**,**G**). n = five animals per group. * $p < 0.05$ vs Lean; $^{\#}$ $p < 0.05$ vs HFD, $^{+}$ $p < 0.05$ vs TQ, and $^{\&}$ $p < 0.05$ vs ω3.

3.8. Effect of TQ and ω3 on the Phosphorylation of the Insulin Receptor in the Liver of HFD Mice

Phosphorylation of the insulin receptor increases glucose utilization and insulin sensitivity. As shown in Figure 7A–C, HFD mice exhibited significantly lower levels of insulin receptor phosphorylation in tyr^{972} (pIR tyr^{972}) and insulin receptor substrate 1 phosphorylation on Ser^{307} (IRS1 Ser^{307}) in the liver. Combination treatment with TQ and ω3 significantly increased the protein expression of pIR tyr^{972} and IRS1 Ser^{307} in the liver of HFD mice.

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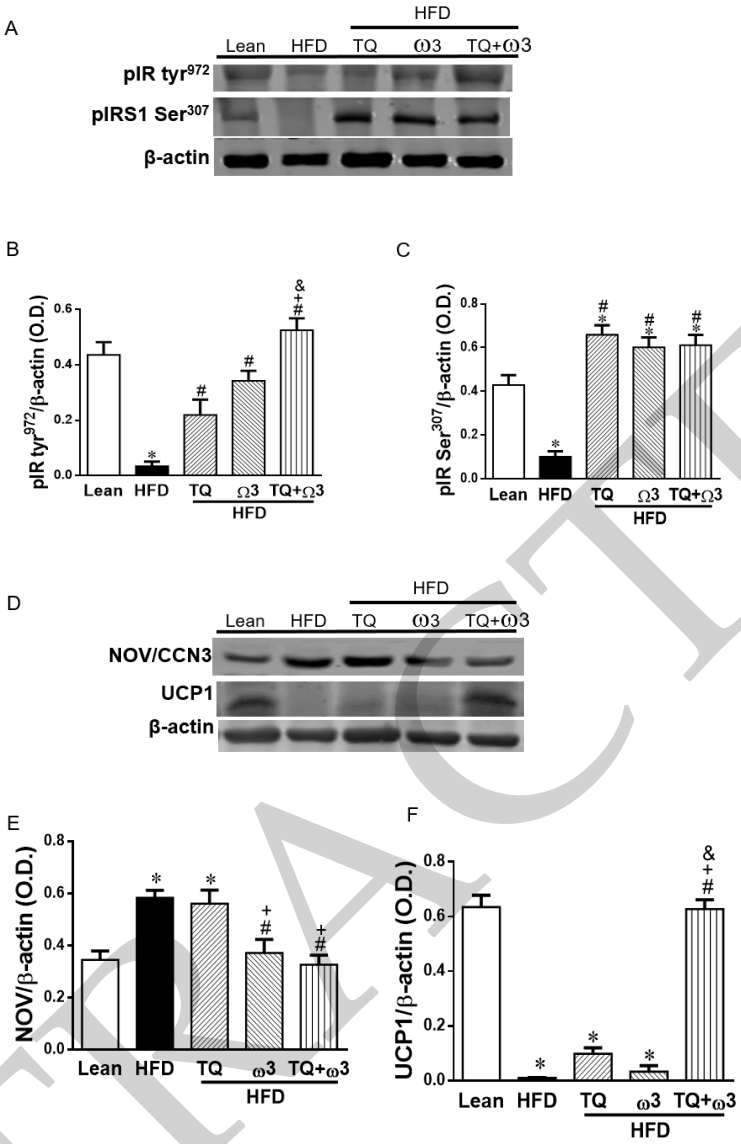


Figure 7. Effects of thymoquinone (TQ) and omega-3 (ω3) treatment on insulin receptor phosphorylation, inflammatory Nephroblastoma Overexpressed (NOV/CCN3), and browning marker uncoupling protein -1 (UCP-1) in the liver of mice fed a high-fat diet (HFD). Representative Western blots (**A**) and densitometry analysis of phosphorylated insulin receptor (pIR tyr^{972}) (**B**) and phosphorylated insulin receptor substrate (pIRS1 Ser^{307}) (**C**); Representative Western blots (**D**) and densitometry analysis of NOV/CCN3 (**E**) and UCP1 (**F**); n = five animals per group. * $p < 0.05$ vs Lean; $^{\#}$ $p < 0.05$ vs HFD, $^{+}$ $p < 0.05$ vs TQ, and $^{\&}$ $p < 0.05$ vs ω3.

3.9. Effect of TQ and ω3 on NOV/CCN3 Expression in the Liver of HFD Mice

Consistent with the results in the WAT, the inflammatory adipokine NOV/CCN3 levels were significantly elevated in the liver of HFD mice compared to the Lean group, which were drastically reversed by the combination of TQ and ω3 (Figure 7D,E).

3.10. Effect of TQ and ω3 on UCP1 Expression in the Liver of HFD Mice

UCP1 expression was significantly attenuated in the HFD group compared to the Lean group (Figure 7F,G). TQ or ω3 treatment alone did not change the protein expression of UCP1 compared to the HFD group. TQ in combination with ω3 significantly upregulated UCP1 expression in HFD mice.

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4. Discussion

In the present study, we showed that the combination treatment of TQ and ω3 reduced lipid droplet size and increased mitochondrial biogenesis, mitochondrial fusion genes, WAT browning, and insulin receptor phosphorylation, which were associated with antioxidant enzyme HO-1 induction in vitro (3T3L1 adipocytes) as well as in vivo (C57/BL6 mice). Furthermore, the in vivo study indicated that the HFD induced adipocyte hypertrophy and subsequent HIF1α expression, contributing to increased secretion of inflammatory adipokines, such as NOV/CCN3 and TWIST2. These deleterious effects were dramatically attenuated by TQ and ω3 treatment and may be associated with the induction of HO-1. Notably, TQ and ω3 up-regulated mitochondrial biogenesis/fusion and brown fat markers in white fat through the activation of the AMPK pathway. TQ and ω3 treatment also increased the phosphorylation of insulin receptors pIR tyr^{972} and pIRS1 Ser^{307} and the downstream signaling of pAKT in the adipose tissue and liver. These results suggest that the reduction of inflammatory responses, enhanced conversion of large unhealthy white to small healthy beige adipocytes, and the restoration of mitochondrial function appear to contribute to the improvement of obesity and related insulin resistance.

We showed that TQ and ω3 decreased the lipid content and adipocyte expansion in 3T3-L1cells, which is consistent with the in vivo results that TQ and ω3 downregulated HIF1α expression in WAT of HFD-fed mice. This supports the hypothesis that the increased conversion of large adipocytes to small adipocytes. Small adipocytes are healthier, adiponectin-secreting, and more insulin sensitive. Additionally, in agreement with our recent report that adipocyte-specific HO-1 upregulation converts large unhealthy adipocytes into small healthy and sensitive adipocytes [34], this reprograming of the adipocyte phenotype is anti-inflammatory and beneficial to reversing the development of metabolic disease.

It is well-established that low-grade chronic inflammation of WAT in obesity plays an important role in the pathogenesis of insulin resistance and metabolic disorders [39]. HFD feeding is known to induce adipocyte hypertrophy and hepatic steatosis, which may result in the upregulation of mRNA and plasma levels of adipokine NOV/CCN3 [40]. A previous report demonstrated that global deletion of NOV/CCN3 in mice with HFD exhibited improved glucose tolerance, insulin sensitivity, and metabolic parameters [4], suggesting that NOV/CCN3 is involved in obesity-associated insulin resistance and can be a novel target for obesity management. Inflammatory NOV levels were elevated in obese mice, and this proinflammatory adipokine decreased HO-1 levels and resulted in non-alcoholic steatohepatitis (NASH) [5]. Epoxyeicosatetraenoic acids (EETs) have previously been shown to decrease adipocyte size, decrease NOV levels, improve mitochondrial function, and reprogram white to beige fat [41]. EETs have also been shown to upregulate HO-1, thermogenic gene levels, and mitochondrial function to prevent obesity-induced cardiomyopathy in obese mice [3,42]. EETs have been shown to improve non-alcoholic fatty liver disease (NAFLD) in mice by increasing PGC-1α-HO-1, reducing fatty acid accumulation and fibrosis, and increasing insulin receptor phosphorylation [43,44]. Conversely, downregulation of PGC-1α prevents the beneficial effect of EET-HO-1 on mitochondrial integrity in obese mice [33]. EETs have been shown to regulate adipocyte differentiation via PGC-1α activation, contributing to the browning of white adipose tissue [43]. Adipocyte size and differentiation are also regulated by HO-1 upregulation. L4F, an HDL mimetic-like peptide, has been shown to increase adiponectin and insulin sensitivity and reduce the amount of hepatic fat [45].

In the present study, we showed that a combination of TQ and ω3 administration in HFD mice enhanced the reduction of NOV/CCN3 and TWIST2 expression in both adipose and liver tissue, suggesting a potential capacity of the combination of TQ and ω3 in attenuating hypoxia-induced inflammatory responses, eventually contributing to the amelioration of obesity-associated insulin resistance. Additionally, HO-1 is considered a strong antioxidant enzyme that protects against oxidative stress and maintains cellular homeostasis in various inflammatory diseases [16]. Omega-3 also increased HO-1 production through the activation of nuclear factor erythroid 2–related factor 2 (Nrf2) and prevented H_2O_2-elicited increases in oxidative stress [46]. In the present study, we showed

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that TQ and ω3 treatment rescued HO-1 expression in the adipose tissue of HFD mice, indicating that induction of HO-1 played an important role in mediating the antioxidant and anti-inflammatory effects of TQ and ω3.

Browning of white to beige fat takes on a multilocular lipid droplet appearance and increases energy expenditure and metabolic activity by promoting mitochondrial biogenesis and UCP1 function [47]. Dietary supplementation with ω3 has been reported to increase BAT thermogenic capacity and oxygen consumption by upregulation of UCP1 in mitochondria [48]. Whether TQ or TQ in combination with ω3 elicits similar effects in WAT has not been fully evaluated. The enhanced energy expenditure of TQ and ω3 administration is primarily mediated by the induction of beige adipocytes through an increase in the expression of the key regulators UCP1, PRDM16, and FGF21 as well as mitochondria biogenesis genes PGC1α, Sitr1, and mitofusin 2 (Mfn2). These beneficial effects appear to be associated with the activation of AMPK signaling cascades. In addition, HO-1 induction increased mitochondrial fusion-regulated protein and mitochondrial quality control that were associated with the conversion of energy-storing white to energy-dissipating beige adipocytes [49].

AMPK is a key regulator of energy metabolism and mitochondrial biogenesis [50] and has therapeutic importance for treating obesity. Activation of AMPK increases fatty acid oxidation through phosphorylation and inhibition of ACC, the rate-limiting enzyme for fatty acid synthesis. Intriguingly, AMPK has been shown to be essential for Sirt1-dependent deacetylation of PGC-1α [15,51]. This orchestrated network translates into enhanced mitochondrial activity and oxidative metabolism, and in turn, plays a major role in energy homeostasis. Our results indicated that TQ and ω3 administration enhanced phosphorylation of AMPK, Sirt1, and PGC-1α expression in WAT, further elucidating the interplay of TQ and ω3 in promoting mitochondrial function and eventually contributing to a marked improvement in obesity and insulin resistance of HFD mice.

5. Conclusions

We provide evidence that intervention with TQ and ω3 (icosapent ethyl) opposes the effects of obesity and markers of insulin resistance by reducing adipocyte hypertrophy-induced inflammation, enhancing AMPK activation, fatty acid metabolism and mitochondrial function as well as reprogramming unhealthy white to healthy beige adipocytes. Reprogramming white to beige fat offers a viable and safe alternative for new interventions in the fight against obesity and its consequent metabolic disorders. There are a number of published studies showing the health benefits induced by the "browning" of white adipose tissue [52–55]. In addition, there are published studies of Omega-3 fish oil converting WAT to beige fat [56] and even novel food compounds assisting in the browning of WAT [57]. Our group has focused on the upregulation of heme oxygenase by eicosanoids in vivo [3,58] and in vitro [44], which has been very effective in the conversion of WAT to beige fat as well as the amelioration of NAFLD and NASH in the liver [5,43]. More recently, our group showed the cardioprotective effect of HO-1- PGC1α in epicardial fat, reducing cardiovascular risk in humans and in obese mice. This novel study showed that upregulation of HO-1-PGC1α increased mitochondrial function and restored inflammatory epicardial fat to beige fat [37]. In conclusion, this current study shows the remarkable effect of thymoquinone in combination with the purified form of Omega-3, icosapent ethyl, in the browning of WAT. This combination is very effective in the browning of WAT and is likely safer than anything currently on the market. This novel discovery needs to be confirmed in human clinical trials.

Author Contributions: H.H.S., L.B., A.C., L.L., L.G., A.S., J.T., A.R. were involved in sample collection, extraction, data collection and analysis as well as manuscript editing. R.R. performed the histological sample preparation, data collection and analysis as well as manuscript editing. S.J.P., D.E.S., N.G.A. were involved in study design and conception. S.J.P. performed manuscript editing. D.E.S. and N.G.A. wrote the first draft of the manuscript. D.E.S. and N.G.A. were involved in funding application and research supervision. All authors have read and agreed to the final published version of the manuscript.

Funding: This work was supported by the National Institutes of Health R 56-139561 (NGA), P01 HL05197-11, and 1R01DK121748-01A1 (D.E.S.) and the National Institute of General Medical Sciences, P20GM104357-02 (D.E.S.).

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Conflicts of Interest: The authors declare no conflict of interest.

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