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Beneficial Effects of Thymoquinone on Metabolic Function and Fatty Liver in a Murine Model of Obesity

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Abstract

Aim: *Nigella sativa* seeds contain a high amount of Thymoquinone (TQ), an antioxidant. We therefore hypothesized that *Nigella sativa* oil would, through the antioxidant properties of TQ ameliorate obesity-induced hyperglycemia and decrease blood pressure and OX-LDL in obese mice.

Methods: Commencing at eight weeks of age, C57B16 male mice were fed a high fat diet (HF) for 20 weeks. Mice were divided into three groups of five animals each as follows: group 1) Lean, group 2) HF diet, group 3) HF diet treated for the last 8 weeks with 3%TQ. Inflammatory biomarkers, antioxidant biomarkers, mitochondrial biogenesis and tissue fat accumulation and hepatic steatosis were determined.

Results: 3% TQ treatment resulted in an increase of oxygen consumption decreased fasting glucose and blood pressure ($P<0.05$) as compared in obese mice. TQ treatment increased both the quantity of hepatic HO^{-1}, and HO activity in response to 3% TQ. Additionally, mitochondrial Mfn2, PGC1α, insulin receptor phosphorylation in response to TQ while decreased LDL and OX-LDL ($P<0.05$) and haptic lipid accumulation.

Conclusion: Fundamentally, TQ intervention attenuated the obesity-mediated decrease of oxygen consumption, fasting glucose, improved mitochondrial biogenesis through an increase and in levels of HO-1 that is associated with ablated HF-induced LDL. Our findings indicate a potential clinical role for TQ in the prevention of obesity-related steatosis in metabolic disease.

Keywords: Thymoquinone; OX-LDL; OX-HDL PGC-1a; Mfn1; Mfn2

Abbreviations

CVD: Cardiovascular Disease; FBS: Fetal Bovine Serum; FFA: Free Fatty Acids; Fis-1: Mitochondrial Fission 1 Protein; HbA1c: Glycated Hemoglobin A1C; HO-1: Heme Oxygenase 1; HO-2: Heme Oxygenase 2; HDL: High Density Lipoprotein; HFD: High Fat Diet; LDL: Low Density Lipoprotein; MetS: Metabolic Syndrome; MFN-1: Mitofusin 1; MFN-2: Mitofusin 2; NADPH: Dihydronicotinamide-Adenine Dinucleotide Phosphate; NAFLD: Non-Alcoholic Fatty Liver Disease; NASH: Non-Alcoholic Steato-Hepatitis; NOV: Nephroblastoma Overexpressed; NRF2: Nuclear Factor (erythroid-derived)-like 2; OPA-1: Optic Atrophy 1; OX-HDL: Oxidized HDL; OX-LDL: Oxidized LDL; PMSF: Phenylmethylsulfonyl Fluoride; ROS: Reactive Oxygen Species; T2DM: Type 2 Diabetes Mellitus; TQ: Thymoquinone.

Introduction

According to the World Health Organization, annually over 2 million people die worldwide from the complications of excessive body fat. An altered adipose tissue function is characterized by an impaired lipid buffering capacity and subsequently by a systemic lipid over flow and ectopic lipid accumulation in several insulin sensitive peripheral tissues such as skeletal muscle, liver, pancreas, heart and kidneys [1,2]. This obesity trend is followed in men and women, both having a similar pattern, being around 40-45% obese in middle age around 35% obese when younger [3]. The ectopic deposition of triglycerides triggers a series of cardiometabolic perturbations, which are grouped into a diagnosis of metabolic syndrome (MetS). This disorder is not only associated with a higher risk of appearance of type 2 diabetes and cardiovascular events but impacts the liver [4]. Recent data suggest that nonalcoholic fatty liver disease (NAFLD), considered the hepatic manifestation of the MetS, precedes the development of MetS [5]. NAFLD is associated with a number of metabolic diseases including diabetes mellitus, obesity and hypertension. In a five-year retrospective review, individuals with NAFLD had a higher incidence of impaired fasting glucose and type 2 diabetes mellitus (T2DM) compared with NAFLD-free controls [6]. In the last few decades, a higher frequency of obesity, T2DM, and MetS have occurred as a result of various dietary changes [7]. Furthermore, individuals with NAFLD have a higher probability of liver failure and, eventually, cirrhosis [8-10]. Epidemiological results suggest that insulin resistance is a common pathogenic factor for all these obesity-related conditions and that it can be both reversed and prevented by a healthy lifestyle and a wholesome diet [11]. In this regard, beneficial effects have been reported for curcumin and Resveratrol [12] which increase the antioxidant gene and heme oxygenase-1 (HO-1). Resveratrol upregulates HO-1 expression, NAD(P)H, quinone oxidoreductase 1,

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through activation of nuclear factor (erythroid-derived)-like 2 (Nrf2) target genes. Resveratrol as well as other HO-1 inducers prevent CVD [13]. Importantly, HO-1 induction is regulated by levels of glucose; while glucose deprivation induces HO-1 gene expression [14], elevated levels of glucose suppress HO-1 gene expression [15,16]. HO-1 levels is affected by obesity and glucose levels (reviewed in [3,17]).

Thymoquinone, present in *Nigella sativa* (NS), has been proposed, based on its anti-oxidant properties, as a protective factor against several metabolic diseases. *Nigella sativa Linn.* (family Ranunculaceae), commonly known as black seed or black cumin, is an herbal plant that has been cultivated for thousands of years in the Middle and South East Asia. Black cumin seed is composed of fixed (stable) and essential (volatile). The Essential oil extracted from black cumin contains a rich volatile fraction comprising Thymoquinone (TQ) and Thymohydroquinone (THQ) [18,19]. Thymoquinone (TQ) is the main pharmacologically active compound of NS and is thought responsible for many therapeutic properties, including anti-inflammatory, antioxidant and anti-hyperglycemic effects. The protective effect of TQ is related to its ability to scavenge reactive oxygen species (ROS), including superoxide and hydroxyl free radicals [20], to block lipid peroxidation and to enhance levels of antioxidant enzymes [21,22]. The aim of this study was to demonstrate the effects of black seed oil, with a high content of TQ, on the metabolic profile, including adipose-mediated release in inflammatory adipokines such as NOV, mitochondrial biogenesis, LDL, Ox-HDL and hepatic steatosis in a murine model of obesity.

Materials and Methods

Animal protocols

Eight-week-old C57B16 male mice were fed western diets with 51% fat content while control mice fed regular diets, high fat diets (Harlan, Teklad Lab animal diets, Indianapolis, IN) (HFD) for 20 weeks. Mice were divided into three treatment groups of five animals each as follows: group 1) Lean, group 2) HFD, group 3) HFD treated for the last 8 weeks with HFD treated for the last 8 weeks with black seed-cold press oil formulation containing thymoquinone (TQ) between 3-3.1% obtained from TriNutra Israel. Formulation of TQ oil is as follows; TQ 3.14 %, p-Cymene, 1.24%, Carvacrol 0.08%, FFA 1,29%, Oleic Acid 21.53%, palmitic acid 11.31%, linoleic acid 57.44%, other fatty acid 1.98% and TPGS, 0.8%. TQ oil was mixed into the HFD food and made into pellets using a mixer. At the end of the experiment, mice were euthanized, assessed for total body weight, fat content and liver fibrosis. All animal experiments followed the NYMC IACUC institutionally approved protocol in accordance with NIH guidelines.

Fasting blood glucose, glucose tolerance testing

Fasting blood glucose and glucose tolerance were measured from tail blood following a 6 h fast. Blood pressure was measured by the tail-cuff method using the CODA tail-cuff System (Kent Scientific, CT, Torrington) as we previously described [23-25].

Determination of oxygen consumption

The C57 mice groups were allowed to acclimatize in the oxygen consumption chambers over a three-week period. Adaptation periods for the three-week duration were executed in two-hour increments, three times a week. The Oxylet gas analyzer and air flow unit (Oxylet, Panlab-Bioseb, Vitrolles, France) were used to determine mouse oxygen consumption (VO2). Each mouse was placed individually in the machine and VO2, VCO2 and respiratory quotient (RQ) was calculated as VCO2/VO2. The data for VO2 are expressed as the consumed oxygen per Kilogram body weight per minute (ml/kg/min) [23-25].

Measurement of HO activity

Liver microsomal HO activity was assayed by the method of Abraham et al. in which liver tissues was homogenates in phosphate buffer, pH 7.8, 0.1 mM EDTA and 1mM PMSF. HO activity was measured in presence of 20 uM heme, glucose 6 phosphate (G-6P), glucose 6 phosphate dehydrogenase (G6PDH), NADPH, at 37°C for 60 minutes. Bilirubin, the product of HO degradation was extracted with chloroform, spin down and leave overnight in the freezer. Samples defrost; spin the samples for 20 minutes and with pasture pipets remove the lower layer which has chloroform. Bilirubin concentration in chloroform determined spectrophotometrically (Perkin-Elmer (Norwalk, CT) Dual UV/VIS Beam Spectrophotometer) using the difference in absorbance at wavelength from λ 460 to λ 530 nm with an absorption coefficient of 40 mM^{-1} and cm^{-1}.

Western blot analysis

For protein expression analyses, liver tissues were lysed in RIPA lysis buffer supplemented with protease and phosphatase inhibitors (CompleteTM Mini and PhosSTOPTM, Roche Diagnostics, Indianapolis, IN) Frozen mouse adipose tissue was ground under liquid nitrogen and suspended in homogenization buffer (comprising mmol/L :10 phosphate buffer, 250 sucrose, 1.0 EDTA, 0.1 PMSF and 0.1%v/v tergitol, pH 7.5). For *In vitro* Western blot analysis pelleted cells were lysed and HO-1, HO-2, OPA1, MFN1, MFN2 and NOV proteins were measured. Protein detection was 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 Tec) and quantified by densitometric analysis performed after normalization with β-actin. Results were expressed as arbitrary units (AU).

Histopathological examination of liver tissue

Liver samples from each experimental group were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin wax, and sectioned (6 μm thick). The main liver histopathological features commonly described in NAFLD including steatosis, inflammation, hepatocyte ballooning, and fibrosis were scored according to the NAFLD histologic activity score (NASH) system, and lipid droplet analysis was performed as previously described [26].

Cell culture and adipocyte cell differentiation

3T3-L1 murine pre-adipocytes, were purchased from American Type Culture Collection (Rockville, MD, USA). After thawing, 3T3-L1 cells were resuspended in DMEM, supplemented with 10% heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% antibiotic/antimycotic solution (Invitrogen). The medium was replaced with adipogenic medium, and the cells were cultured for an additional 6 days. Differentiating 3T3-L1 pre-adipocytes were treated for 6 days with 3% TQ (2, 4, 6 M).

Oil red O staining

Staining was performed using 0.21% Oil Red O in 100% isopropanol (Sigma-Aldrich, St. Louis, MO, USA). Briefly, adipocytes were fixed in 10% formaldehyde, stained with Oil Red O for 10 minutes, rinsed with 60% isopropanol (Sigma-Aldrich), and the Oil Red O eluted by adding 100% isopropanol for 10 minutes and the optical density (OD) measured at 490 nm, for 0.5 sec reading.

Statistical analysis

Data are expressed as means ± S.E.M. Bonferroni's post -test analysis for multiple comparisons was used to calculate the significance of mean value differences using one-way analysis of variance. The null hypothesis was rejected at $p<0.05$.

Results

Effects of TQ on body weight, blood pressure, fasting blood glucose and oxygen consumption

We examined the effect of TQ in mice fed a HFD for 20 weeks (Figures 1 and 2). Blood pressure and fasting blood glucose levels were increased in mice fed a HFD as compared to control animals (Figure 1A and 1B). TQ reduced blood pressure and fasting blood glucose levels in mice fed a HFD. Mice on a HFD displayed a decrease in VO_2 consumption. In contrast, TQ produced a significant ($p<0.05$) increase in oxygen consumption (Figure 1C). As shown in Figure 2, weight of the HFD group was increased ($p<0.05$) compared to Lean, but no difference occurred between the HFD and TQ groups.

Effect of TQ on adipogenesis *in vitro*

TQ decreased large lipid droplet content in differentiated adipocytes compared with differentiated control cells ($p< 0.05$) (Figure 3A and 3B). Furthermore, TQ3 3% decreases oil lipid accumulation seen clearly between differentiation cell and cells treatment with TQ 3% at 6 M, suggesting that TQ decreased adipocyte terminal differentiation preventing the conversion of small "healthy" adipocytes to large adipocytes. TQ decreased lipid content in a dose-dependent manner (Figure 3B). Sardana and Kappas reported that the increase in HO-1mRNA and protein are several orders of magnitude higher than the increase in liver HO activity [27], therefore, we measured the consequence of TQ treatment on liver HO activity and generation of bilirubin anti-oxidant effect. Since HO-1 converts heme to equimolar amounts of CO and bilirubin (20), we measured HO activity by formation of bilirubin. HO activity in control liver tissues was 0.81 ± 0.16 nmol bilirubin formed/mg protein/hour and decreased to 0.49 ± 0.12 nmol bilirubin formed/mg protein/hour in high fat liver ($p<0.05$) (Figure 3B-3F). The stimulatory effect of 3%TQ on HO^{-1} protein was associated with an increase in HO activity (Figure 3G) to 0.78 ± 0.12 nmol bilirubin/mg/hour ($p<0.05$).

Effects of TQ on protein expression in adipose tissue

Western blot analysis of fat tissue showed significant differences in protein expression levels of pIR972, HO-1, Fis-1, Mfn2 and NOV in obese mice compared to control mice. Untreated obese animals exhibited a significant ($p<0.05$) decrease in insulin receptor phosphorylation levels and HO-1 when compared to age-matched lean mice. TQ increased both pIR_{972} mitochondrial fusion protein and HO-1 levels in obese mice (Figure 4A-4E). A HFD resulted in a decrease in Mfn2 ($p<0.05$) and an increase in FIS-1 a fission protein ($p<0.05$). TQ treatment reversed the negative effect on mitochondrial protein as seen by the increased in the levels of MFN2 ($p<0.05$) and decreased FIS-1 ($p<0.05$) compared to HF mice (Figure 4B and 4D). As seen in Figure 4D, levels of adipose tissue derived NOV, a pro-inflammatory protein in lean group are significantly ($p<0.05$) lower than in the HFD group. As shown in Figure 4C, TQ treatment decreased NOV protein expression compared to mice fed a HFD alone.

TQ intervention decreased level lipid, steatosis and fibrosis

Liver of lean mice exhibited no significant steatosis, no inflammatory foci and no fibrosis (Figures 5 and 6). Livers of HFD mice had elevated steatosis, moderate lobular inflammatory loci, hepatocyte ballooning, and fibrosis. Lipid content (Figure 5) was significantly increased ($p<0.05$) in mice fed a HFD as compared to control mice. TQ treatment decreased lipid content as compared to mice on a HFD alone. Morphometric analysis of liver lipid droplets showed that TQ decreased lipid droplet diameter compared to the HFD group ($p<0.05$). As seen in Figure 6, non-treated HFD mice display more fibrosis than HFD mice treated with TQ. TQ reduced HFD-induced fibrosis and collagen deposition ($p<0.05$).

Effect of TQ on MFN-1, MFN-2, OPA1, NOV, HO-2 and HO-1 protein expression

Control obese mice exhibited lower hepatic protein expression of MFN-1, MFN-2, OPA1 and HO-1. TQ produced a significant ($p< 0.05$) increase in the hepatic levels of MFN-1, MFN-2, OPA1 and HO-1 (Figure 7). TQ prevented the HFD-mediated increase in NOV expression (Figure 7). No significant changes were observed on HO-2 among the different groups.

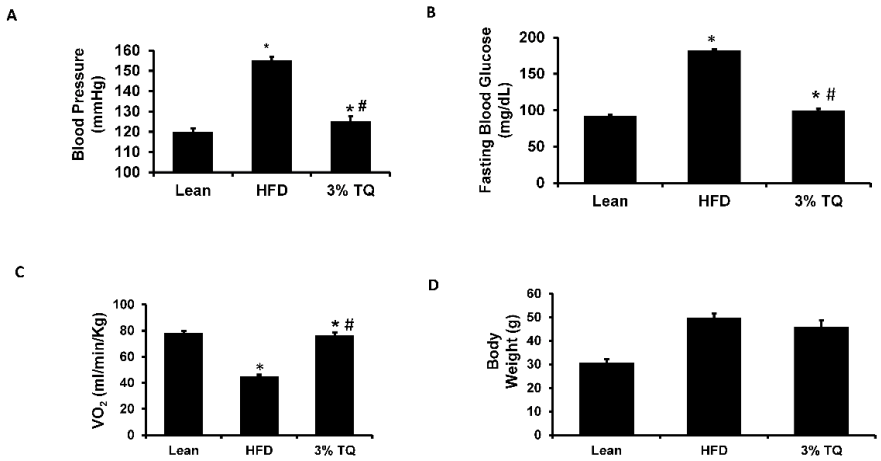

Figure 1: Effect of TQ on blood pressure, blood glucose, oxygen consumption and body weight. Results are mean +/- SE n=6, *$p<0.05$ *vs* lean mice, #$p<0.05$ *vs* HFD mice.

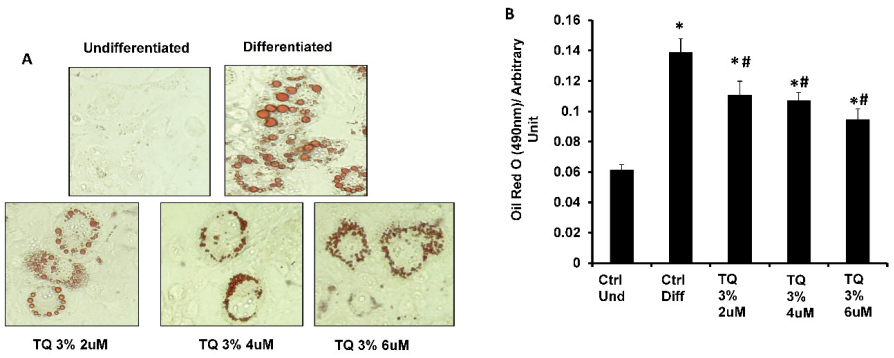

Figure 2: Effect of TQ on oil droplets formation in 3T3 adipocytes. We measured the effect of 3% TQ administration on adipogenesis. Daily supplementation of TQ was effective on adipogenesis suppression at 6 days. TQ treatment showed a significant ($p<0.05$) reduction of lipid droplets formation in 3T3 adipocytes. (n=4), #$p<0.05$ *vs.* control, ** $p<0.05$ *vs.* control.

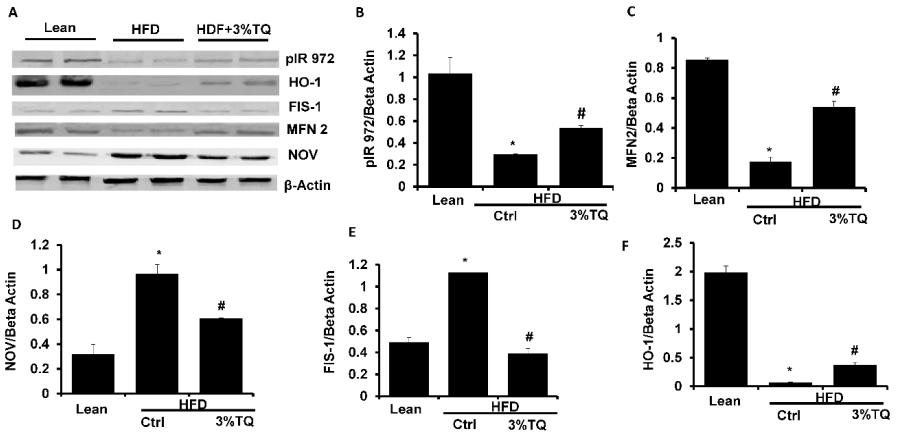

Figure 3: Effect of TQ administration on levels of HO-1, Fis1, MFN2, NOV and pIR972 in adipose tissue on Lean, HFD and HFD +3% TQ. Representative western blots; (A) and densitometry analysis of (B) pIR972, (C) MFN2, (D) NOV, (E) Fis1, (F) HO-1 of Lean, HFD and HFD +3% TQ. Results are mean ± SE, n=6, *$p<0.05$ *vs.* Lean, #$p<0.05$ *vs.* HFD.

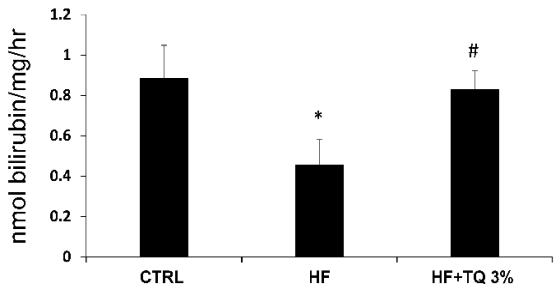

Figure 3G: HO activity in control, HF untreated and HF-treated with 3%TQ treated mice. HO activity was determined and results are mean±SE, n= 3, *$p<0.05$ *vs.* control, # $p<0.05$ *vs.* HF mice.

Effect of TQ on serum levels of Oxidized LDL, OX-LDL and HDL

Plasma from obese mice displayed an increase in LDL and OX-LDL and a decrease in HDL levels. TQ reduced the levels of LDL and oxidized LDL ($p<0.05$), HDL levels were unaffected.

Discussion

TQ is an active component of TriNutra's™ Nigella seed oil and is considered responsible for most of the latter therapeutic potential. The plant *Nigella sativa (N. sativa)* has been used throughout the world in various traditional systems of medicine as a therapy for many different ailments and conditions. The key finding of the present study highlights the hepato-protective effects of TQ in a rodent model of NAFLD. TQ administration for 8-weeks reduced hepatic fat accumulation preventing the development of NASH and liver fibrosis in 36-week study of obese mice. NAFLD affects ~ 25% of the adult population and is the most common cause of chronic liver disease in the Western World. Concomitantly it is associated with obesity, type II diabetes and hyperlipidemia, and may serve as a marker of increased morbidity and mortality from cardiovascular disease. While the mechanism of NAFLD has not been elucidated, it is manifest as tissue injury as a result of fat accumulation. In this process oxidative stress results in mitochondrial damage [28,29] and tissue dysfunction manifest as hepatocellular oxidative damage leading to hepatic inflammation (non-alcoholic steatohepatitis). Hepatic dysfunction leads to fibrosis followed by cirrhosis, liver failure and hepatocellular carcinoma.

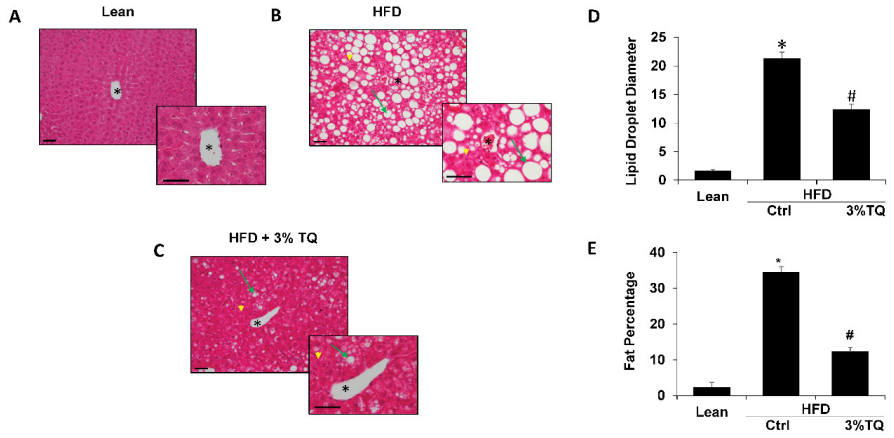

Figure 4: Haematoxylin-eosin staining of liver of lean (A), HFD (B), HFD treated with 1% TQ (C), and HFD treated with 3% TQ (D) mice. Graphs summarize the morphometrical analysis of liver lipid droplet diameter (F) and adipose tissue percentage (G). * p< 0.05 versus lean; # p< 0.05 versus HFD. Bar 50 μm. Yellow arrowheads show inflammatory cells, green arrows indicate adipose tissue and * denote centrolobular vein.

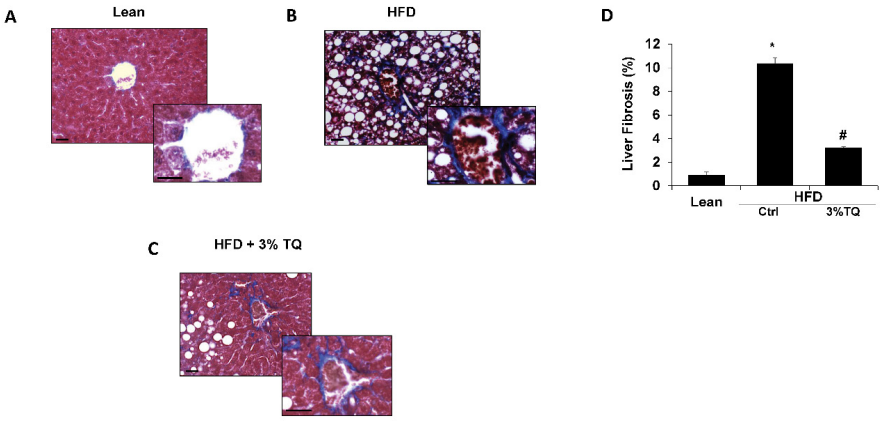

Figure 5: Masson's trichrome staining of liver and presence of fibrosis, lean (A), HF diet (B), HFD treated with 1% TQ (C), and HF diet treated with 3% TQ (D) mice. Graph summarizes the morphometrical analysis of fibrosis percentage (F). *p<0.05 *versus* lean; # p<0.05 *versus* HFD. Bar 50 μm.

Treatment of obese mice with TQ improves mitochondrial function in adipose and hepatic steatosis by decreasing levels of adipocyte derived NOV, and increasing, an antioxidant gene, HO-1 expression, resulting in increased mitochondrial biogenesis, function, and fusion potential, leading to an improvement in oxidative stress and inflammation in obese mice. The following key findings substantiate this conclusion. A HFD increased the expression of the genes regulating mitochondrial fission in mice, while concomitantly reducing the expression of the genes responsible for mitochondrial quality control and fusion processes in adipose and hepatic tissue. We further investigated whether TQ treatment positively affect signaling proteins. TQ positively increased HO-1 and insulin receptor phosphorylation in liver adipose tissue (Figures 7 and 8). Similar effect is seen in heart and kidney signaling protein (data not shown). A HFD enhanced FFA generation and increased mitochondrial dysfunction and ROS levels [30,31]. Mitochondrial dysfunction results in a decrease in beta oxidation in the liver allowing fat to accumulate resulting in a "fatty liver" [26,32,33]. TQ reduced mitochondrial fission potential and normalized an enhanced expression of mitochondrial fusion-associated genes in mice fed a HFD. TQ is a natural antioxidant and hypoglycemic compound that may prove advantageous therapeutically when compared to the high cost and the adverse effects of pharmacological drugs.

NOV expression in obese mice was increased when compared to lean mice, the levels of NOV in HFD-fed mice treated with TQ were lower than mice fed a HFD alone. Increased NOV levels are linked to increased levels of inflammatory cytokines which deleteriously affect insulin signaling, resulting in insulin resistance and eventually obesity [34,35]. In contrast, downregulation of NOV is associated with a reduction in adipose tissue deposition and inflammatory cytokines, as well as enhanced insulin sensitivity in obese mice [23,26].

Figures 4 and 5 showed that TQ improved hepatic steatosis, fibrosis and metabolic balance in obese mice. More importantly, ingestion of TQ in HF mice led to a reversal of this trend and a resultant increase in both the level and activity of HO-1, which strongly suggests a role for HO-1 and HO activity in the antioxidant and anti-inflammatory effect of TQ. Other report in agreement with our finding that induction of HO-1 suppresses adiposity and diabetes [36,37]. Further, sex-depends

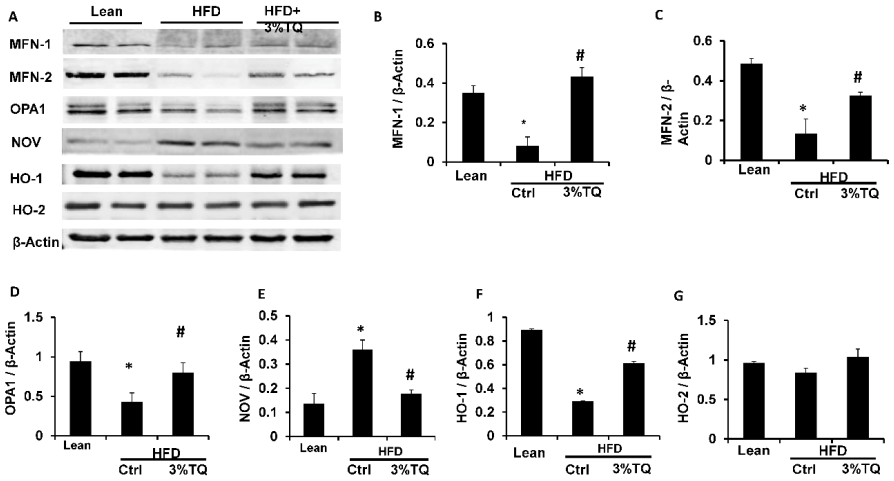

Figure 6: Effect of TQ administration increases mitochondrial function, antioxidant HO-1 and decreases cytokine NOV in liver tissue on obese mice. Representative western blots; (A) and densitometry analysis of (B) MFN1, (C) MFN2, (D) OPA1, (E) NOV, (F) HO-1 and (G) HO-2 of Lean, HFD and HFD +3% TQ. Results are mean ± SE, n=6, *p<0.05 *vs.* Lean, #p<0.05 *vs.* HFD.

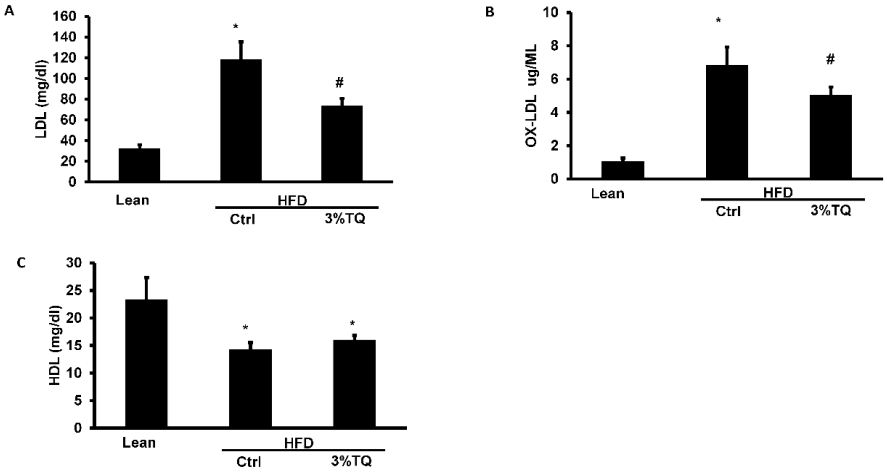

Figure 7: Analysis on plasma levels of (A) LDL, (B) OX-LDL and (C) HDL in Lean, HFD and HFD + 3% TQ mice respectively. Results are mean ± SE, n=6, *p<0.05 *vs.* Lean, #p<0.05 *vs.* HFD diet.

effect of HO-1 in adipose is well described [38], in which expression of HO-1 in adipose tissue may have a greater protective role in female as compared to male [38].

Further, HO-1 is considered a novel target for the treatment of hypertension and obesity [39]. More importantly beneficial effect of HO-1 is seen in human liver transplant biopsies; subjects with higher HO-1 levels showed decreased hepatocellular damage and improved outcomes [40].

Additionally, it appears that the increase of HO-1 levels, decrease in pro-inflammatory NOV expression and the normalization of mitochondrial function rescue liver function in obese mice. The beneficial effects of TQ on hepatic protein expression suggest an anti-steatosis effect that prevents disease progression to steatohepatitis in our animal model support the effect on decrease fasting glucose and oxygen consumption.

It appears that TQ was capable of reprogramming the adipocyte phenotype by regulating energy gene and mitochondrial function and HO-1 expression, leading to an increase in "healthy", i.e. small, adipocytes and a decrease in large adipocyte qualitatively and in terminal differentiation as evidence suggesting that increase in activity as evidence of increase in oxygen consumption, may maintain healthier adipocytes in obese mice. This occurred without body weight change, further, TQ improved the metabolic profile of obese mice by lowering fasting glucose, BP and hypertension, and increasing oxygen consumption compared to non-treated obese mice. One plausible explanation for body weight remaining unchanged could be the direct effect of TQ on adipocyte hyperplasia. This supports the hypothesis that the expansion of adipocytes may lead to an increased number of adipocytes of smaller size; smaller adipocytes are considered to be "healthy", insulin-sensitive adipocyte cells that are capable of producing adiponectin [41,42]. There is a tight link exists between adipocyte hypertrophy and inflammation; followed by a reduction in adipocyte size leading to amelioration of metabolic functions [43-46]. In our current study we show that TQ decreased lipid content. In agreement

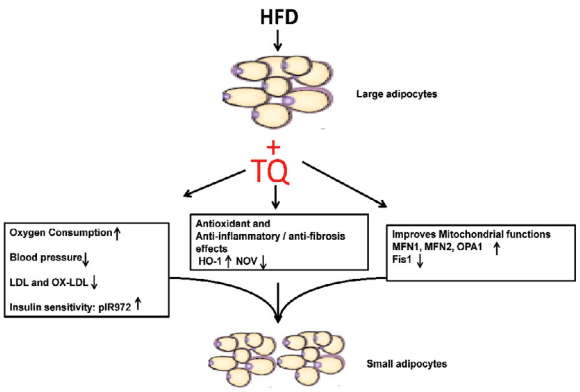

Figure 8: Schematic representation of TQ effects on reprogram adipocyte phenotype from naïve state to healthy adipocyte that increase activity of HO-1, mitochondrial proteins and insulin receptor phosphorylation. TQ treatment change the quality of fat from sick fat, adiposopathy, to healthy fat, i.e., metabolically active, that express more mitochondrial signaling, increase oxygen consumption and increase insulin receptor phosphorylation.

with our *in vivo* results and previously published reports, the increase of HO-1 levels in adipocytes turns large unhealthy adipocytes into small healthy insulin-sensitive adipocytes [47]. In addition, the decrease in pro-inflammatory adipocyte NOV expression, the increase of HO-1 levels and the increased levels of insulin receptor phosphorylation in adipose tissue lead to the normalization of mitochondrial function and a reversal of adipocyte phenotype from an inflammatory to a healthy functional status. Together, these results clearly indicate that activation of the HO-1 antioxidant response is crucial to the beneficial effects of TQ on mitochondrial biogenesis and on the reduction of fission and increase of fusion-associated processes in both adipose and hepatic tissue.

Importantly, in obesity, it is well established that there is association of elevation in HDL and OxHDL in obese animals. Obese mice treated with TQ demonstrated a significant decrease in LDL and OX-LDL levels. LDL oxidation, as well as HDL oxidation, is critical in the development of atherosclerosis and NAFLD has many features in common with cardiovascular disease, including lipid accumulation, macrophage activation and infiltration, and inflammation [48-50]. The activation of Kupffer cells by OX-LDL leads to a rapid release of various inflammatory mediators and signaling molecules such as cytokines, ROS, proteases, and lipid mediators that contribute to hepatic inflammation [51]. Fundamentally, TQ ingestion that result in decrease in OX-LDL and inflammatory molecule, NOV and increase in mitochondrial biogenesis and attenuates liver steatosis and NASH will contribute to an increase in insulin sensitivity and organ protection, indicates the potential of this nutraceutical approach to prevent disease progression in an animal model of metabolic syndrome. TQ intervention that contributes to lower blood pressure, fasting glucose may be beneficial to obese and non-obese subjects, may involve the increase of HO-1. HO-1 induction shown to lower blood pressure in hypertensive and obese animal models [52-55]. The beneficial effects of TQ in the pathogenesis of NAFLD in a murine model of obesity offer a portal into therapeutic approaches to the treatment of this and other obesity-related diseases.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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