ORIGINAL ARTICLE

Effects of Simvastatin and Omega-3 on Autophagic Flux and Adipogenicity Marker (PPARγ) in Obese Male Wistar Rat Model Induced with High-fat Diet

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ABSTRACT

Prescription of the lipid-lowering drug simvastatin, and omega-3 have been well-documented to possess anti-inflammatory, cardioprotective, and triglyceride-lowering properties. Their co-administration may demonstrate a complementary effect in lowering patients' triglycerides and total cholesterol; these traditional therapies were used for many years to treat atherosclerosis and prevent myocardial infarction and stroke. But their effects as potential anti-obesity therapy and/or protection against obesity are not yet known through the inhibition of autophagy.

Aim: The study aims to evaluate the effect of simvastatin on autophagic genes, including (P62) and on the adipogenicity marker (PPAR-γ), using quantitative real-time polymerase chain reaction (PCR).

Method: One hundred twenty (120) male Wistar rats (5-6 weeks age and weighing 100-150g) were allocated into five groups: treated with two different doses of simvastatin, omega-3, and mixed treatment in addition to high-fat diet group which considered as a control group. Treatments were given for eight weeks. Three rats from each group were weekly authenticated along the 60 days interscapular brown adipose tissue, and inguinal white adipose tissues were obtained.

Results: showed that well-known hypolipidemic drug-simvastatin has an obvious activation of P62/SQSTMI genes. This reflects a decrease in autophagic flux and consequent activation of PPARγ with its activation of thermogenic genes in adipose tissue in obese high-fat diet rats. Treatment with omega 3 alone and its co-administration with simvastatin produced no reduction in the autophagic flux, but there was a synergistic increase in the thermogenic mechanism.

Conclusion: This study gave hope for the utilization of simvastatin and omega-3 as anti-obesity therapy; but, each of them acts by different mechanisms; where, simvastatin act through its reduction of autophagic genes that consequently activate the adipogenic PPARγ gene, which co-operates with thermogenic genes in white and brown adipose tissues that results in adipogenesis reduction and a consequent weight loss; while omega-3 caused a significant body weight reduction without down-regulating autophagy process and its co-administration with simvastatin enhance its thermogenesis process.

Keywords: Adipogenic PPARγ genes, Autophagy gene, Beige adipocytes, Brown adipocyte, High-fat diet, Omega-3, P6, 2 LC3I/LC3II, Simvastatin.

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INTRODUCTION

The autophagy process is a well-known internal recycling mechanism that is responsible for maintaining cellular homeostasis through degradation of damaged and unwanted harmful components in the cell.[1] The degraded substrate can be recycled and used as energy substrates or for building new cellular components.[2,3] Obesity is considered as a kind of metabolic disease which contributes for the induction of adipose tissue remodeling and impairing of adipogenesis.[4,5] Inhibition of autophagy is harmful in some clinical conditions like atherosclerosis, sepsis, hepatic steatosis, and cachexia through an increase in free fatty acid (FFA) and glycerol release from white adipose tissue (WAT). While the emerging concept of conversion white adipose tissue to beige/brown adipose tissue through the browning mechanism has a controversial effect on obesity, facilitates weight loss, and

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improves metabolic health.[6] The P62, a protein also known as sequestosome-1 (Sqstm1), has been proposed as a predictor of the autophagic process, where it is required to remove waste through the packing and delivery of dysfunctional organelle and misfolded proteins. A study showed that the P62 has many other functions which extend beyond autophagy. It can interact with different key signaling-proteins through various structural elements and coordinates the processes required for metabolic homeostasis; this can happen, in part, through its connections with autophagy[7] since P62 does not only bind to proteins for disposal by autophagy, but it also degraded by autophagy. Studies approved that the inactivation of P62 resulted in a reduction of energy expenditure and, consequently, the development of obesity.[6-10] Moreover, P62 is considered as regulator thermogenesis in brown adipose tissue through its regulation of energy metabolism and controlling mitochondrial function. Therefore, adipocyte P62 is considered as a significant regulator of energy balance and adiposity.[11] Simvastatin, one of the traditional lipid-lowering[12,13] might alleviate excessive autophagy-that characterized by a high LC3II/LC3I ratio and low level of P62- and finally, exert beneficial effects on cardio-protection against pressure overload.[14] Other studies showed controversial results through treatment of glioma cell lines with simvastatin; since results showed the appearance of auto-phagolysosome and the induction of autophagy genes "LC3I/LC3II and Beclin-1" as well as down-regulation of P62 autophagic target.[15] Omega-3 fatty acids, which is known as ω-3 long-chain polyunsaturated fatty acids (ω-3 PUFAs), composed of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), elucidated an anti-inflammatory and cytoprotective against oxidative stress through activation of autophagy-associated gene including microtubule-associated protein light chain 3 (LC3) where its expression was found to be increased after treatment with DHA and EPA.[16,17] While another study showed a controversial result. The application of DHA and EPA on A549 lung cancer cells has been found to interfere with the autophagosome formation by activating the Akt/mTOR signaling pathway and reducing autophagic flux by decreasing Beclin-1 expression level and increasing P62 levels; thus, it may act as a possible anticancer drug.[18,19] The present work aims to evaluate the effect of simvastatin and omega-3 as ant obesity drugs through their effect on the most common autophagic markers "P62/SQSTMI, LC3I/LC3II "and the adipogenesis marker" PPARγ genes" using quantitative real time PCR in white and brown adipocyte of obese male Wistar rat model where the obesity had been induced using high fat diet.

MATERIAL AND METHODS

Animals and Experimental Design

The local research ethics committee approved the research protocol and animal care procedures, College of Pharmacy, University of Baghdad, Iraq, and in accordance with the standard requirements for the care and the use of experimental animals reported elsewhere.

One hundred and twenty (120) male Wistar rats, age of five- to six-week-old weighing 100 to 150 g, were obtained from the local bred of the Animal House, Department of Pharmacology and Toxicology, University of Baghdad, and housed under light/dark cycle (12/12 hours) and controlled room temperature (24ºC ± 2) with standard chow and drinking water *ad libitum*.

After a week of acclimatization, all animals were fed for eight weeks with a high-fat diet [(HFD) (standard chow contains 30% lard)] specially prepared for this purpose[20] to induce obesity and create an obese rat model. After that, when rats get obese according to their Lee index and body parameter;[21] since, Lee index refers to fat accumulation in the various region in the body, mainly in the abdomen region:

- Lee index¼cube root of body weight (g) / nose-to-anus length (cm),[22,23] which refer to accumulation of body fats.
- Lee index = [$\sqrt[3]{}$body weight in gram ÷ naso – anal length in mm] X 10000

Rats were randomly allocated into five groups (each containing twenty-four rats). Four of them received drugs, and one group continued feeding with HFD without any treatment and was considered a control (Group I). Twelve rats were housed /caged to reduce their activity inside the cage and reduce energy expenditure. Rats groups were treated as follows: Group II: Obese (HFD) rats administered pure simvastatin powder [Ph. Eur. Artemis Biotech (9 mg/kg/day)] via 14-inch oral gavage needle within 2cc running water;[24] Group III: Obese HFD rats given a double dose of pure simvastatin powder (18 mg/kg/day) via 14-inch oral gavage needle within 2cc running water.[24] Group IV: Obese, HFD rats, given omega-3 fatty acids (oral dose 1-mL/day) [Green Field Nutrition, Inc. Chicago, IL.60625 USA. Fish Oil =1000 mg, EPA = 180 mg, DHA =120 mg] with 14 inch oral gavage needle, and the Group V: Obese, HFD rats, given a combination of simvastatin (9 mg/kg) and omega-3 fatty acids (1-mL/day) with 14 inch oral gavage needle.[25] Fifteen rats were weekly-euthanized (3 rats/group) by diethyl ether (Romia pure chemistry/Cambridge/UK) and the interscapular brown adipose tissue (iBAT) and inguinal adipose tissue were dissected. The P62 autophagic gene and the PPAR-γ gene expression were measured using quantitative real-time PCR, and triplicate PCR amplification has been done for all genes to reduce bias.

STATISTICAL ANALYSIS

Data were analyzed using SPSS/IBM version 24. The numeric data were expressed as mean ± standard error of the mean (SEM). The statistical significance of each group in comparison with the obese/high-fat diet control group was determined by an independent t-test, while comparison among all groups was tested by the utilization of one way- ANOVA test. *p*-values less than 0.05 ($p < 0.05$) were considered significant for all data presented in this study.

RESULTS

Detection of P62 mRNA in Brown Adipose Tissue

Using quantitative PCR to evaluate the level of P62 mRNA in brown adipose tissue, results showed that treatment with

simvastatin 9 mg/kg/day (Group II) produced a significant increase at week five when compared to obese HFD (Group I) rats (mean=1509.5 ±S EM=10.5, $p < 0.05$). In contrast, in rats treated with simvastatin 18 mg/kg/day (Group III), there was a significant increase in the level of P62 mRNA in brown adipose tissue at the fourth week of treatment (mean = 735.53± SEM=14.25, $p < 0.05$). Furthermore, results showed that treatment with the omega-3 alone (Group IV), and a combination of omega-3 (1-mL/day) and simvastatin (3 mg/kg/day) (Group V) there were non-significant value ($p > 0.05$) of P62 mRNA level in brown adipose tissue during all eight weeks of treatment when each compared to obese HFD (Group I) rats as shown in Table 1. While by the utilization of the one-way ANOVA test, results showed a significant increase among all groups of rats from the first week of therapy. Table 1.

Detection of P62 mRNA in White Adipose Tissue

Results of Table 2 showed that rats treated with simvastatin 9 mg/kg/day (Group II), there was a significant increase in P62 mRNA level in white adipose tissue of rats at week 5 of treatment (mean = 257.9 ± SEM = 8.7 $p < 0.05$). In contrast, at the dose of simvastatin 18 mg/kg/day (Group III), there was a significant increase at the fourth week of treatment (mean = 385.8 ± 9.15 $P < 0.05$). However, non-significant results occur in therapy with omega-3 1-mL/day (Group IV) and its combinations at all 8 weeks of treatment ($p > 0.05$). While the one-way ANOVA test gave a significant increase ($p < 0.05$) in P62 mRNA level in white adipose tissue among all groups from the first week of therapy compared to obese HFD control (Group I) as shown in Table 2.

Detection of PPARγ Gene in Brown Adipose Tissue

Results showed that a significant increase started from 3rd week of simvastatin 9 mg/kg/day treatment (Group II) when compared to obese HFD (Group I) rats (mean = 313.5 ± SEM=18.29 $p < 0.05$); while, obvious significant increase occurs from the first week of starting simvastatin at18mg/kg/day therapy (Group III) rats (mean = 151.58 ± SEM = 8.3 $p < 0.05$); and omega-3 (1-mL/day) (Group IV) (mean = 149.2 ± SEM=5.9 $p < 0.05$), while mixed dose of simvastatin and omega-3 (Group V) showed a significant increase at all weeks except the second week of therapy for unknown cause (mean = 90.5 ± SEM 7.75 $p > 0.05$) as shown in Figure 1.

Table 1: Effects of two different doses of simvastatin, omega-3, and combination of simvastatin and omega3 on fold change expression of P62 mRNA values using quantitative real-time PCR in brown adipose tissue

Time of treatments in weeks	*Obese High-fat diet Group I*	*Simvastatin 9 mg Group II*	*Simvastatin 18 mg Group III*	*Omega-3 Group IV*	*Omega-3+ Simvastatin Group V*
Week 1	554.25 ± 42.05	31.9 ± 2.1	308.1 ± 0.5	62.8 ± 5.5	38.05 ± 1.05
Week 2	334.2 ± 21.8	150.5 ± 2.5	418.45 ± 0.05	99.95 ± 0.55	88.7 ± 0.5
Week 3	469.15 ± 7.15	282.5 ± 26.5	529 ± 0.5	176.2 ± 35.6	192.55 ± 28.7
Week 4	236.5 ± 0.5	632.5 ± 33.5	735.35 ± 14.2*	209.3 ± 7.7	308.08 ± 7.08
Week 5	302 ± 2	1509.5 ± 10.5*	967.9 ± 7.6*	337.3 ± 25.8	370.65 ± 36.3
Week 6	367.5 ± 1.5	1651 ± 12*	2341.5 ± 5.5*	473.7 ± 12.32	644.8 ± 44.9
Week 7	247.6 ± 3.6	1751.25 ± 19.2*	4167.9 ± 4.8*	585.05 ± 34.25	1150.9 ± 100.9
Week 8	564.1 ± 3.1	1811.1 ± 3.4*	4209.4 ± 1.6*	734.023 ± 35.7	1336.5 ± 216.5

Values are expressed as mean ± SEM; n=24 rats in each group; (*) refers to a significant difference in groups ($P < 0.05$) compared to Group I; time represents treatment in each week.

Table 2: Effects of two different doses of simvastatin, omega-3, and combination of simvastatin and omega3 on fold change expression of P62 PCR fold change of expression in white adipose tissue.

Time of treatments in weeks	*Obese High-fat diet Group I*	*Simvastatin 9 mg Group II*	*Simvastatin18mg Group III*	*Omega-3 Group IV*	*Omega-3+ Simvastatin Group V*
Week 1	21.9 ± 0.2	29.2 ± 2.2	82.2 ± .6.6	17.09 ± 5.51	18.555 ± 0.885
Week 2	25.8 ± 7.5	67.06 ± 4.06	117 ± 0.8	33.8 ± 4.9	37.75 ± 3.95
Week 3	22.1 ± 0.13	107.05 ± 7.95	147.72 ± 12.2	30.5 ± 1.25	39.86 ± 1.64
Week 4	37.3 ± 4.3	155.4 ± 14.3	385.8 ± 9.15*	62.3 ± 5.5	57.7 ± 9.1
Week 5	34.9 ± 4.79	257.9 ± 8.7*	422.96 ± 11.46*	34.8 ± 2.2	37.2 ± 2.65
Week 6	61.35 ± 1.65	384.5 ± 11.5*	362.38 ± 10.32*	43.35 ± 5.55	52.14 ± 4.24
Week 7	28.7 ± 8.3	474.7 ± 9.1*	478.5 ± 3.3*	42.4 ± 1.4	74.5 ± 9.05
Week 8	20.46 ± 1.03	485.5 ± 5.7*	431.7 ± 9.7*	53.35 ± 7.6	130.1 ± 8.3

Values are expressed as mean ± SEM; n = 24 rats in each group; (*) refers to a significant difference in groups ($p < 0.05$) compared to Group I; time represents treatment in each week.

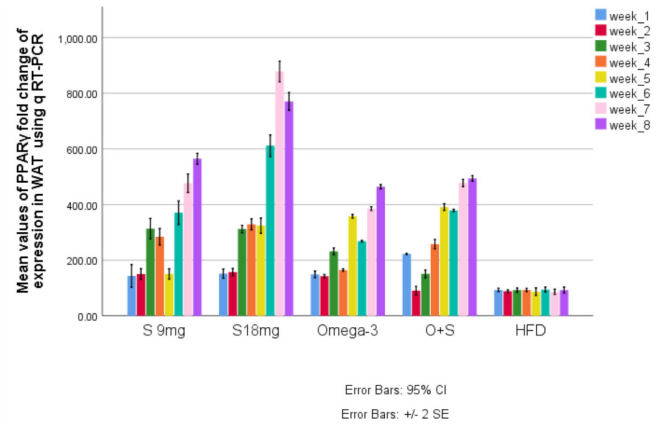

Figure 1: Effects of two different doses of simvastatin, omega-3, and combination of simvastatin and omega3 on PPARγ genes in brown adipose tissue (BAT).

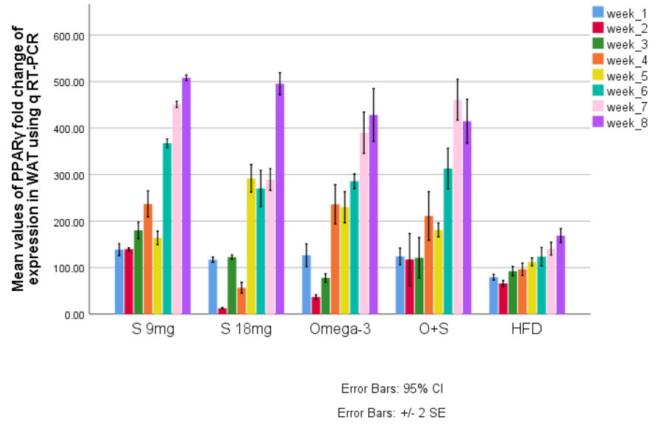

Figure 2: Effects of two-different doses of simvastatin, omega-3, and combination of simvastatin and omega3 on PPARγ genes in white adipose tissue (WAT).

Moreover, the ANOVA test gave a significant increase among all groups from the first week of treatment which explained activation of PPARγ gene in BAT from the first week of therapy.

Detection of PPARγ Gene in White Adipose Tissue

Results showed a clear significant increase for simvastatin treatment at both doses from the first week 9 mg and 18 mg/kg/day (Group II and Group III**)** (mean=138.84 ± SEM 6.39 $p < 0.05$) for Group II and (mean=117.2 ± SEM=2.87 $p < 0.05$) for Group III respectively. Moreover, treatment with omega-3 **(**Group IV**)** gave a significant increase in PPAR γ expression in week 6 and 7 (mean = 285 ± SEM=7.9 $P < 0.05$) (mean = 390.1 ± SEM = 22.2 $p < 0.05$). In contrast, omega-3 combined with simvastatin (9 mg/kg/day) (Group V) gave significant expression in PPAR γ from the fifth week of treatment (mean=181± SEM=7.4 $p < 0.05$) as in Figure 2. Furthermore, there was a significant increase ($p < 0.05$) in PPAR γ expression among all groups from the first week of treatment**.**

DISCUSSION

This study focused on the role of simvastatin that utilized in two different doses (9 mg/kg/day) and its double dose (18 mg/kg/day) on the level of the main autophagic markers (P62) gene in brown and white adipocyte and PPARγ genes in an attempt to correlate between autophagy process and thermogenesis program using simvastatin in an intent to discover a new possible therapeutic effect for this drug rather than treating high cholesterol level and atherosclerosis. For this aim, the induction of obesity in male rats was done with HFD (Group I) for eight weeks before starting the experiment. Male rat is preferred in such experimental model more than female, because induction of obesity is more easier in male since estrogen hormones in female rats found to protect against fat adipose tissue accumulation through its action on estrogen α (ERα) and estrogen β (ERβ).[26] Many previous studies mentioned that hyperactive autophagy occurs in the WAT of obese rats and humans (through an increase in LC3I/LC3II and reduction in P62 gene levels) to generate more fats from recycled energy in adipose tissue of obese patients.[27-29] Moreover, other studies have suggested that stimulating browning in WAT would be beneficial in slowing obesity, diabetes, and even the aging process.[30] This study revealed that simvastatin 9 mg/kg/day and 18 mg/kg/day led to significant decrease ($p < 0.05$) in autophagic flux in brown and white adipose tissue when compared to obese rats (HFD) (Group I) through an increase in P62 gene expression from the fifth week of treatment with simvastatin 9 mg/kg/day (Group II) rats and the fourth week (for simvastatin 18 mg/kg/day) (Group III) rats. Furthermore, up-regulation of PPARγ has been detected for simvastatin; since, at its dose 9 mg/kg/day (Group II) led to significant PPARγ gene expression from the third week of the experiment, and simvastatin 18 mg/kg/day (Group III) gave an obvious gene expression from the first week of therapy. In consistent with clinical reports, the increase in expression of P62 genes reflects a reduction in the expression of LC3I/LC3II autophagic genes with subsequent activation of PPARγ genes; where the peroxisome proliferator-activated receptor-gamma (PPARγ) that is a member of the nuclear receptor PPARs family, is a well-established and considered as a central player in coordinating numerous molecular events that ensure the normal physiological function of white, brown and beige adipocytes.[31,32] The PPARγ is mostly expressed in adipose tissue, both white and brown adipocyte, where it is involved in the developing and maintenance of the mature adipocyte phenotype.[33] The PPARγ exert a pleiotropic effect including (1) adipocyte differentiation and lipid storage[2] Acquisition of brown /beige adipocyte identity (3) maintenance of brown /beige adipocyte thermogenic capacity (4) Brown/beige adipocyte functional decline in aging.[34] Moreover, PPARγ is considered a master transcription factor in brown adipocyte's general differentiation program and induces uncoupling protein1 (UCP1) expression during adipogenesis.[35] Activation of non-shivering thermogenesis emerge a promising approach to lower body weight in obesity; where a previous study showed that reduction of P62 in adipocyte results in a reduction in systemic energy expenditure; furthermore, the P62 is considered as a critical regulator of energy balance and adiposity, and it may

regulate adaptive thermogenesis through ATF2 nuclear target which activates in its turn UCP1 and Pgc1α and results in down-regulation of adipogenesis.[36] In this study, the increased thermogenic and mitochondrial activity characteristic of P62 up-regulation is due to the increased activation of the PPARγ transcriptional program. This may speculate that activation of thermogenesis genes, mainly" UCP1" as a consequence of a multi-component protein complex; where P62 protein can form a complex with PPARγ in the brown and white adipose tissues and accelerate the activity of thermogenic genes (UCP1, Pgc1α).[35] Omega-3 fatty acids supplements provide various health benefits in a tissue-specific manner; since many studies have been shown that administration of omega-3 results in increased energy expenditure in muscle[37] and decreased inflammation in immune cells.[38] Furthermore, the metabolic benefits of PUFA derived from fish oil resemble the adaptive metabolic responses upon brown/beige fat activation through activation of adaptive thermogenesis process. For this reason, n-3 PUFA intake has gained attention as a dietary regimen to promote thermogenesis.[39] Therefore, omega-3 can exert dual benefits in obesity by reducing lipid accumulation in WAT with consequent activation of thermogenesis and reducing lipogenesis in BAT.[40-42] The use of omega-3 PUFA showed an oscillatory effect (increase and decrease) in autophagy flux in BAT throughout 8 weeks of an experiment when compared to obese HFD (Group I) rats. Moreover, this study showed that P62 gene expression post-omega-3 administration (Group IV) produced a non-significant increase ($p > 0.05$) with the high autophagic flux of obese rats. While the gene expression of PPARγ in adipocytes showed a clear significant increase ($p < 0.05$) when compared to obese HFD (Group I). This may explain the increase in the thermogenesis process in white and brown adipose tissues. While results of co-therapy of omega-3 and simvastatin 9 mg/kg/day (Group V rats) compared to obese HFD (Group I) rats showed an obvious non-significant increase ($p > 0.05$) in autophagic gene expression P62. Furthermore, the PPARγ gene expression gave a significant increase ($p < 0.05$) along the therapy process. While the PPARγ gene expression in WAT showed an oscillatory increase and decrease in expression in Group IV rats along with the experiment. Furthermore, in rats treated with omega 3 plus simvastatin (Group V), an obvious increase in expression of PPARγ gene expression started from the 5th week of the experiment in WAT. This may explain the increase in thermogenesis process in this rats' group with a clear reduction in adipogenesis and body weight. To our knowledge, no study was conducted to study the effect of simvastatin, omega 3, and its co-treatment. Thus, we cannot have a chance to compare the results of this study with others. Moreover, the current study opens the door for further research in this field.

CONCLUSION

The present study showed a hope for the utilization of simvastatin and omega-3 as anti-obesity therapy; but each of them acts by different mechanisms as simvastatin alone utilized at two different therapeutic doses results in a reduction in the autophagy flux with activation of PPARγ gene expression and subsequent activation of thermogenic genes in brown and white adipose tissue with a consequent weight loss. While omega-3 caused a significant body weight reduction without down-regulating the autophagy process, its co-administration with simvastatin enhances the thermogenesis process synergistically.

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