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Original Article

ANTI-OBESITY EFFECT OF RESVERATROL IN RATS ON HIGH FAT DIET THROUGH REGULATION OF GENE EXPRESSION OF VISCERAL WHITE ADIPOSE TISSUE

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ABSTRACT

Objective: Obesity is a chronic disease associated with serious health complications including inflammation, insulin resistance, metabolic syndrome, cardiovascular complications and several types of cancers. So this study was carried out to look for effective intervention, especially with its rising prevalence in adults and children and lack of well-tolerated therapy.

Methods: Rats received high-fat diet for four months to induce obesity. Animals which had ≥30 % increase in body weight were selected in this study. Obese rats were treated for 8 w with calorie restriction (25 % food restriction of commercial chow) and/or resveratrol (30 mg/kg/day orally).

Results: Obese rats demonstrated a significant elevation in the body and visceral white adipose tissue (WAT) weight, serum total cholesterol (TC), triacylglycerol (TAG) and low-density lipoprotein cholesterol (LDL-c). Visceral WAT gene expression of fatty acid synthase (FAS) significantly increased. A remarkable decrease in gene expression of peroxisome proliferator-activated receptor gamma (PPARɣ), lipoprotein lipase (LPL) and silent information regulation 2 homolog 1 (Sirt1) was recorded. The combination significantly decreased body and visceral WAT weights, TC, TAG, LDL-C and FAS mRNA expression and increased adiponectin level, the mRNA expression of PPARɣ and Sirt1as compared to individual treatments. While all treatment strategies showed a similar increase in LPL mRNA expression.

Conclusion: Calorie restriction supported with resveratrol has beneficial effects in terms of reducing body weight, and appears to reverse high fat diet-induced dysregulation of expression of the genes responsible for healthy visceral WAT.

Keywords: Obesity, Visceral white adipose tissue, Calorie restriction, Resveratrol.

INTRODUCTION

Obesity is a metabolic disease that arises from energy imbalance when energy intake exceeds energy expenditure. According to the data from the World Health Organization in 2014, 13 % of adults aged ≥18 y old were obese, and thus, nearly 2 billion adults worldwide are overweight and, of these, more than half a billion are obese[1]. Obesity is no longer a cosmetic problem, on the contrary, it is a serious medical condition associated with cluster of chronic diseases including, insulin resistance, inflammation, type 2 diabetes mellitus, dyslipidemia, metabolic syndrome, heart diseases, as well as, several types of cancers, so increasing the burden on the society concerning health care costs for obesity and its related complications [2, 3].

White adipose tissue (WAT), was considered in the past as a static organ that is hormonally inert and only serves as a site for energy storage, however, recently WAT has attracted attention as a dynamic organ that represents the largest endocrine organ in the body. The WAT is the major source of obesity-related complications, as obesity causes WAT hypertrophy, dysregulated expression of several enzymes and adipokines e. g. adiponectin and leptin, and elevated expression of pro-inflammatory molecules such as TNFα that causes inflammation leading to insulin resistance and metabolic. Although both subcutaneous and visceral WAT are linked to metabolic risk profile, visceral adipose tissue is more associated with metabolic syndrome [4, 5]. Accordingly our study will focus on the visceral white adipose tissue.

Calorie restriction, defined as reducing caloric intake below usual ad libitum intake while maintaining adequate nutrition, represents one alternative, and it has been found to extend lifespan in mammals, slow aging and reduce the incidence of major diseases like type 2 diabetes, cardiovascular diseases, and cancer [6]. However, it is sometimes difficult to maintain long-term calorie restriction in modern society. Consequently, there has been an increased interest in developing pharmacological agents that act as calorie restriction mimetic as resveratrol (3, 5, 4 -*trans*-trihydroxystilbene), naturally abundant poly phenolic compound, found in grapes, berries, peanuts and red wine [7]. A growing body of literature has provided evidence for the multi-faceted properties of resveratrol and suggests that resveratrol may have potential therapeutic implications including anti-inflammatory, antioxidant, cardioprotective, and beneficial properties against aging, metabolic diseases and obesity [8, 9].

After the withdrawal of sibutramine and rimonabant, as weight loss drugs, because the former was found to be associated with increased cardiovascular complications and strokes, and the latter; for its psychiatric side effects. Orlistat became the only approved drug for long-term treatment of obesity which is often not well tolerated for its gastrointestinal upsets, and also needs kind of limitation for fat rich diets as it prevents dietary fat absorption by only 30 %, thus rises an indeed need to have other interventions to obesity management [10].

Therefore, the present study was undertaken to investigate the common therapeutic mechanisms between calorie restriction and resveratrol on metabolic changes induced by high-fat diet, and the possible synergistic effect of the combination of calorie restriction and resveratrol as a promising alternative for obesity management.

MATERIALS AND METHODS

Animals and experimental protocol

Forty mature male wistar albino rats weighing 150±15 g, supplied from the Egyptian Organization for Biological Products and Vaccines (Cairo, Egypt), were housed in stainless steel rodent cages at room temperature (25±2 °C) with a 12 h dark/light cycle. Animals were fed rodent chow (El-Nasr Pharmaceuticals and Chemicals Industry, Egypt), and allowed free access to drinking water. All animal experiments received approval from the Ethical Committee of the Faculty of Pharmacy, Zagazig University, Egypt (No. P14/1/2014).

After one week of acclimatization, rats were assigned to two groups, one kept on normal chow diet (n=8) and the other switched to high-fat diet (25% total fat (including 11% unsaturated fat), 44%

carbohydrate, 18% protein, and 13% fiber, ash and other ingredients)(n=32) for four months to induce obesity[11]. The latter group was subdivided to four groups of eight animals as following: obese control group, calorie restricted group (25% food restriction of commercial chow), resveratrol (supplied from Mega Resveratrol and Candlewood Stars Inc., USA) group (receiving 30 mg/kg daily orally) and (calorie restriction+resveratrol) group for 8 w.

Sample collection and storage

At the end of the experimental period, body weight was determined for all groups then rats fasted overnight, blood was obtained via retro-orbital bleeding in dry centrifuge tubes and centrifuged at 3000 rpm for 15 min for serum separation to analyze biochemical parameters, and animals were sacrificed. The visceral white adipose tissues were removed and weighed for evaluation of abdominal fat content. All tissue samples were frozen until analysis.

For histological study, visceral fat specimens were kept in 10 % formalin for at least 1 w, and then the specimens were dehydrated with a series of ascending grade ethanol from 75 to 100 %. Tissues were placed thereafter in xylol and embedded in paraffin. Cross sections of about 4 μm thickness were processed on slides and stained with hematoxylin and eosin (H&E) stain.

Estimation of biochemical parameters

Serum levels of TC, TAG and HDL-c, were determined using commercially available kits (Spin react), and LDL-c was calculated according to Friedewald formula

$$LDL\text{-}c\ (mg/dl) = TC - [HDL\text{-}c + TAG/5]$$

Reverse transcription–polymerase chain reaction

Total RNA was extracted from visceral white adipose tissue using the acid guanidinium thiocyanate–phenol–chloroform method. RNA concentration and purity were measured using a UV spectrophotometer, with A260/A280 ratios of 1.8 to 2.0 considered acceptable. Reverse transcription–polymerase chain reaction (RT-PCR) was performed using the extracted RNA for evaluation of PPARɣ, LPL, FAS, and Sirt1 gene expressions. β-actin expression level was used as a loading control for each sample. For amplification of target genes, RT-PCR was run as 2 separate steps. Briefly, equal amounts of total RNA were denatured thermally and reverse transcribed using the Moloney murine leukemia virus reverse transcriptase (Promega, Madison, Wisconsin), ribonuclease inhibitor (Promega), deoxynucleoside 50 triphosphate and oligo-dT primer. The reactions were terminated by heating to 95 ˚C for 10 min, followed by cooling to 4 ˚C. The complementary DNA samples were amplified in the presence of Taq DNA polymerase (Promega), deoxynucleoside 50 triphosphate, and the appropriate primer pairs (primers, annealing temperatures, and product sizes are listed in table 1). All PCR products were separated on 2% agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator. Semi-quantification was performed using a gel documentation system (Biodoc analyzer) supplied by Biometra (Gottingen, Germany). The relative expression level of each studied gene (R) was calculated according to the following formula: R = Densitometric units of gene/Densitometric units of β-actin.

Table 1: Primer sequences, annealing temperature and product size for the studied genes

Gene	Primer sequence	Annealing temp.	Product size
PPARγ	Forward primer: 5'-GATGACCACTCCCATTCCTTTG-3' Reverse primer: 5'-GATGCTTTATCCCCACAGACTC-3'	55 °C	281 bp
LPL	Forward primer: 5′-GAGATTTCTCTGTATGGCACC-3' Reverse primer: 5′-CTGCAAATGAGACACTTTCTC-3'	57 °C	275 bp
Sirt1	Forward primer: 5′-AGCTGGGGTTTCTGTTTCCT-3' Reverse primer: 5′-TGCTGAGTTGCTGGATTTTG-3'	55 °C	224 bp
FAS	Forward primer: 5'-GGCCTGGACTCGCTCATGGG-3' Reverse primer: 5'-TGGGCCTGCAGCTGGGAGCA-3'	60 °C	514 bp
Beta actin	Forward primer: 5'-AACCCTAAGGCCAACCGTGAAA-3' Reverse primer: 5'-TCATGAGGTAGTCTGTCAGGTC-3'	60 °C	265 bp

Abbreviations: PPARɣ, Peroxisome proliferator-activated receptor gamma; LPL, Lipoprotein lipase; Sirt1, Silent information regulation 2 homolog 1; FAS, Fatty acid synthase.

Measurement of adiponectin by enzyme-linked immunosorbent assay

Visceral WAT adiponectin concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (*In vitro* gen) according to the manufacturer's instructions.

Statistical analysis

All results were expressed as mean±SD. Statistical analysis and correlation were performed using GraphPad Prism software. Student "t" test and the analysis of variance (one-way ANOVA) followed by Tukey's post hoc test were used for comparison between groups. The correlations between the studied parameters were assessed using the Pearson's correlation coefficient (r).

RESULTS

Body and visceral white adipose tissue weights

Body and visceral WAT weights increased significantly in the obese group by 145.5 % and 780.8 % respectively after the four months high fat diet compared to the normal group, while significant decreases were observed after 8 w in the calorie restriction, resveratrol, and calorie restriction+resveratrol groups. The calorie restriction+ resveratrol group showed a significant decrease in body and visceral WAT weight compared to calorie restriction group (fig. 1A and 1B).

Serum parameters

Comparison between normal and obese groups (table 2) has shown that the obese group had significantly higher total cholesterol, triacylglycerol and LDL-c ($p<0.001$) and lower levels of HDL-c. These

alterations in lipid profile were significantly ameliorated in all treated groups.

Co-administration of resveratrol with calorie restriction significantly lowered total cholesterol, triacylglycerol and LDL-c by 17.9 %, 27.5 %, and 29 % respectively, as compared to calorie restricted group ($p<0.05$). The HDL-c showed non-significant change.

Tissue parameters

Messenger RNA expression of PPARɣ, LPL, Sirt1 and FAS in visceral WAT

There was a significant decrease in visceral WAT PPARɣ, LPL, and Sirt1 messenger RNA (mRNA) expression with a concomitant increase of FAS mRNA expression in obese rats as compared to normal ($P<0.001$). Calorie restriction, RSV and their combination are induced a marked up regulation of PPARɣ and LPL genes and downregulation of FAS gene. However, CR and its combination with RSV showed significant up regulation for Sirt1 gene. In comparison to CR group, the combination demonstrated a marked up regulation of PPARɣ and Sirt1 genes and downregulation of FAS gene (fig. 2A, 2B,2C and 2D).

Adiponectin

Figure3. Illustrate that high-fat diet and subsequent obesity-induced a significant decrease in adiponectin content in white adipose tissue as compared to the normal control group ($p<0.001$). Treatment with calorie restriction or resveratrol either individually or in combination significantly increased adiponectin as compared to the obese control group ($p<0.05$).

In comparison to CR rats, the combination revealed a significant increase in WAT adiponectin content ($p<0.05$).

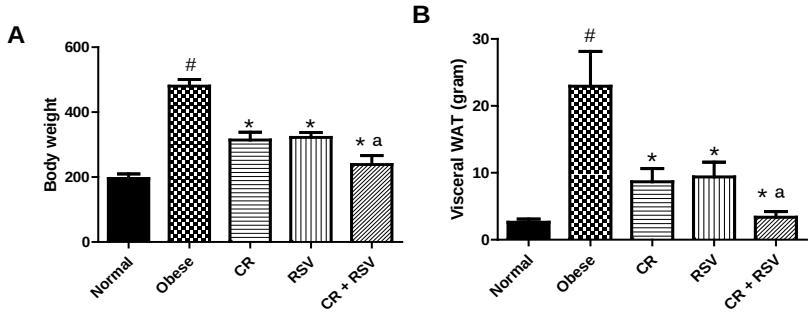
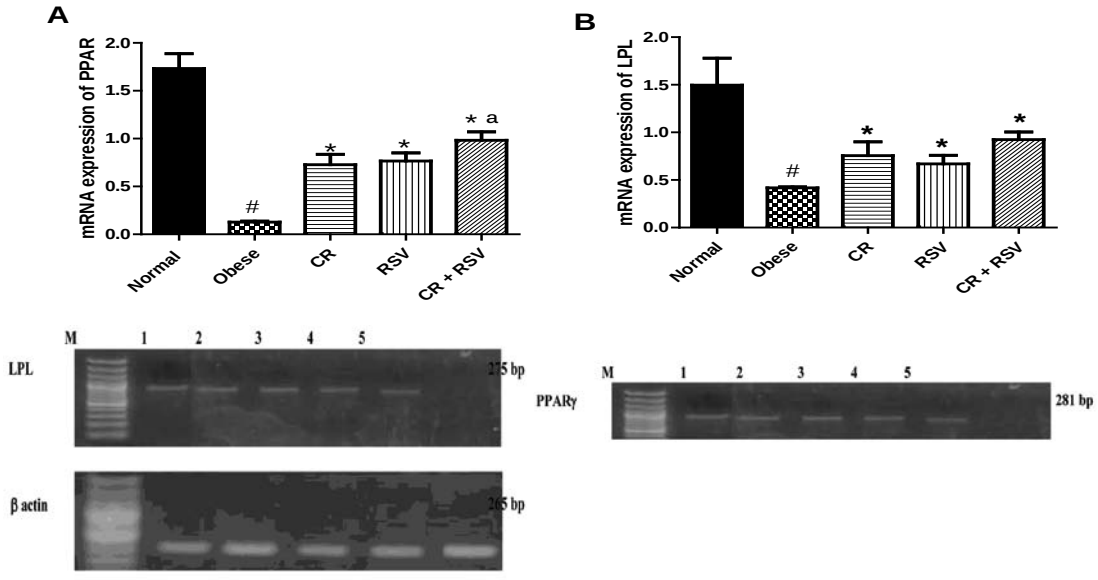


Fig. 1: Effects of CR, RSV and their combination on Body weight (A) and visceral WAT weight (B); Values are expressed as mean±SD (n=8), # significantly different from normal group at p<0.001, *significantly different from obese group at p<0.05, ª significantly different from calorie restricted group at p<0.05. CR, calorie restriction; RSV, resveratrol; WAT, White adipose tissue

Table 2: Serum parameters of obese rats with calorie restriction, resveratrol (30 mg/kg/day) either individually or in combination with calorie restriction for 8 w

	Normal	Obese	CR	RSV	CR+RSV
TC	85.4±4.4	145.6±7.2 #	108.1±10.4*	109.8±14.5*	88.8±7* ª
TAG	61.1±4.1	110±9.4 #	86.8±7*	73.9±2.8* ª	62.9±8.6* ª
HDL-C	36.5±4.2	24.3±4.3 #	35.6±5.6*	34.2±5.9*	37.1±3.8*
LDL-C	36.7±1	99.3±1.6 #	55.1±3.9*	60.8±9.9*	39.1±1.9* ª

Values are expressed as mean±SD (n=8), # significantly different from the normal group at $p<0.001$, * significantly different from the obese group at $p<0.05$, ª significantly different from calorie restricted group at $p<0.05$. CR, calorie restriction; RSV, resveratrol; TC, total cholesterol; TAG, triacylglycerol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol



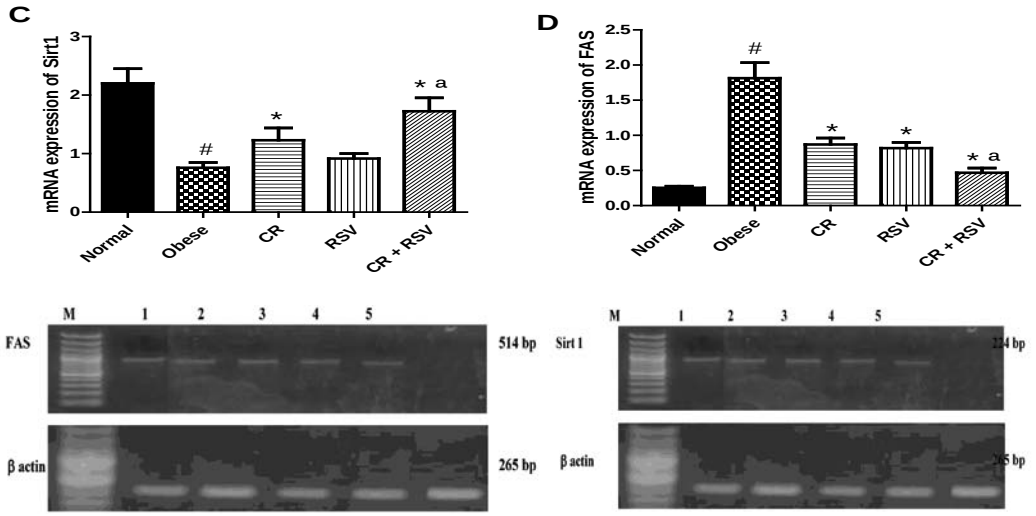


Fig. 2: Effects of CR, RSV and their combination on mRNA expression of PPARɣ (A), LPL (B), Sirt1 (C) and FAS (D). # Significantly different from normal group at p<0.001,* Significantly different from obese group at p<0.05,ª Significantly different from calorie restricted group at p<0.05, CR, calorie restriction; RSV, resveratrol; PPARγ, Peroxisome proliferator-activated receptor gamma; LPL, Lipoprotein lipase; Sirt1, Silent information regulation 2 homologs 1; FAS, Fatty acid synthase

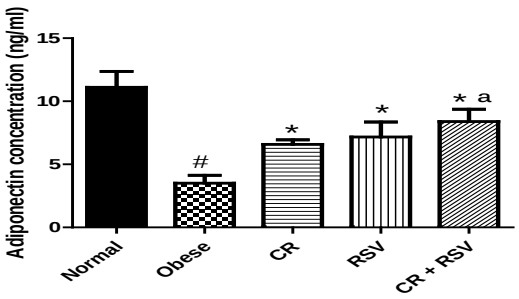


Fig. 3: Effects of CR, RSV and their combination on Adiponectin concentration. Values are expressed as mean±SD (n=6), # significantly different from the normal group at p<0.001, *significantly different from the obese group at p<0.05, ª significantly different from calorie restricted group at p<0.05. CR, calorie restriction; RSV, resveratrol

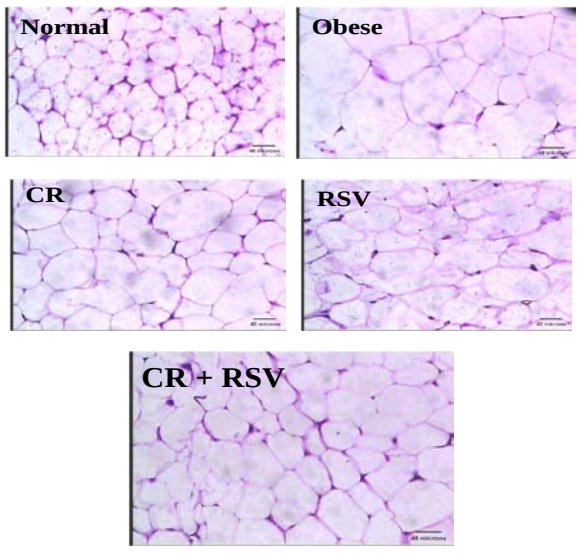


Fig. 4: A photomicrograph of sections in visceral WAT (H&E X100) from different groups showing many adipocytes with very big size in obese rats, and smaller size in CR, RSV and their combination. CR, calorie restriction; RSV, resveratrol

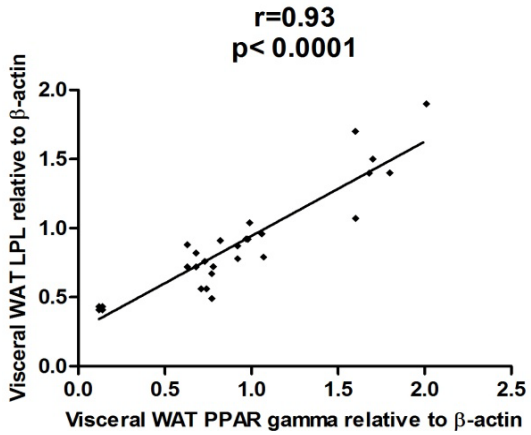


Fig. 5: Correlation between visceral WAT LPL gene expression and visceral WAT PPARγ gene expression. WAT, white adipose tissue; PPAR gamma, Peroxisome proliferator-activated receptor gamma; LPL, Lipoprotein lipase

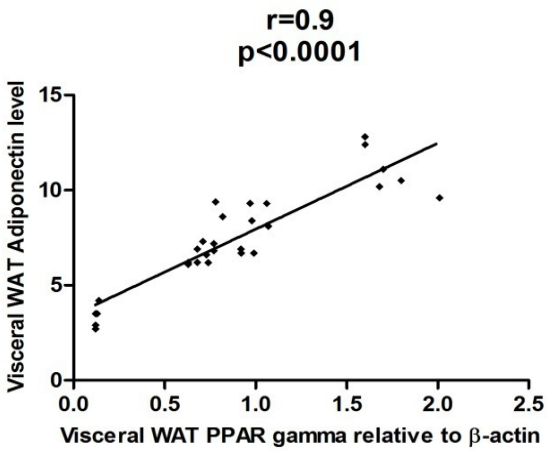


Fig. 6: Correlation between visceral WAT PPARγ gene expression and visceral WAT adiponectin level. WAT, white adipose tissue; PPAR gamma, Peroxisome proliferator-activated receptor gamma

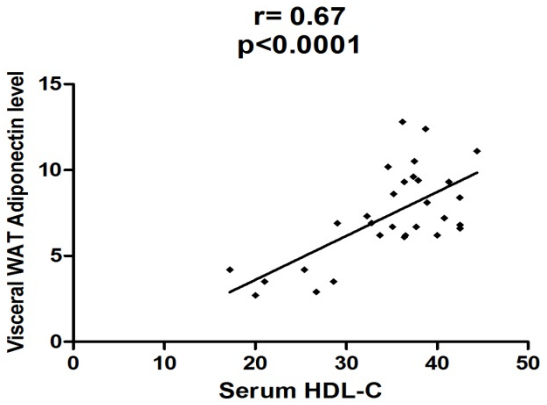


Fig. 7: Correlation between serum HDL-C and visceral WAT adiponectin level. WAT, white adipose tissue; HDL-c, high-density lipoprotein cholesterol

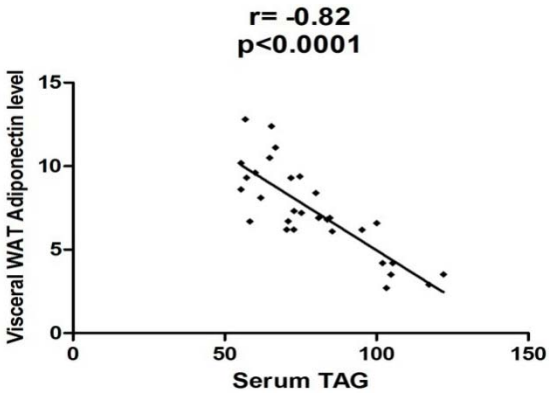


Fig. 8: Correlation between serum TAG and visceral WAT adiponectin level. WAT, white adipose tissue; TAG, triacylglycerol

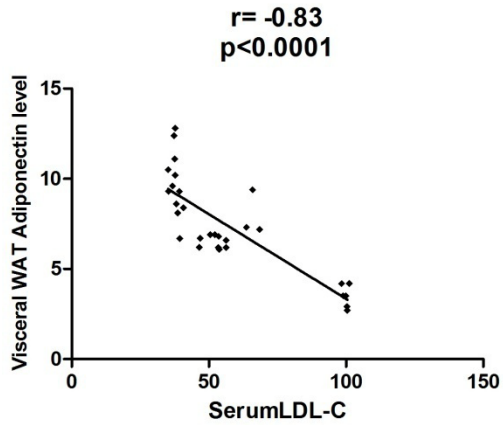


Fig. 9: Correlation between serum LDL-C and visceral WAT adiponectin level. WAT, white adipose tissue; LDL-c, low-density lipoprotein cholesterol

The morphological changes in visceral WAT

Microscopic examination of H&E stained sections of visceral WAT of the normal group revealed small size adipocytes. However, the obese group showed extremely enlarged adipocytes.

All strategies of treatment either dietary (CR) or therapeutic (Resveratrol) or combination showed an observed decrease in the size of adipocytes, relatively similar to that of normal rats.

DISCUSSION

The present study provides some evidence for a high fat diet-induced metabolic disturbances and protective effect of maintaining negative energy balance through calorie restriction or administration of resveratrol (30 mg/kg/day), as well as, their combination.

The current work handled the effect of high-fat diet in the induction of obesity. The latter was manifested in a marked increase in body weight and visceral WAT mass. Moreover, dyslipidemia was reported with a dramatic increase in serum TC, TAG and LDL-c and decrease in HDL-c.

Additionally, the study demonstrated a significant reduction in visceral WAT mRNA expression of PPARγ and LPL in obese rats compared to normal one which, in part, could be explained by the increase of inflammatory mediators associated with obesity including TNFα, leading to CCAAT interactions with PPARγ and LPL promoter regions. Our results are in good accordance with those published by other authors [12, 13]. Moreover, this study showed a positive correlation between PPARγ and LPL. This result is in consistency with another study [14]and this provides evidence that LPL gene is a target for the action of PPARγ.

The study presented here stated a significant reduction in adiponectin level in obese rats compared to normal one. Large adipocytes found in obese rats are known to produce higher levels of pro-inflammatory cytokines including TNFα, these inflammatory cytokines, and hypoxia that results in endoplasmic reticulum stress due to the accumulation of unfolded proteins in the endoplasmic reticulum can induce a reduction in adiponectin level [15, 16]. In addition decrease in PPARγ, which is involved in enhancing adiponectin levels through endoplasmic reticulum oxidoreductase (Erol-Lα) and disulfide-bond A oxidoreductase-like protein (DsbA-L), could contribute to this reduction[17]**.** This study clearly demonstrated a positive correlation between PPARγ and adiponectin.

Our data suggested the presence of insulin resistance state which has been previously reported being associated with decreased expression of PPARγ, LPL and adiponectin [18-20]**.**

With regard to FAS, the central enzyme in de novo lipogenesis, the present study showed a significant rise in its visceral WAT expression in high-fat diet supplemented rats, which is significantly related to obesity and visceral fat accumulation.

The Sirt1, a nicotinamide adenine dinucleotide (NAD^+) dependent protein deacetylase causing either activation or inactivation of target proteins, has been extensively studied for its multiple beneficial effects including anti-obesity effect through decreasing adipogenesis and lipogenesis and increasing mitochondrion genesis [21]. In the present study, it was found that high-fat diet significantly downregulates the expression of Sirt1 in visceral WAT.

Both consumptions of calorie restricted diet and resveratrol showed a significant increase in mRNA expression of PPARγ and consequently LPL as one of its target genes and thus protect from many detrimental effects of obesity. This action is obtained through maintaining the differentiated state of adipocytes and the growing number of small adipocytes resulting in increasing insulin sensitivity, and possibly by causing apoptosis of large fat cells [22, 23]**.**

Adiponectin, which is a protein greatly expressed by adipose tissue and involved in lipid metabolism by increasing fatty acids oxidation and lowering free fatty acids, has an anti-obesity activity possibly through activation of AMPK and PPARα pathways[24]. Adiponectin was significantly increased in CR and resveratrol groups contributing to their body weight-lowering effects and causing metabolic improvements by reducing serum TAG and fat cell size. Another possible mechanism for the action of adiponectin proposed by [25] is decreasing the secretion of tissue inhibitor of metalloproteinase (TIMP), essential for adipogenesis, thus reducing adipocyte hypertrophy and fat accumulation.

As a result of the significant increase in gene expression of genes related to lipid processing in adipose tissue as LPL and the significant increase in adiponectin level, in which its subnormal

levels are known to be related to dyslipidemia [26], an improvement of the lipid profile represented by significant decrease in serum levels of TC, TAG, LDL-c with a significant increase in HDL-c was observed in treated rats. These results were supported by correlation studies.

Beneficial effects of CR in the protection against diet-induced obesity and its adverse consequences as insulin resistance can also be attributed to the significant increase in visceral WAT expression of Sirt1. This effect may be through several proposed mechanisms including de acetylation and activation ofPGC-1α, a master regulator of mitochondrial biogenesis and fatty acid oxidation and could promote energy expenditure[27]. De acetylation and activation of FoxO1 that up-regulates adipose triglyceride lipase (ATGL) which activates lipolysis in adipocytes and reduces TAG stores[28], and by reducing the expression of lipogenic enzymes [29].

By contrast to some publications that stated sirt1 activation as one mechanism by which resveratrol produces its beneficial effects on health[30-32], our results showed that resveratrol alone exert only weak non-significant increase in sirt1 expression, while upon combining with CR, a sirt1 activator, this group expressed a significant increase when compared with both obese and CR groups.

Growing evidence has proposed that resveratrol treatment mimics CR in numerous health aspects, including its metabolic effects [6, 33].

The present study shed light on the beneficial effects of using a combination between CR and resveratrol compared to CR alone. The results demonstrated a synergistic effect for this combination represented as a significant decrease in body weight, visceral WAT weight, and adipocyte size in the group receiving CR+resveratrol when compared to the group on CR alone.

Additionally, better improvement in the lipid profile, significant increase in visceral WAT mRNA expression of PPARγ, Sirt1 and levels of adiponectin and significant decrease in visceral WAT mRNA expression of FAS were observed in the group receiving CR+resveratrol, when compared to the group on CR alone.

CONCLUSION

Dietary treatment of obesity by restricting calorie intake can suppress body weight gain and improve the metabolic effects caused by obesity. Antioxidant, anticancer and cardioprotective effect of resveratrol (as therapeutic treatment of obesity) can potentiate the favorable effect of CR diet. Therefore, this study recommended such regimens especially the combination to reduce adverse metabolic syndrome of obesity. Further clinical studies are an urgent need to validate these results.

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CONFLICT OF INTERESTS

Declared none

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