Original Article

# Beneficial Effect of Metformin, Quercetin, and Resveratrol Combination on High Glucose-Induced lipogenesis in HepG2 Cells

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## ABSTRACT

**Objectives:** It has been reported that Metformin (MET), Resveratrol (RSV), and Quercetin (QRS) possess anti-lipogenic effects. This study aimed to investigate the combined effects of these compounds on lipid accumulation in HepG2 cells treated with high glucose (HG).

**Methods:** HepG2 cells were treated with HG (33 mM), and different concentrations of MET, QRS, and RSV. The cytotoxic effects of these compounds were determined by an MTT assay. Changes in total lipid content and triglyceride (TG) levels were measured using Oil Red O staining and a triglyceride assay kit, respectively. The expression of fatty acid synthetase (FAS) and sterol regulatory element-binding protein 1c (SREBP-1c) was evaluated by quantitative real-time PCR.

**Results:** MET at doses 1, 2, and 5 mM, QRS at doses 5, 10, and 20 µM, and RSV at doses 25 and 50 µM could decrease total lipid content and triglyceride levels in HepG2 cells. The EC50 (half maximal effective concentration) from Oil red O staining results were MET: 1.786 mM, QRS: 8.132 µM, and RSV: 10.9 µM. The combination of MET (mM), QRS (µM), and RSV (µM) at ratios of (2:20:50), (1:10:25), and (0.5:5:10), could reduce lipogenesis greater than that observed with each of the individual compounds of MET, QRS, or RSV or the double combinations of MET+QRS or MET+RSV. In addition, combined treatment of MET (0.5mM), QRS (5 µM), and RSV (10 µM) was able to decrease SREBP-1c and FAS genes expression in HG-treated cells.

**Conclusion:** The combination of MET, QRS, and RSV could inhibit lipid accumulation in HepG2 cells by reducing total lipid content, triglyceride levels, and the expression of the genes involved in lipogenesis.

**Keywords:** Diabetes, Lipogenesis, Metformin, Quercetin, Resveratrol, HepG2, Combination, Liver, Non-alcoholic fatty liver disease

**Abbreviations:** FAS: fatty acid synthase, FFA: free fatty acid, HG: high glucose, NAFLD: nonalcoholic fatty liver disease, NG: normal glucose, SREBP: sterol regulatory element-binding protein 1c, MET: Metformin, RSV: resveratrol and ORS: Quercetin

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## Introduction

The increasing prevalence of diabetes worldwide is one of the most serious and challenging health problems in the 21st Century. The World Health Organization estimates that the number of people with diabetes will double by 2030 (1). Defects in insulin action (insulin resistance) and insulin secretion (β cell dysfunction) are two key players in the pathophysiology of type 2 diabetes (T2D) (2, 3). Insulin resistance is characterized by an impaired effect of insulin to reduce hepatic glucose production and to promote glucose uptake in peripheral tissues (4). In the liver, insulin resistance is an important underlying cause of dyslipidemia in subjects with non-alcoholic fatty liver disease (NAFLD) and T2D (4, 5). This dyslipidemia is characterized by increased levels of plasma triglycerides and small, dense low density lipoproteins (sdLDL), and decreased levels of high-density lipoprotein (HDL) cholesterol (4). Insulin resistance controls hepatic very low density lipoprotein (VLDL) production, especially VLDL1, by affecting the rate of apoB synthesis and degradation and hepatic de novo lipogenesis (6), as well as indirectly by modulating free fatty acid (FFA) flux from adipose tissue into the liver (7). These conditions result in excessive lipid accumulation in hepatocytes leading to hepatic steatosis and inflammation. In this regard, any strategy to decrease lipid accumulation in the liver may provide a protective effect against NAFLD and T2D. Metformin (MET), the most widely used anti-diabetic drug, is an insulin-sensitizing agent that provides glycemic control, especially in obese individuals (8). Metformin has been reported to have the ability to reduce hepatic de novo lipogenesis (9, 10). Metformin is typically required at higher doses for optimal effects (11). Moreover, this drug exhibits several side effects such as hypoglycemia, liver toxicity, lactic acidosis, diarrhea (12). For many patients, metformin monotherapy is insufficient to achieve glycemic targets, and therefore, additional therapies are advised (11). Recent evidence highlights the potential value of polyphenols abundantly found in some plants and common dietary preparations, in helping relieve clinical complications in subjects with diabetes and NAFLD. Polyphenols have been proposed to be useful as adjuvant therapy for their potential anti-diabetic, anti-obesity, anti-oxidant, anti-inflammatory and lipid-lowering effects (13, 14). For example, quercetin (QRS), a polyphenol belonging to the class of flavonoids, was reported to have a large spectrum of well-characterized biological effects such as anti-oxidant, lowering blood pressure, and ameliorating hyperglycemia-related disorders (14). Studies have also highlighted the beneficial effects of QRS in ameliorating hyperlipidemia in animal models and also reducing lipid accumulation in hepatic cells (15). Resveratrol (RSV) (trans-3,5,4 0-trihydroxystilbene), a member of polyphenols, was also reported to exhibit a wide range of pharmacological effects, including anti-tumorigenic, anti-oxidant, anti-inflammatory, anti-angiogenic, and hepatoprotective effects (16). Studies have suggested that RSV exerts a hypolipidemic effect on hepatocytes (17, 18). Most investigations on anti-diabetic, anti-obesity, antioxidant, anti-inflammatory and lipid-lowering effects of polyphenols were conducted with individual compounds. However, the present study was designed to determine whether a blend of RSV and QRS would act with MET to control high glucose (HG)-induced lipogenesis in vitro. The intention was to develop a formulation that uses very low level of MET in combination with the RSV and QRS blend in order to lower the effective dose of MET required for management of diabetes and NAFLD. Herein is reported the design, mechanism of action, and application of a potentiated form of Metformin-Quercetin-Resveratrol (MQR), as a promising therapeutic agent for control of lipid accumulation in HepG2 cells.

## Methods and Materials

### Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin EDTA were purchased from Gibco (Gibco, Germany). Tissue culture flasks and disposable plastic ware were purchased from Greiner Bio-One (Frickenhausen, Germany). Metformin, Quercetin, and Resveratrol were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, United States). Phenyl methyl sulfonyl fluoride (PMSF), and protease inhibitor cocktail were from Roche (Mannheim, Germany). All primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, Massachusetts, United States). Polyvinylidene difluoride (PVDF) membrane was from Millipore (Schwalbach, Germany). ECL reagents were from Amersham Pharmacia Corp. (Piscataway, NJ, USA). All other reagents and chemicals were from Sigma Aldrich.

### Cell culture

HepG2 cells were purchased from the Iranian Biological Resource Center (IBRC). Cells were cultured at 37˚C (in an atmosphere of 5% CO2) in DMEM supplemented with 10% FBS, and 1% penicillin-streptomycin. To induce insulin resistance, HepG2 cells were stimulated with high glucose (HG) 33 mM D-glucose. D-mannitol (27.5 mM mannitol) was added to normal glucose (NG) (5.5mM glucose) treated cells as an osmotic control. The time and dose of glucose treatment were selected based on previously published studies (18-20). HepG2 cells were pretreated with MET, QRS, and RSV alone and in combination for 2 hours.

### Oil red O staining

To determine the effect of the combinations on lipid content, HepG2 cells were washed in cold phosphate-buffered saline (PBS) and then fixed with a 4% paraformaldehyde solution for 30 minutes at room temperature. The cells were then washed with distilled water and incubated for 10 minutes in 60% isopropanol. Subsequently, the cells were stained for 20 minutes with fresh oil red O working solution (300 mg of oil red O powder added to 100 ml of 99% isopropanol). Stained cells were washed thoroughly with distilled water and then imaged using an Olympus camera mounted on an Olympus upright microscope. To quantify, 250 µl DMSO was added to the dried plates and then the optical density was measured at 510 nm. The protein concentration was determined by the bicinchoninic acid assay (BCA) method. The results were normalized against the total protein level.

### Determination of triglycerides

Treated cells were washed twice with PBS, and lysed on ice with RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, and 0.2% sodium deoxycholate, 0.2% SDS, 1 mM Na-EDTA, and 1 mM PMSF) supplemented with a protease inhibitor cocktail buffer for 30 minutes. After centrifugation at 12000 g for 20 minutes at 4°C, the supernatant was transferred to a new tube. Intracellular triglycerides content was measured with the Biovision triglyceride quantification Colorimetric/Fluorimetric kit according to the manufacturer's instructions. The protein concentration was determined by the BCA method. The results were normalized against the total protein level.

### Real-time PCR

RNA was extracted using the GeneAll RibospinTM total RNA purification kit (GeneAll Biotechnology, South Korea). Complementary DNA (cDNA) was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Gene expression was quantified using specific primers for fatty acid synthase (FAS), and sterol regulatory element-binding protein 1c (SREBP-1c) using SYBR Green RealQ Plus 2x Master Mix Green (Ampliqon) on Applied Biosystems Real-Time PCR (Thermo Fisher Scientific). The levels of the target gene transcripts were normalized relative to β-actin.

## Results

### Combination of metformin, quercetin, and resveratrol decreases lipid content in HepG2 cells treated with high glucose

The cytotoxic effects of the selected compounds on HepG2 cells were first evaluated using an MTT assay. The cells were treated with various concentrations of MET, QRS, and RSV for 24 hours. As shown in Fig.1, the IC50s (half maximal inhibitory concentration) values obtained from the results were MET: 9.819mM, QRS: 68.43 µM, RSV: 192.7 µM.

To determine the effect of the selected compounds on lipid content, Oil Red O staining was performed in HepG2 cells treated with 33mM glucose. As shown in Fig.2, the compounds individually could significantly decrease HG-induced total lipid content in HepG2 cells. The EC50 (half maximal effective concentration) from the Oil red O staining results were MET: 1.786 mM, QRS: 8.132 µM, RSV: 10.9 µM.

### MQR is more potent than individual compounds in reducing total lipid in HepG2 cells

To investigate whether QRS and RSV could potentiate the effect of a low dose of MET on HG-induced lipogenesis in HepG2 cells, several experiments were

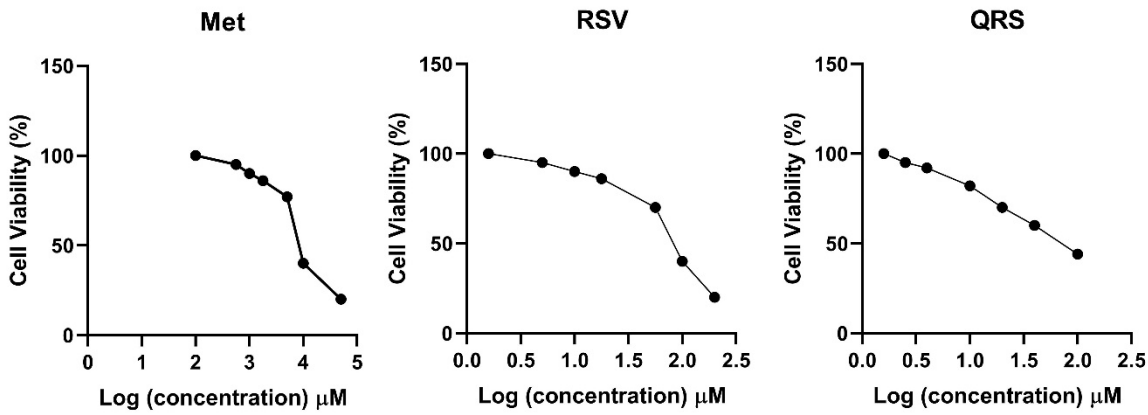


**Figure 1:** The cytotoxicity effect of MET, QRS and RSV on HepG2 cells. Cell growth activity was measured by MTT assay. Represented data are from three independent experiments and are means ± SEM. MET: Metformin; QRS: Quercetin, RSV: Resveratrol.

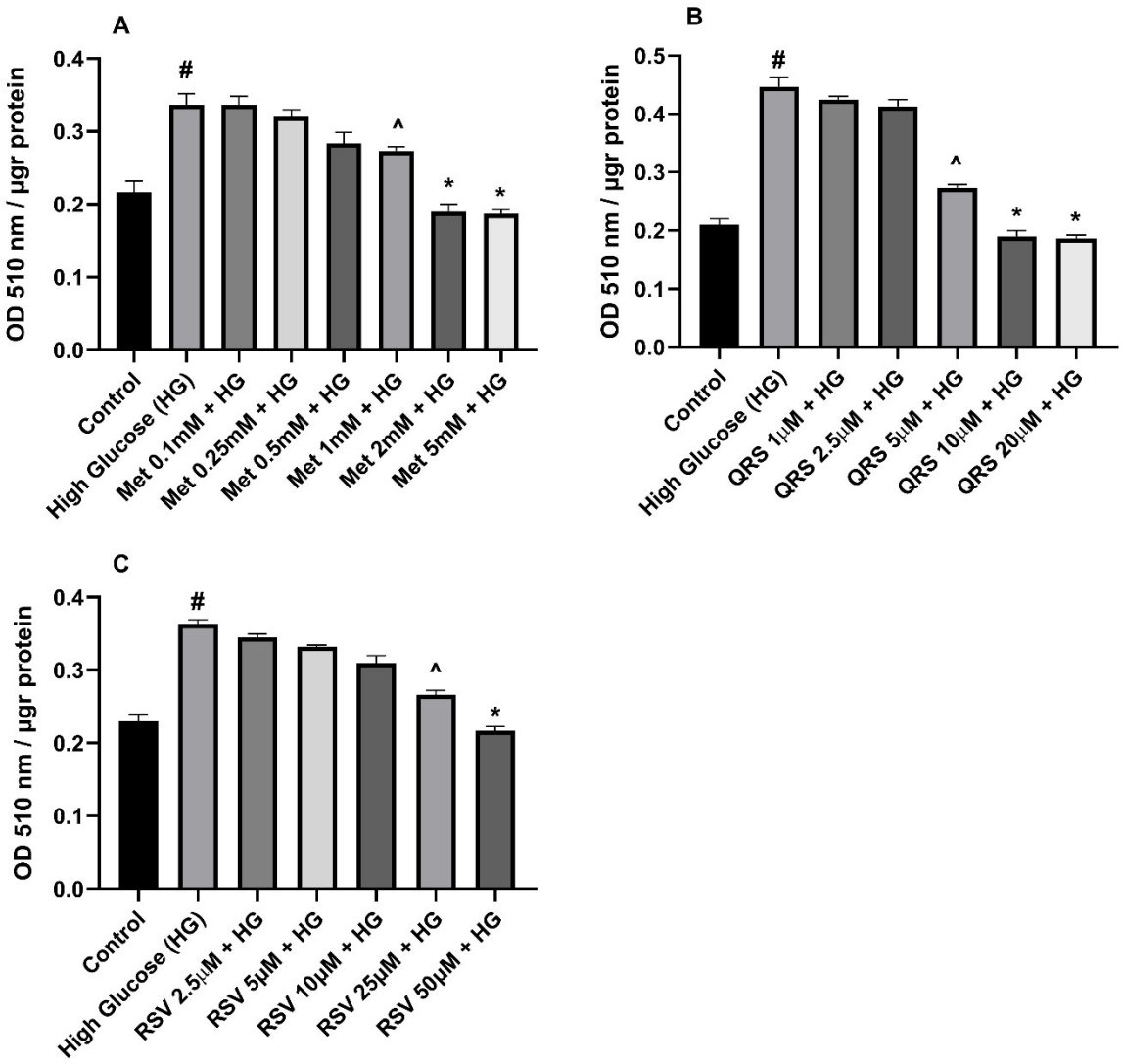


**Figure 2:** Total lipid content of cells treated with different concentrations of MET, QRS, and RSV. Represented data are from three independent experiments and are means ± SEM. #: Control vs High glucose, *: Treatments vs HG with $p < 0.0001$, ^: Treatments vs HG with $p < 0.05$. Control: D-mannitol (27.5 mM mannitol) in normal glucose (NG) (5.5mM glucose), HG: High (33 mM glucose), MET: Metformin, QRS: Quercetin, RSV: Resveratrol.

conducted to determine the anti-lipogenic effect of the MET, QRS, and RSV combination. Initial experiments using a mixture of MET (2mM), QRS (20µM), and RSV (50µM) at their maximum concentrations revealed the antilipogenic effects of both the combination and individual compounds (Fig. 3). Since the aim of this study was to obtain a combination formula with the lowest dose and the highest effect, Oil-red O staining was performed using sub-concentrations of these compounds in several steps. It was found that MET (mM), QRS (µM), and RSV (µM) at doses of (2:20:50), (1:10:25), and (0.5:5:10) had more potency to ameliorate HG-induced lipogenesis when compared to individual compounds. Importantly, MET (mM), QRS (µM), and RSV (µM) at doses of 0.25: 2.5: 5 showed significant anti-lipogenic effect, whereas the individual compounds had no effect. This combination was named as MQR. The data also showed that MET (mM), QRS (µM), RSV(µM) at doses of 0.1:1:2.5 had no significant anti-lipogenic effect in HepG2 cells.

## MQR is significantly more potent than metformin in reducing triglyceride level in HepG2 cells.

The above data was confirmed by measuring TG concentrations. When 0.25 mM MET was combined with 2.5 µM QRS and 5 µM RSV, the TG level decreased significantly despite undetectable changes with individual doses (Fig. 3D), and this effect was conserved when using higher concentrations of MET (Fig 4). As expected, the inhibitory effects on hepatocyte lipogenesis were unaltered in the presence of low doses

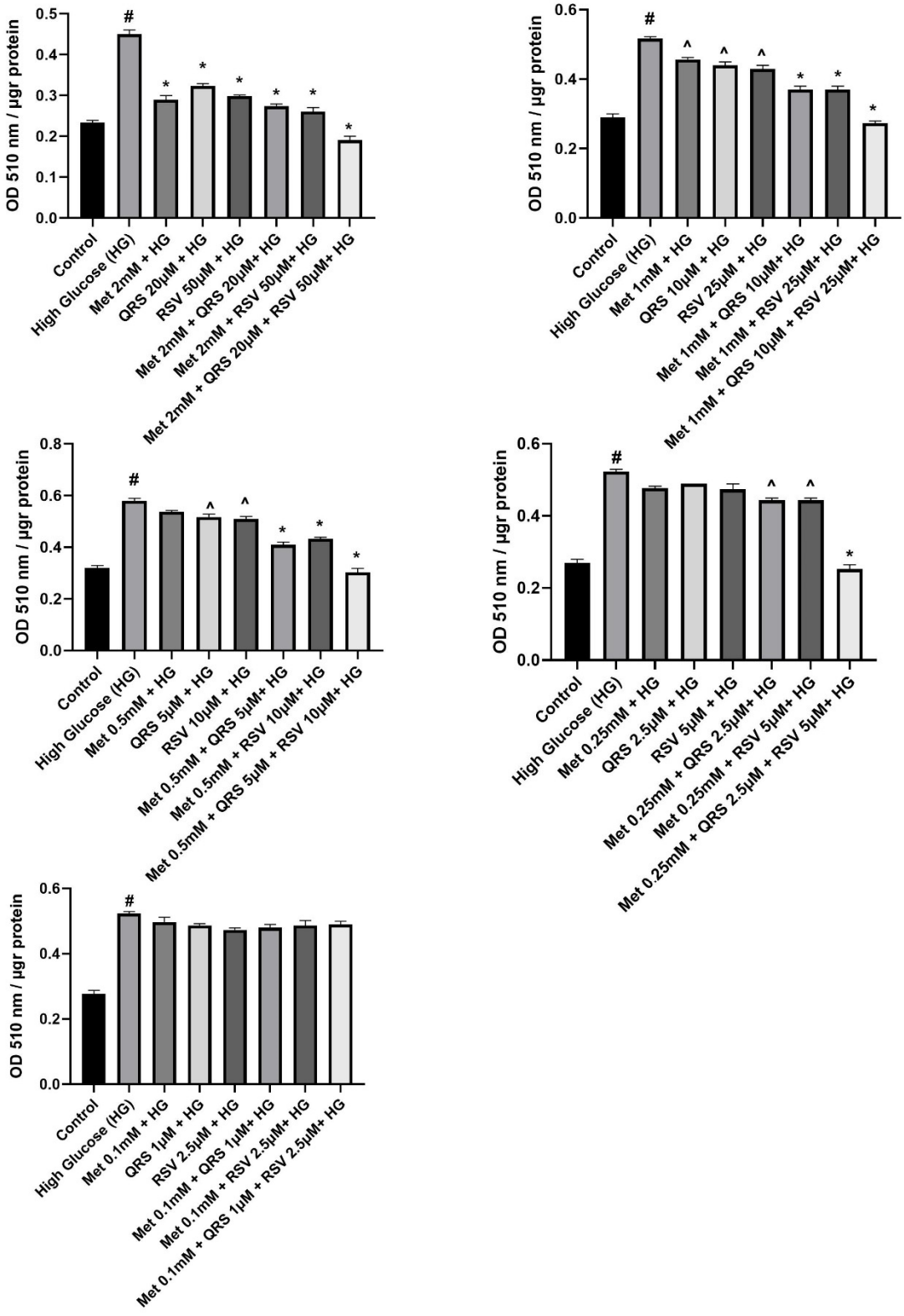


**Figure 3:** Total lipid content of cells treated with MET, QRS, RSV, MET+QRS, MET+RSV, and MET+QRS+RSV. Represented data are from three independent experiments and are means ± SEM. #: Control vs HG, *: Treatments vs HG with $p < 0.0001$, ^: Treatments vs HG with $p < 0.05$. Control: D-mannitol (27.5 mM mannitol) in normal glucose (NG) (5.5mM glucose), HG: High (33 mM glucose), MET: Metformin, QRS: Quercetin, RSV: Resveratrol.

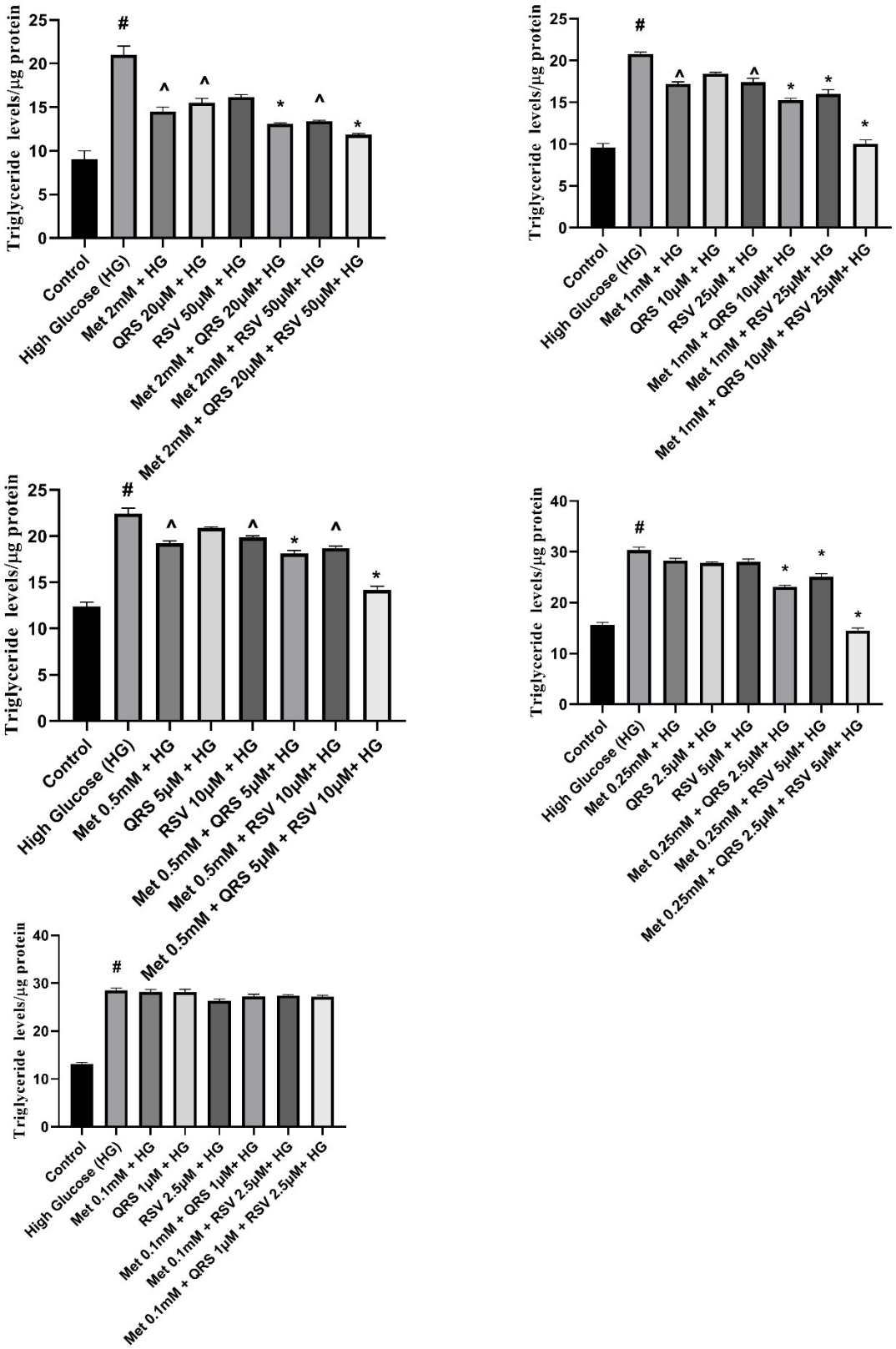


**Figure 4:** TG levels in the cells treated with MET, QRS, RSV, MET+QRS, MET+RSV, and MET+QRS+RSV. Represented data are from three independent experiments and are means ± SEM. #: Control vs HG, *: Treatments vs HG with $p < 0.0001$, ^: Treatments vs HG with $p < 0.05$. Control: D-mannitol (27.5 mM mannitol) in normal glucose (NG) (5.5mM glucose), HG: High (33 mM glucose), MET: Metformin, QRS: Quercetin, RSV: Resveratrol.

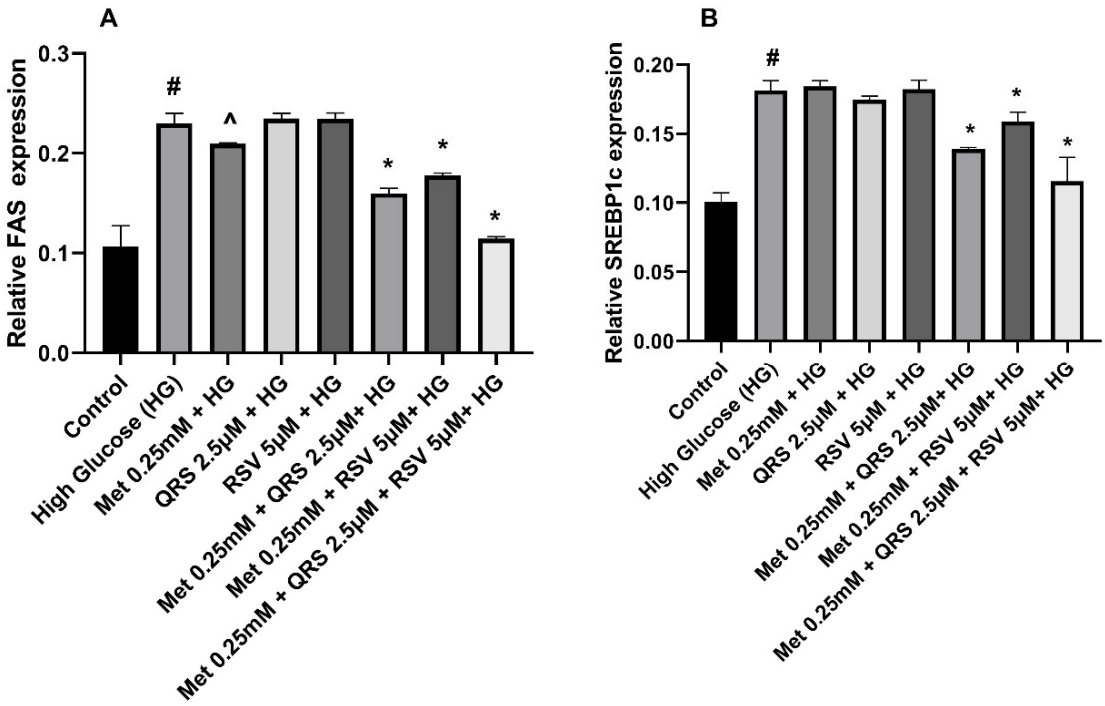


**Figure 5:** The expression of the key lipogenic genes SREBP1c and FAS in the cells treated with MET, QRS, RSV, MET+QRS, MET+RSV, and MET+QRS+RSV. Represented data are from three independent experiments and are means ± SEM. #: Control vs HG, *: Treatments vs HG with $p < 0.0001$, ^: Treatments vs HG with $p < 0.05$. Control: D-mannitol (27.5 mM mannitol) in normal glucose (NG) (5.5mM glucose), HG: High (33 mM glucose), MET: Metformin, QRS: Quercetin, RSV: Resveratrol.

of MET (0.1 mM) or QRS (1 µM) or RSV (2.5 µM) alone or in combination (Fig. 3E). Taken together, these results suggest that MQR is significantly more potent than MET in reducing TG level in HepG2 cells.

### MQR treatment suppresses SREBP-1c and FAS expression in HepG2 cells.

To investigate the molecular mechanisms by which Metformin-Quercetin-Resveratrol (MQR) could ameliorate HG-induced lipogenesis, the expression levels of SREBP-1c and FAS in the cells treated with either single or combination of MET (0.25 mM), QRS (2.5µM), and RSV (5µM) (MQR) were evaluated. The results showed that HG increases the expression of SREBP-1c (68%), and FAS (130%) genes compared to control. In this experiment, only a partial reduction of FAS at 0.25 mM MET alone (17%) was observed. However, delivering 0.25 mM MET along with 2.5µM QRS and 5µM RSV (MQR) caused a more dramatic reduction of SREBP-1c (37%), and FAS (120%) within 24 hours (Fig. 3). The combination of MET+QRS and MET+RSV caused a reduction of FAS, but the reduction of FAS was most significant in MQR treatment (Fig.5). Concomitantly, a sharp decrease in SREBP-1c expression was observed in the HepG2 cells following treatment with MQR or MET+QRS (Fig.5).

## Discussion

Metformin has been reported to exhibit several beneficial effects in NAFLD and T2D (21). Despite many therapeutic effects, the combination therapy of MET with other drugs is commonly used. It has been reported that MET in combination with other drugs such as insulin improve glycemic control in adults with type 2 diabetes (21). Recently, many researches have focused on the combination of MET with dietary polyphenols (22, 23). Consequently, this study was designed to determine whether a blend of QRS-RSV would act with MET to ameliorate lipid accumulation in HepG2 cells. The aim was to develop a formulation that uses very low levels of MET in combination with the QRS-RSV blend in order to lower the effective dose of MET.

After obtaining the dose response of three compounds, we proceed to use the Oil red O staining and TG levels to discover a new combination that is more potent than the EC50 concentrations of any individual compound. In fact, we wanted to check the possibility of producing a mixture using three compounds at reduced concentrations but higher potency on lipid accumulation in HepG2 cells. This will allow us to use lower drug dosages, thereby reducing the associated costs. Our results demonstrate the potentiation of the effect of MET, using a mixture of QRS and RSV, on lipid accumulation in a model of

NAFLD. These effects were found with concentrations of each compound that were too low to exert significant independent effects, demonstrating a synergistic action. The proportion of Met (mM): QRS (μM): RSV (μM): (2:20:50) and (1:10:25) had strong synergistic inhibitory effects on lipid accumulation. Interestingly, combining these compounds at the lowest doses at a unique proportion Met (mM), QRS(μM), RSV(μM) (0.25:2.5:5) named MQR, yield a synergistic inhibitory effect on lipid accumulation in HepG2 cells treated with HG. In previous studies, it has been reported that combination of MET and RSV may have a greater efficacy in protecting against diabetes (24). In another study by Bruckbauer et al. it has been reported the synergistic effects of a mixture of RSV with a low dose of MET on increased insulin sensitivity and reduced adiposity in mice (22).

Hepatic de novo lipogenesis plays an important role in the accumulation of lipids in the liver during NAFLD (25). Hepatic de novo lipogenesis is a fundamental biosynthetic pathway within the liver, contributing to the lipids that are stored and secreted by hepatocytes (26). This process is mainly regulated by transcriptional factor SREBP-1c. SREBP-1c primarily regulates the expression of genes involved in de novo lipogenesis and TG synthesis such as FAS (27). To investigate whether the beneficial effects of our combined treatment (MQR) on lipid accumulation is mediated through regulating genes involved in lipogenesis, we measured the expression levels of SREBP-1c and FAS in HepG2 cells treated with Met (mM), QRS(μM), RSV(μM) (0.25:2.5:5). According to the data, our QRS and RSV combination act synergistically with MET to lower lipid accumulation in HepG2 cells through inhibiting the expression of two genes SREBP-1c and FAS. The inhibitory effects of these compounds individually have been previously reported (9, 28, 29).

In conclusion, the data of this study provide evidence on the individual and combined effects of MET, RSV and QRS on HG-induced lipogenesis in HepG2 cells. Treatment with a combination of these compounds in a unique formula (MQR) decreased de novo lipogenesis, and this combination significantly potentiated the effects of low-dose MET on lipid accumulation, resulting in efficacy comparable with that of treatment with higher doses of MET. Therefore, this combination formula may be a useful approach to lower lipid accumulation in the liver of patients with NAFLD and T2D.

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## Conflict of Interest

Authors have nothing to declare.

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