

ORIGINAL ARTICLE

## Additive Effects of Resveratrol and Resveratrol/Quercetin in Prevention of Hyperglycemia-Mediated Cell Death through Downregulation of NADPH Oxidase and RAGE Expression

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### ABSTRACT

**Objective** Oxidative stress has been postulated to be a significant mechanism in the progression of diabetic nephropathy. **Methods** The cytoprotective effects of resveratrol, quercetin and the mixture of RSV/Q in high glucose-treated HEK-293 cells was evaluated by lactate dehydrogenase release and MTT assay. The levels of gene expression of nicotinamide adenine dinucleotide phosphate hydrogen oxidase as the main source of reactive oxygen species generation, RAGE, and SMP30 in high glucose media with or without RSV, Q, or RSV/Q were estimated by real-time PCR. The results showed that RSV, Q, and especially RSV/Q increased cell viability in a dose-dependent manner. Gene expression also showed that RSV, Q, and notably RSV/Q treatment attenuated nicotinamide adenine dinucleotide phosphate hydrogen oxidase and RAGE expression and improved SMP30 levels when compared to high glucose medium alone. **Conclusion** It was concluded that RSV, Q, and RSV/Q effectively attenuate hyperglycemia-mediated cytotoxicity in HEK-293 cells and should be considered a new therapeutic strategy for the treatment of diabetic nephropathy.

### INTRODUCTION

Diabetes mellitus is a common chronic disease that has been increasing in prevalence. It afflicts 7.7% of the population aged 20-79 years. In 2010, it was estimated that about 285 million people worldwide were diabetic; this is expected to increase to 439 million by 2030 [1].

Diabetic nephropathy is a serious complication of diabetes and the most common cause of end-stage renal disease in most developed countries [2]. Hyperglycemia is a crucial factor in the development of microvascular diabetic complications [3] and related mechanisms induce disorders in kidney cells that result in diabetic nephropathy [2].

Evidence suggests that oxidative stress plays a vital role in the pathogenesis of diabetic nephropathy [3]. NADPH oxidase (NOX) is a major source of reactive oxygen species (ROS) generation and is strongly expressed in the kidney, especially in renal vessels, mesangial, and podocyte

cells and is implicated in diabetic nephropathy [4, 5]. Advanced glycation end-products (AGEs) are products of nonenzymatic glycation and oxidation of proteins and lipids, the generation of which increases significantly in various tissues under diabetic conditions [2].

AGEs interact with their cell surface receptors (RAGE) and induce oxidative stress that contributes to progression of diabetic renal disease [6]. NOX and RAGE contribute in the induction of oxidative stress and the resulting kidney damage associated with diabetes. One of the proteins that are affected by hyperglycemia and has a role in oxidative stress is senescence marker protein-30 (SMP30). SMP30 is an aging marker molecule for which expression decreases with age in a sex-independent fashion [7]. SMP30 binds with Ca<sup>2+</sup> and regulation of its intracellular concentration plays a key role in cell protection against high intracellular Ca<sup>2+</sup> injury, especially in renal tubule cells [8]. Recent reports demonstrate that SMP30 controls antioxidant enzyme activity and its age-dependent attenuation is closely associated with oxidative stress [9].

Antioxidants decrease oxidative stress and are a potential source of therapy for improving kidney function in diabetic patients [10]. Flavonoids are phenolic compounds with antioxidant properties and are widely-distributed secondary metabolites in plants. Quercetin (3,5,7,3',4'-pentahydroxyflavone; Q) is a flavonoid present in red wine and several types of fruit, vegetables, and nuts [11]. Recent studies have shown that Q is a potent scavenger of free radicals that affect antioxidant pathways

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**Abbreviations** LDH lactate dehydrogenase

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both *in vivo* and *in vitro* [12]. Resveratrol (trans-3,5,4'-trihydroxystilbene; RSV) is a polyphenolic phytoalexin found in red grapes and numerous plant species having beneficial health properties. RSV is a potent antioxidant [13] that can directly scavenge ROS and has anti-inflammatory properties [14]. The present study evaluated and compared the protective effects of RSV, Q, and a mixture of the two (RSV/Q) against hyperglycemia-mediated oxidative stress in a human embryonic kidney cell line (HEK-293) to elucidate the molecular mechanism of RSV and Q under high glucose conditions.

### Chemicals

Dulbecco Modified Eagle Medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and 0.05% trypsin/EDTA (1X) solution were purchased from Gibco (USA). MTT powder (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), antibiotics (penicillin and streptomycin), RSV, and Q powder were purchased from Sigma (USA), dimethylsulfoxid (DMSO) were purchased from MP Biomedical (USA), and primer sequences for quantitative real-time PCR were purchased from Bioneer (Korea).

### *In vitro* experiments

HEK-293 cells were acquired from Pasteur Institute of Iran (Tehran) and were cultured in DMEM supplemented with 10% FBS and 1% streptomycin and penicillin. The cells were incubated in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The culture medium was refreshed every 48 h.

### Cell viability assay

HEK-293 cells with 80% confluence were trypsinized and seeded in 96-well cell culture microplates at a density of 7×10<sup>3</sup> cell/well in DMEM supplemented with 2% FBS and 100 mM glucose for the high glucose group. DMEM with 25 mM glucose served as the control. After 6-8 h, the cells in the high glucose condition were treated with different concentrations of RSV, Q, and RSV/Q for 24 and 48 h. The treatments used in this study are listed in **Table 1**. Cell viability under normal and high glucose conditions before and after treatment with RSV and Q were evaluated by MTT assay. MTT solution (5 mg/ml in PBS) was added to the wells. After 4h of incubation in the dark at 37°C, the media was aspirated and insoluble crystal formazan was dissolved in DMSO (100 µL/well). Absorbance was measured at 570 nm by a plate reader (BioTek; Epoch; USA).

### Cytotoxicity Assay

The ability of RSV, Q and RSV/Q to support the cells from hyperglycemia-induced cell injury was assessed by lactate dehydrogenase (LDH) release. A Pierce LDH cytotoxicity assay kit (No. 88953) was used to measure LDH release. Cells (2×10<sup>5</sup> cells/well) were plated in 24-well plates the day before the experiments. After treatment for 48 h, the medium from each well was collected to measure the amount of LDH released. The cells then were exposed to lysis buffer (9% Triton X100) for 30 min at 37°C and the media was collected to measure the total amount of cellular

LDH. Optical density was measured at a wavelength of 490 nm and the percentage of released LDH versus total intracellular LDH was calculated.

### Determination of Cell Oxidant and Antioxidant Status

Reduced and oxidized glutathione (GSH and GSSG) levels were determined by HPLC chromatography having a fluorescent detector (Ex: 385 Em: 515 nm) using commercial kits (Chrom systems Diagnostics; Germany) according to manufacturer instructions. Thioredoxin as an intracellular antioxidant was measured using solid phase sandwich ELISA (Human Thioredoxin Assay Kit; IBL) according to manufacturer instructions. Ferric reducing antioxidant power (FRAP) assay was carried out as recommended by Benzie and Strain (1996) to evaluate total antioxidant power in the cell lysate of the different groups [15]. To evaluate lipid peroxidation, the malondialdehyde (MDA) level in the kidney cell lysate was measured using the TBARS method [16].

Intracellular production of ROS was assessed using 2',7'-dichlorofluorescein diacetate (DCF-DA; Molecular Probes; Sigma) in a flat-bottomed 96-well cell culture microplates. HEK-293 cells (3×10<sup>5</sup> cells/well) were treated with different concentrations of RSV, Q, and RSV/Q under high glucose conditions. They were then exposed to 10 µM DCFDA in PBS<sup>-</sup> for 15 min at 37°C in the dark. The cells were washed with PBS<sup>-</sup> and fluorescence was measured with a Floustar Optima spectrofluorimeter (BMG Laboratories; Germany) using filters with an absorption spectrum of 480 nm and emission of 520 nm.

### RNA Extraction and qRT-PCR Analysis

The total RNA from the HEK-293 cell line was isolated using TRIzol extraction reagent (Bioneer; Korea) according to manufacturer recommendations. The integrity of the mRNA was confirmed by electrophoresis in denaturing

**Table 1.** The list of different treatments used for HEK-293 cell line.

Groups	Status and Treatment
Control	HEK-293 cells with DMEM+ 25 mM Glucose
High Glucose	HEK-293 cells with DMEM+ 100 mM Glucose
RSV1	HEK-293 cells with DMEM+ 100 mM Glucose under treatment with Resveratrol (2.5 µM)
RSV2	HEK-293 cells with DMEM+ 100 mM Glucose under treatment with Resveratrol (5 µM)
RSV3	HEK-293 cells with DMEM+ 100 mM Glucose under treatment with Resveratrol (10 µM)
Q1	HEK-293 cells with DMEM+ 100 mM Glucose under treatment with Quercetin (3 µM)
Q2	HEK-293 cells with DMEM+ 100 mM Glucose under treatment with Quercetin (6 µM)
Q3	HEK-293 cells with DMEM+ 100 mM Glucose under treatment with Quercetin (12µM)
RSV/Q1	HEK-293 cells with DMEM+ 100 mM Glucose under treatment with Resveratrol (2.5 mM) and Quercetin (3 µM)
RSV/Q2	HEK-293 cells with DMEM+ 100mM Glucose under treatment with Resveratrol (5 mM) and Quercetin (6 µM)
RSV/Q3	HEK-293 cells with DMEM+ 100mM Glucose under treatment with Resveratrol (10 mM) and Quercetin (12 µM)

1% agarose gel. The Revert Aid H Minus First Strand cDNA synthesis kit (Thermo Scientific; USA) was used to reverse-transcribe 1µg of RNA in a final volume of 20 µL. The qRT-PCR of β-actin (reference gene), NOX4, RAGE, and SMP30 was carried out using the primers identified in **Table 2**.

The 20 µL reaction mixture consisted of 2× ABI SYBR Green PCR Master Mix, 1 µL cDNA, and 1 µL of each primer. Amplification was performed using the ABI StepOne Real-Time PCR System (Applied Biosystems; USA) with 40 cycles of denaturation at 95°C for 30 s, annealing and extension at 60°C for 30 s, and data collection at 80°C for 20 s. The intensities of the mRNA levels were normalized to those of the β-actin product as ratios producing arbitrary units of relative abundance. Relative gene expression ( $2^{-\Delta\Delta CT}$ ) between the RSV, Q, and RSV/Q treated samples and the control group was assayed using the  $C_T$  method [17].

### STATISTICAL ANALYSIS

All experiments were carried out in triplicate and the data was expressed as mean ± standard deviation (SD) and analyzed using SPSS v.16 software (SPSS: USA). The statistical significance of the difference between mean levels of LDH and GSH of the control and treated groups was evaluated using student's t-test. The results were considered significant at  $P < 0.05$ . The graphs were drawn with GraphPad Prism version 5 (GraphPad; USA).

### RESULTS

#### RSV And Q Had Protective Effect on HEK-293 Cells under Hyperglycemic Condition

**Figure 1a** shows that the high glucose condition dramatically decreased cell viability (control: 100; high glucose:  $50.1 \pm 1.6$ ;  $P \leq 0.05$ ) and increased cell injury by LDH assay (control: 100; high glucose:  $580 \pm 3.1$ ;  $P \leq 0.05$ ) (**Figure 1b**). The results presented in **Figure 1a** and **b** indicate that treatment with RSV and Q increased cell viability and decreased LDH release. This protective effect was notable for the RSV/Q mixture.

#### Effects of RSV and Q on Oxidative Stress Status in Kidney Cells

The results summarized in **Table 3** show that the high glucose condition decreased the level of GSH and the GSH/GSSG ratio. Treatment of kidney cells with RSV and Q increased GSH and improved the GSH/GSSG ratio. The RSV had a considerably greater effect than Q. The RSV/Q mixture at all doses applied was more effective than RSV and Q alone (**Table 3**). As shown in **Table 3**, hyperglycemia decreased thioredoxin level and treatment with RSV, Q and RSV/Q increased thioredoxin to the normal value. This increase was significantly different for RSV3, RSV/Q2 and RSV/Q3 formulations (**Table 3**). RSV, Q, and RSV/Q down-regulated ROS production in HEK-293 cells in less than 60 min at all concentrations applied. Down-regulation by RSV at high concentration and RSV/Q3 was greater than that for Q (**Table 3**). A protective effect for cells by the RSV/Q was considerably greater than for all doses of RSV and Q alone ( $P < 0.05$ ).

The results of the TBARS assay indicated that MDA levels as a marker of lipid peroxidation in the HEK-293 cell lysate increased markedly after treatment with 100 mM glucose compared to those in the control group. In contrast, the MDA levels in the cell lysate having different concentrations of RSV, Q, and RSV/Q decreased significantly in a dose-dependent manner. The combined therapy was more effective than the RSV and Q alone. In fact, at high doses of both antioxidants, the MDA levels decreased to control group level. Treatment with different doses of RSV, Q, and RSV/Q increased antioxidant capacity in the cell lysate of HEK-293 in a dose-dependent-fashion. As shown in **Table 3** high doses of RSV/Q increased the antioxidant values of the treated cells to the level of the control group.

#### RSV And Q Inhibited the Gene Expression of NOX<sub>4</sub> and RAGE and Improved Gene Expression of SMP30

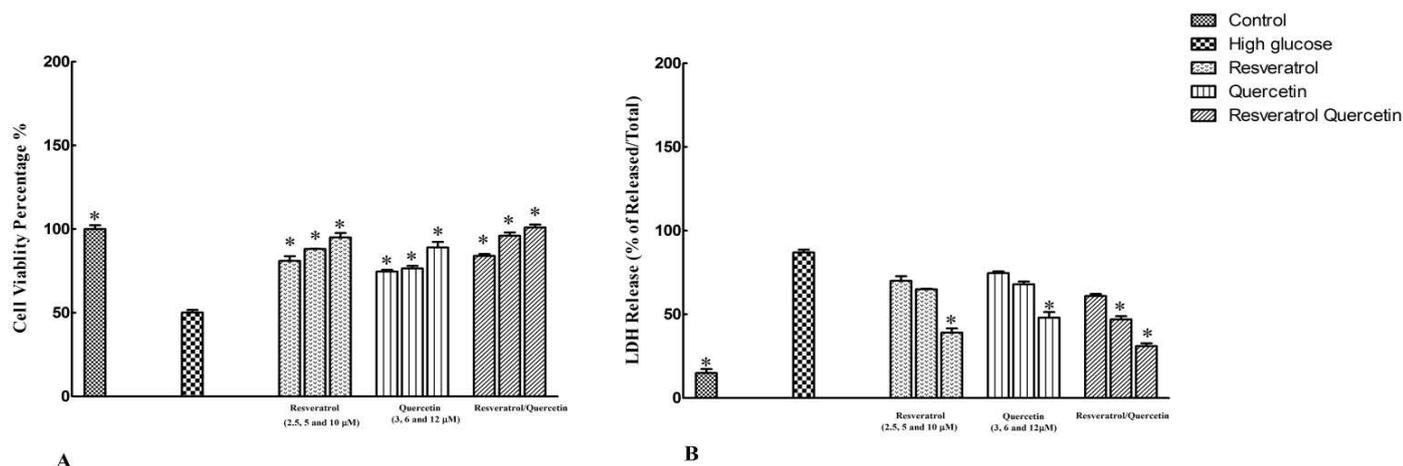
The antioxidant effects of RSV, Q, and RSV/Q on NOX4 and RAGE transcript levels were examined using qRT-PCR analysis. **Figures 2,3** show that the high glucose condition induced expression of NOX4 and RAGE, respectively, in the HEK-293 cell line that could be key factors in the progression of diabetic nephropathy. Treatment with different concentrations of antioxidants reduced expression of NOX4 and RAGE. Additionally, RSV/Q was more effective than RSV or Q alone. Expression of SMP30 anti-aging factor in high glucose medium decreased (**Figure 4**). Treatment with various concentrations of the antioxidants increased expression of SMP30.

### DISCUSSION

Diabetic nephropathy is a leading cause of end-stage renal disease. Hyperglycemia through induction of ROS production [8] and by a decrease in antioxidant defense systems plays an important role in the pathogenesis of diabetic nephropathy. The present investigation showed that maintaining the high glucose condition for 48 h dramatically decreased the viability of HEK-293 cells. In line with these results, studies have shown that hyperglycemic condition has a negative effect on cell viability [18]. The current data demonstrates that co-treatment of hyperglycemic HEK-293 cells with different concentrations of RSV and Q alone and RSV/Q increased cell viability in a dose-dependent manner. Previous studies have confirmed the cytoprotective effect of RSV and Q under high glucose conditions in bEnd.3 cultured cells [12] and dorsal root ganglion neurons of rats [18], respectively.

**Table 2.** Real-time primer sequences.

Gene	Sequences
NOX4	5'- TCCAGCTGTACCTCAGTCAA- 3' (forward)
	5'- ACACCAACTGTTTTCTCTGT-3' (reverse)
RAGE	5'- TCCGTGTCTACCAGATTCT- 3'(forward)
	5'- TAGCTTCCCTCTGACACACA- 3'(reverse)
SMP30	5'-CCCTGTACTCCCTTTCTCTG-3'(forward)
	5'-AACACTTCTGCGTTGGAGAT-3'(reverse)
β-actin	5'-GTCCACCCGCGAGTACAAC-3' (forward)
	5'-GACGACGAGCGAGCGATA-3' (reverse)



**Figure 1.** Effects of RSV, Q and RSV/Q on viability and cytotoxicity in high glucose treated HEK-293 cells. **(a).** Cell viability of HEK-293 cells which was assessed by MTT. **(b).** Cytotoxicity of HEK-293 cells which was assessed by LDH release. (\*Compared with control group,  $p < 0.05$ ,  $n = 3$ )

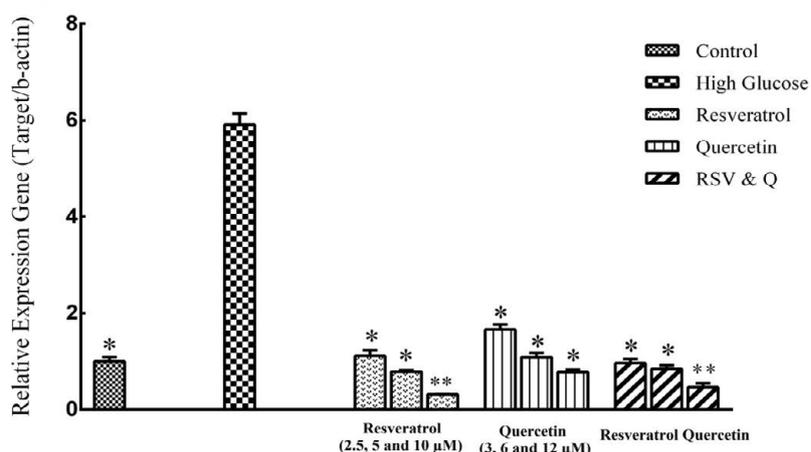
**Table 3.** Effects of quercetin and resveratrol on oxidative stress status in HEK-293 cells.

Experimental Groups†	Thioredoxin (ng/mL)	GSH (mmol/g protein)	GSSG (mmol/g Protein)	GSH/GSSH	Changes in ROS (Fluorescence Intensity)
Control	118±15*	49±3.5*	36±4.1*	1.25±0.3*	2700±31*
High Glucose	72±11	31±2.7	72±5.9	0.54±0.1	4200±24
RSV1	78±9.5	39.5±3.1	70±4.3	0.55±0.1	3450±22
RSV2	84±12	40±7.1	67±4.5	0.59±0.2	3150±27
RSV3	98±8.9*	44±5.6*	59±3.1*	0.72±0.2*	2900±35*
Q1	74±12	38±4.3	71±4.9	0.53±0.1	3830±54
Q2	79±9.4	39±5.2	69±3.6	0.56±0.1	3360±21
Q3	82±10	42±4.7*	65±2.8	0.63±0.2	3080±28*
RSV/Q1	83±13	41±2.9*	64±6.1	0.61±0.1	3100±32*
RSV/Q2	96±9.7*	43±5.3*	61±5.5	0.70±0.2*	2990±31*
RSV/Q3	108±12*	46±6.2*	49±4.7*	0.93±0.2*	2870±37*

† For details of experimental conditions see the text.

‡ Data were expressed as mean ± SD of three repeats in each group.

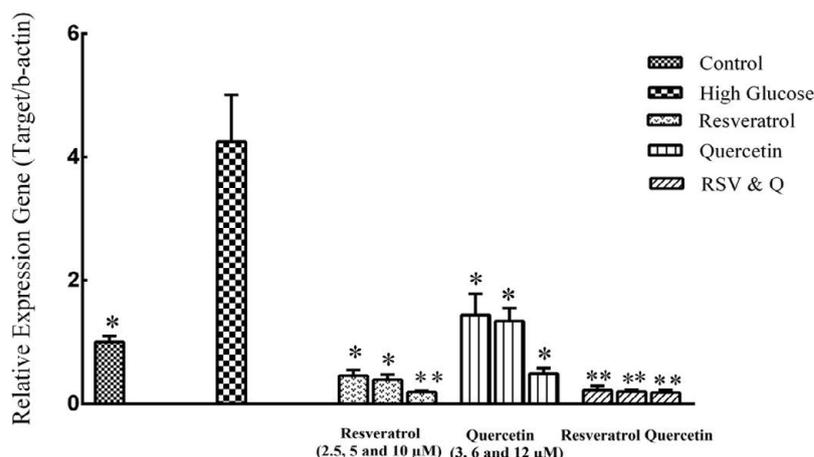
\* was considered significant at  $p < 0.05$  when compared with the high glucose group.



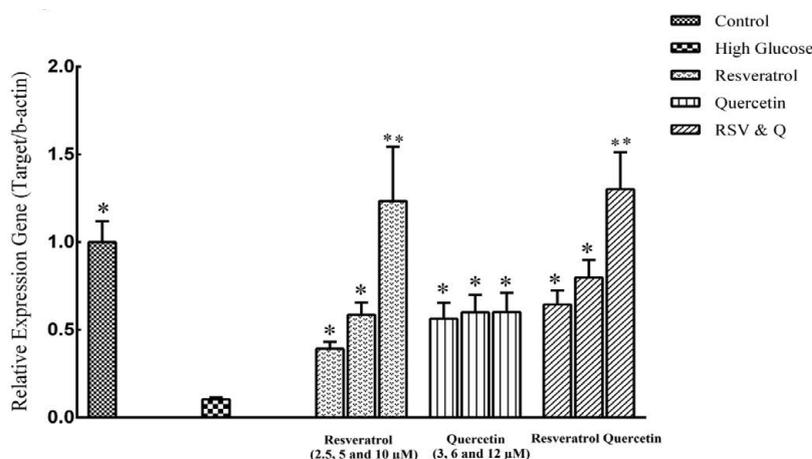
**Figure 2.** Expression of NOX4 mRNA in HEK-293 cells under high glucose condition before and after treatment with RSV, Q and RSV/Q. Treatment with RSV and Q significantly attenuated high glucose-induced NOX4 mRNA expression. The combination effect of RSV/Q showed more effective than RSV and Q alone.

According to the present study, antioxidant activity of thioredoxin decreased in the high glucose condition that is line with other studies results [19]. Studies have also shown that the antioxidant activity of thioredoxin decreases after hyperglycemia-mediated thioredoxin interacting protein (Txnip) induction as a critical mechanism to promote vascular oxidative stress in diabetes mellitus [19].

Improvement of thioredoxin level in RSV, Q and especially RSV/Q treated groups indicates beneficial effects of the flavonoids in kidney cells (Table 3). The glutathione level (as a predictor of cell redox status) and GSH/GSSG ratio dropped in the high glucose condition (Table 3). The reduction in the GSH/GSSG ratio and GSH in patients with type II diabetes has been reported elsewhere [20]. The



**Figure 3.** Effects of RSV, Q and RSV/Q on expression of RAGE mRNA in high glucose treated HEK-293 cells. Treatment with RSV and Q significantly attenuated high glucose-induced RAGE mRNA expression. The combination effect of RSV/Q showed more effective than RSV and Q alone.



**Figure 4.** Expression of SMP30 mRNA in HEK-293 cells under high glucose condition with or without treatment with RSV, Q and RSV/Q. Treatment with different concentrations of RSV, Q and notably RSV/Q induced the mRNA expression of SMP30 in HEK-293 cell.

present study recorded an increase in GSH level and the GSH/GSSG ratio in response to various concentrations of RSV, Q, and especially RSV/Q. MDA levels, as a marker of oxidative damage, increased under high glucose condition that confirmed by recent studies [21]. The results of the present study showed that MDA levels of HEK-293 lysate increased under hyperglycemic conditions and treatment with different concentrations of R, Q, and especially RVS/Q decreased MDA levels in a dose-dependent manner. High glucose condition also decreased total antioxidant capacity in kidney cells and different doses of RSV, Q and especially RSV/Q improved antioxidant status in the cells (**Table 3**). It seems reduced antioxidant capacity in hyperglycemic condition may be through elevated ROS production in the cells.

Treatment with RSV, Q, and RSV/Q downregulated ROS production in kidney cells resulting improved antioxidant capacity. In addition to direct ROS scavenging, studies indicate that RSV induced expression of antioxidants, including thioredoxin. *In vivo* studies have shown that RSV/Q has a greater pharmacological effect and more effectively improves brain antioxidant capacity [22]. In the present study, results obtained from biochemical analysis collectively confirmed beneficial effects of RSV, Q RSV/Q mixture.

The results of the gene expression assay in the present study revealed that RSV, Q, and RSV/Q decreased high glucose-induced RAGE and NADPH oxidase expressions in HEK-293 cells. It has recently been shown that the activity of NADPH oxidase increases under diabetic conditions in the kidney and can induce oxidative stress resulting in diabetic nephropathy [5].

Based on the literature, RSV inhibits NADPH oxidase activity and ROS generation and can prevent high glucose-induced kidney damage in rat renal mesangial cells [3], which agrees with the results of the present study. Studies have shown that RSV can attenuate the expression of NADPH oxidase (NOX4) in endothelial cells [23]. RSV inhibits NADPH oxidase in high glucose-treated cells which protects against cell death [12]. Furthermore, the effect of flavonoids such as Q in down-regulation of NADPH oxidase gene expression in spontaneously hypertensive rats was reported by Sancheza *et al.* [24].

RAGE is another factor in oxidative stress induction and is activated under hyperglycemia conditions [7]. The interaction of RAGE and AGE induces signal transduction and increases cellular oxidative stress through activation of NADPH oxidase [25]. Moreover, RAGE plays a key role in the progression of diabetic nephropathy. Its expression is significantly reduced by RSV in renal tissue

of diabetic rats [26]. Other results indicate that RSV and Q significantly reduce diabetes-induced renal damage in rats by attenuating histopathological changes [27].

It has been reported that SMP30 deficiency induces proximal tubule injury in diabetic mice. A reduction in the SMP30 level increases the risk of chronic kidney disease, including diabetic nephropathy, with age [7]. Studies have shown that NADPH oxidase activity and ROS production increase significantly in the brains of SMP30 knockout mice [9]. The decrease in SMP30 during aging has been related to elevated ROS generation in rat livers and kidneys [9]. It was found that RSV extends the lifespan of various organisms [28] and the activation of antiaging factor (SIRT-1) in HT29 cells extends their lifespan [29].

The cytoprotective effects of RSV and Q were found in the present study as well as the additive effects of RSV. Taken together, this data gives clues for identifying new formulations of antioxidants drugs for treatment of diabetic nephropathy. In the present study, biochemical and gene expression results collectively revealed that Q and especially RSV protect against hyperglycemia-mediated oxidative stress in kidney cells. The protective effects of Q and RSV could occur by modification of RAGE downstream signaling pathways. To better understand the exact mechanisms of Q and RSV in kidney physiology, more genes in the RAGE and NOX4 related signaling pathways should be investigated.

In conclusion, the present study increases understand of the beneficial effects of Q and RSV at the molecular level to induce cytoprotective effects against diabetic nephropathy. The effect of RSV on oxidative stress-related gene expression was notable. Of the doses used, RSV/Q3 formulation provided the most protective effect with no cytotoxicity and should be considered for design of new drugs to increase the life span of the kidney.

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## Conflict of interest

Authors declare that they have no conflict interests.

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