



Cissus quadrangularis extract (CQR-300) inhibits lipid accumulation by downregulating adipogenesis and lipogenesis in 3T3-L1 cells

Hae Jin Lee^a, Bao Le^a, Dong-Ryung Lee^b, Bong-Keun Choi^b, Seung Hwan Yang^{a,*}

^a Department of Biotechnology, Chonnam National University, Yeosu 59626, Republic of Korea

^b Nutrpharm Tech, Jungwon-gu, Seongnam, Gyeonggi 13201, Republic of Korea

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ABSTRACT

The objective of this study was to evaluate the anti-obesity activity and the action mechanism of *Cissus quadrangularis* extracts (CQR-300) in 3T3-L1 adipocytes. *Cissus quadrangularis* was extracted with hot water, resulting in CQR-300. The anti-obesity activity of CQR-300 in 3T3-L1 adipocytes was examined by Oil-red O staining. Possible mechanisms of CQR-300 in 3T3-L1 adipocytes were determined by real-time PCR and western blot. Treatment with CQR-300 inhibited lipid accumulation without showing cytotoxicity to 3T3-L1 adipocytes. Furthermore, CQR-300 decreased adipogenesis/lipogenesis-related mRNA expression levels of fatty acid binding protein (aP2), fatty acid synthase (FAS), lipoprotein lipase (LPL), stearoyl-CoA desaturase-1 (SCD-1), and acetyl-CoA carboxylase (ACC). CQR-300 also down-regulated expression levels of adipogenesis/lipogenesis-associated proteins, including peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer-binding protein α (C/EBP α), sterol regulatory element binding protein-1c (SREBP-1c), and FAS. It's also up-regulated the expression level of phosphorylated-AMPK (p-AMPK). Collectively, these results suggested that CQR-300 might have an anti-obesity effect by its ability to decrease expression levels of adipogenesis/lipogenesis-related genes and proteins.

1. Introduction

Obesity is a serious world-wide health problem. Its occurrence has been increasing in both developed and developing countries [1]. It has been estimated that worldwide overweight and obese population is about 6 billion. This figure is expected to increase to over 10 billion by 2025 [2]. Obesity is a multi-factorial disease. Diet with high calories, genetics, lifestyle, culture, and environment all play important roles in the development of obesity [3]. It can lead to metabolic syndromes such as cardiovascular disease, dyslipidemia, atherosclerosis, stroke, hypertension, dyslipidemia, type II diabetes mellitus, and various types of cancer [1,4,3]. Recently, people have recognized the need to prevent obesity and manage weight [4]. Obesity is characterized by increased adipose tissue mass associated with increased fat cell number and size [5]. Adipocytes are important cellular components of fatty tissues. Excessive amounts of lipid (triglycerides) can accumulate in adipose tissues accompanied by increased expression levels of adipogenesis/

lipogenesis and enhanced the body weight gain [6]. Obesity and its related diseases are closely related to adipocyte differentiation and fat accumulation [7]. 3T3-L1 cells are widely used in anti-obesity studies to investigate key molecular markers of adipocyte differentiation [4,8].

Cissus quadrangularis Linn has been used as a common medicinal plant in Africa and Asia for more than a century [9]. Its stem and leaf have been used in food preparation and raw drug in India for treating various diseases [10]. Several reports have demonstrated the anti-obesity effect of *C. quadrangularis* in animals and humans with lipase, amylase, and α -glucosidase inhibition activities [9,11,12].

However, the mechanisms involved in the effect of *C. quadrangularis* on adipocytes related to adipogenesis and lipogenesis have not been reported yet. Therefore, the objective of the present study was to examine the effect of *C. quadrangularis* extract (CQR-300) on adipocytes differentiation and lipid accumulation and its regulatory mechanisms in 3T3-L1 adipocytes.

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; aP2, fatty acid binding protein (aP2); BCS, bovine calf serum; C/EBP α , CCAAT/enhancer-binding protein α ; CQR-300, *Cissus quadrangularis* extract; DMEM, Dulbecco's modified Eagle's medium; FAS, fatty acid synthase; FAS- α , fatty-acid synthase; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPL, lipoprotein lipase; MDI, medium dependent interface; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; ORO, Oil-red O; p-AMPK, phosphorylated-AMPK; PPAR γ , peroxisome proliferator-activated receptor γ ; RIPD, radioimmunoprecipitation assay buffer; SCD-1, stearoyl-CoA desaturase-1; SREBP-1c, sterol regulatory element binding protein-1c; TG, triglycerides

* Corresponding author.

E-mail address: ymichigan@jnu.ac.kr (S.H. Yang).

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Table 1
Sequences and condition of primers used in real-time quantitative PCR.

Gene	Primer (5' → 3')
aP2	F: 5'-CCAATGAGCAAGTGGCAAGA-3' R: 5'-GATGCCAGGCTCCAGGATAG-3'
FAS	F: 5'-TGGTGGGTTTGGTGAATTGTC-3' R: 5'-GCTTGTCTGCTCTAACTGGAAGT-3'
LPL	F: 5'-GGCCAGATTCATCAACTGGAT-3' R: 5'-GCTCCAAGGCTGTACCCTAAG-3'
ACC	F: 5'-GCGTCGGGTAGATCCAGTT-3' R: 5'-CTCAGTGGGGCTTAGCTCTG-3'
SCD-1	F: 5'-CATGCCTGCTCTACCCTTT-3' R: 5'-GAACTGCGCTTGGAACCTG-3'
GAPDH	F: 5'-AACTTTGGCATTGTGGAAGG-3' R: 5'-ACACATTGGGGGTAGGAACA-3'

2. Materials and methods

2.1. CQR-300 preparation

The CQR-300 was provided from Gateway Health Alliance, INC (Fairfield, CA, USA). The stems and leaves of *Cissus quadrangularis* were washed and extracted by aqueous water for 3 times at 100 °C for 3 h and then filtered. The filtered extract was concentrated at 60 °C for 3 h with vacuum evaporator and then dried. The CQR-300 was dissolved in dimethyl sulfoxide (DMSO) for *in vitro* study.

2.2. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), Bovine Calf Serum (BCS), Fetal Bovine Serum (FBS), and penicillin-streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). 3-isobutylmethylxanthine, insulin, and dexamethasone were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Oil-red O (ORO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT),

and isopropanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibodies against α -actin, PPAR γ , C/EBP α , AMPK, p-AMPK, SREBP-1c, and FAS- α were obtained from Cell signaling (Danvers, MA, USA). Horseradish peroxidase (HRP)-linked anti-rabbit IgG and HRP-linked anti-mouse IgG were purchased from Bio-Rad (CA, USA).

2.3. 3T3-L1 cell culture and differentiation

3T3-L1 mouse preadipocytes were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM supplemented with 10% BCS and 1% penicillin-streptomycin at 37 °C under a humidified atmosphere with 5% CO₂. For differentiation of 3T3-L1 preadipocytes to mature adipocytes, full confluent 3T3-L1 preadipocytes (defined as Day 2) were incubated in differentiation medium containing DMEM, 10% fetal bovine serum, 0.5 mM 3-isobutylmethylxanthine, 5 μ g/ml insulin, and 1 μ M dexamethasone (Wako Pure Chemical Industries Ltd., Osaka, Japan). After two days (Day 4) of culture, cells were switched to DMEM supplemented with 10% FBS and 5 μ g/ml of insulin. The medium was changed every two days. These cells were fully differentiated into mature adipocytes on Day 7.

2.4. Cell viability assay

Effects of CQR-300 on cell viability of 3T3-L1 adipocytes were analyzed by MTT assay. Briefly, cells were incubated with various concentrations (50–200 μ g/ml) of CQR-300 in DMEM containing 10% FBS for 24 h. Sterile (filtered) MTT solution (5 mg/ml) in phosphate-buffered saline (PBS) was added to cells to reach a final concentration of 0.5 mg/ml. After 4 h of incubation, unreacted MTT reagent was removed and insoluble formazan crystals were dissolved in DMSO. Absorbance at 595 nm was measured using a microplate reader (Tecan, Mannedorf, Switzerland).

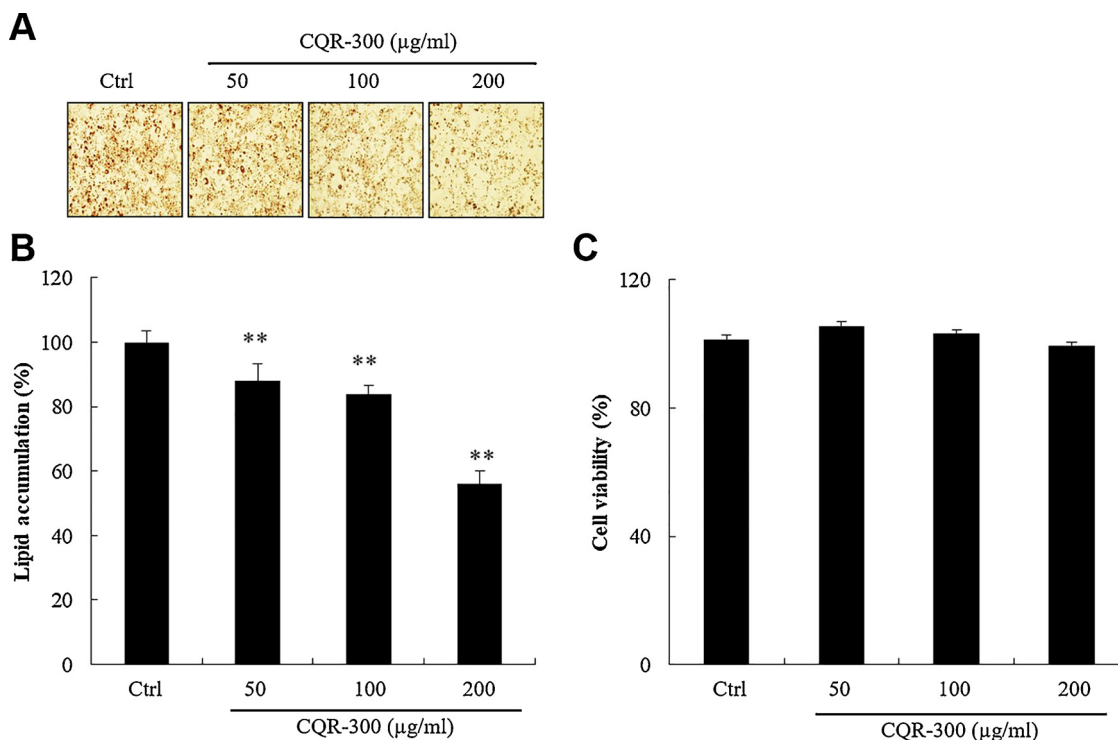


Fig. 1. CQR-300 inhibits adipogenic differentiation in 3T3-L1 cells. 3T3-L1 preadipocytes were induced to differentiate into adipocytes by indicated MDI solution in the presence or absence of CQR-300. (A) Cell viability after treatment with CQR-300 was determined by MTT assay. (B) Lipid droplets were photographed after Oil Red O staining. (C) Stained lipids were quantified by measuring the absorbance at 490 nm. Data are expressed as means \pm SE. ** $p < 0.01$ compared to the control.

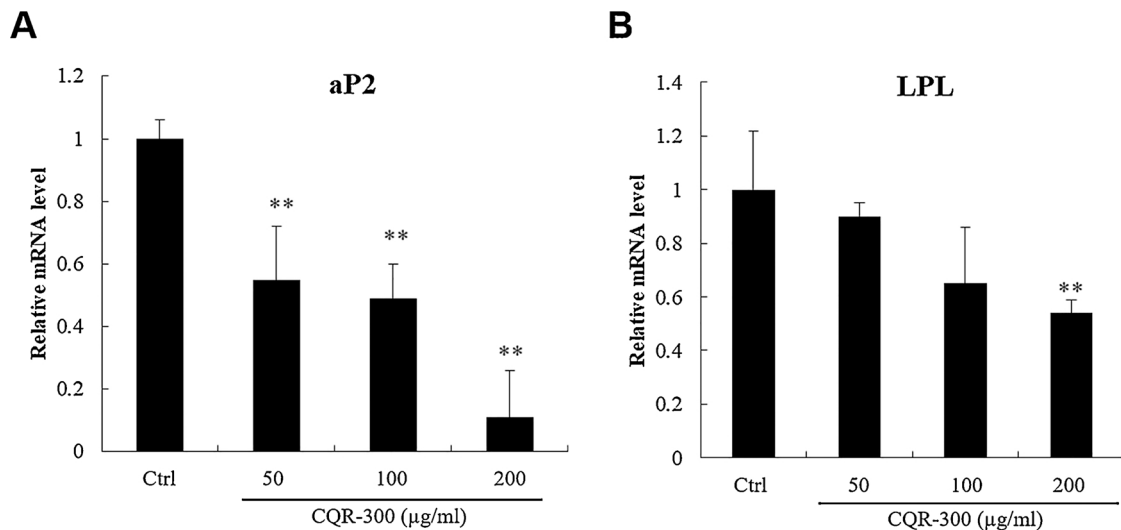


Fig. 2. Effects of CQR-300 on expression levels of adipogenic genes in 3T3-L1 cells. Adipocytes were incubated with CQR-300 and mRNA expression levels of adipogenic related genes were evaluated by quantitative real-time PCR. Data are presented as means \pm SE. ** $p < 0.01$ compared to the control.

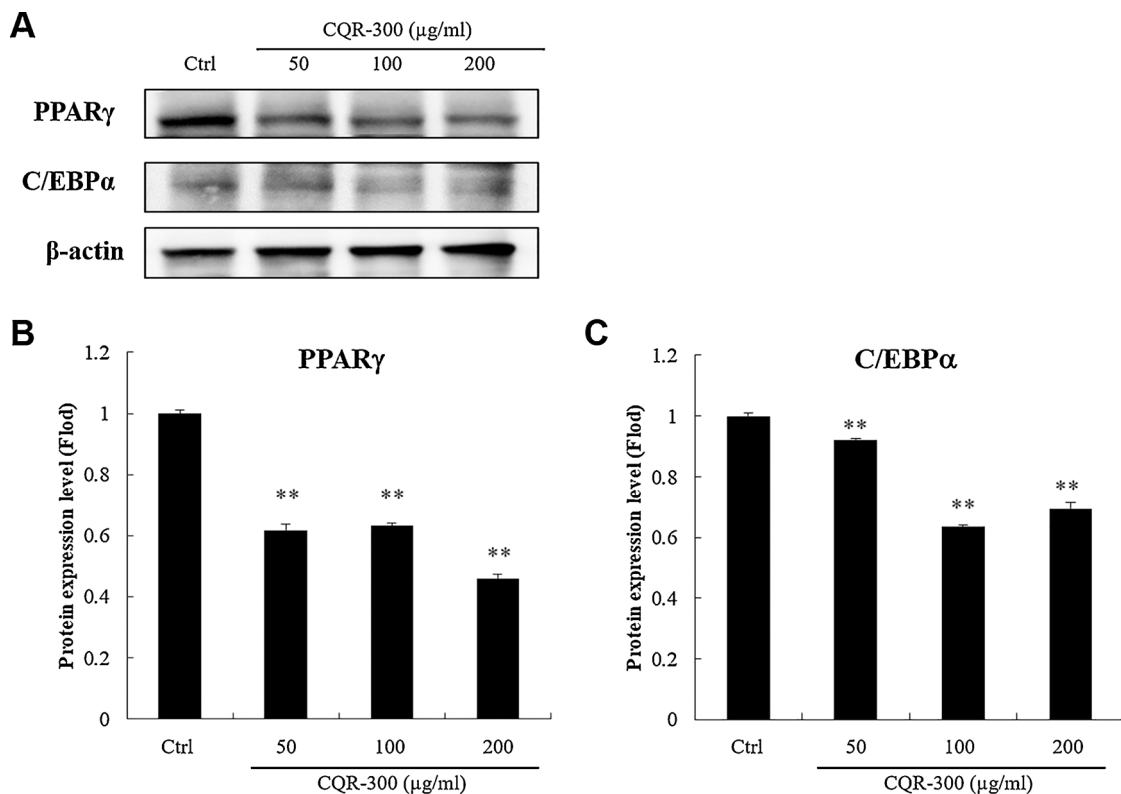


Fig. 3. Effects of CQR-300 on expression of adipogenic proteins in 3T3-L1 cells. (A) Adipocytes treated with various concentrations of CQR-300 were subjected to Western blotting using PPAR γ and C/EBP α antibodies. (B) Their quantitative analysis. Data are presented as means \pm SE. ** $p < 0.01$ compared to the control.

2.5. Oil red O (ORO) staining

For ORO staining, cells were washed gently with phosphate-buffered saline (PBS), fixed with 4% formaldehyde solution in PBS for 1 h, and then stained with a filtered ORO solution (0.5% in 60% isopropanol) for 30 min. The excess ORO staining solution was removed, and cells were washed three times with distilled water and dried. Stained lipid droplets were viewed with an optical microscope (Olympus, Tokyo, Japan). Lipid droplets stained with ORO were extracted with isopropanol and quantified by measuring the absorbance at 520 nm with a microplate reader.

2.6. Isolation of total RNA and quantitative real-time PCR

Total RNA was isolated from 3T3-L1 cells using the RNeasy mini kit (Qiagen, Velno, Netherlands) following the manufacture's protocol. RNA concentration and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo, Germany). Total RNA was then converted to cDNA using Primescript 1st strand cDNA synthesis kit (Takara, Shiga, Japan). The RNA expression level was quantified using a real-time PCR LightCycler 96 system (Roche, Basel, Switzerland) and SYBR[®] Green Master mix (Takara, Shiga, Japan) according to manufacturers' instructions. Gene-specific primers used in this experiment

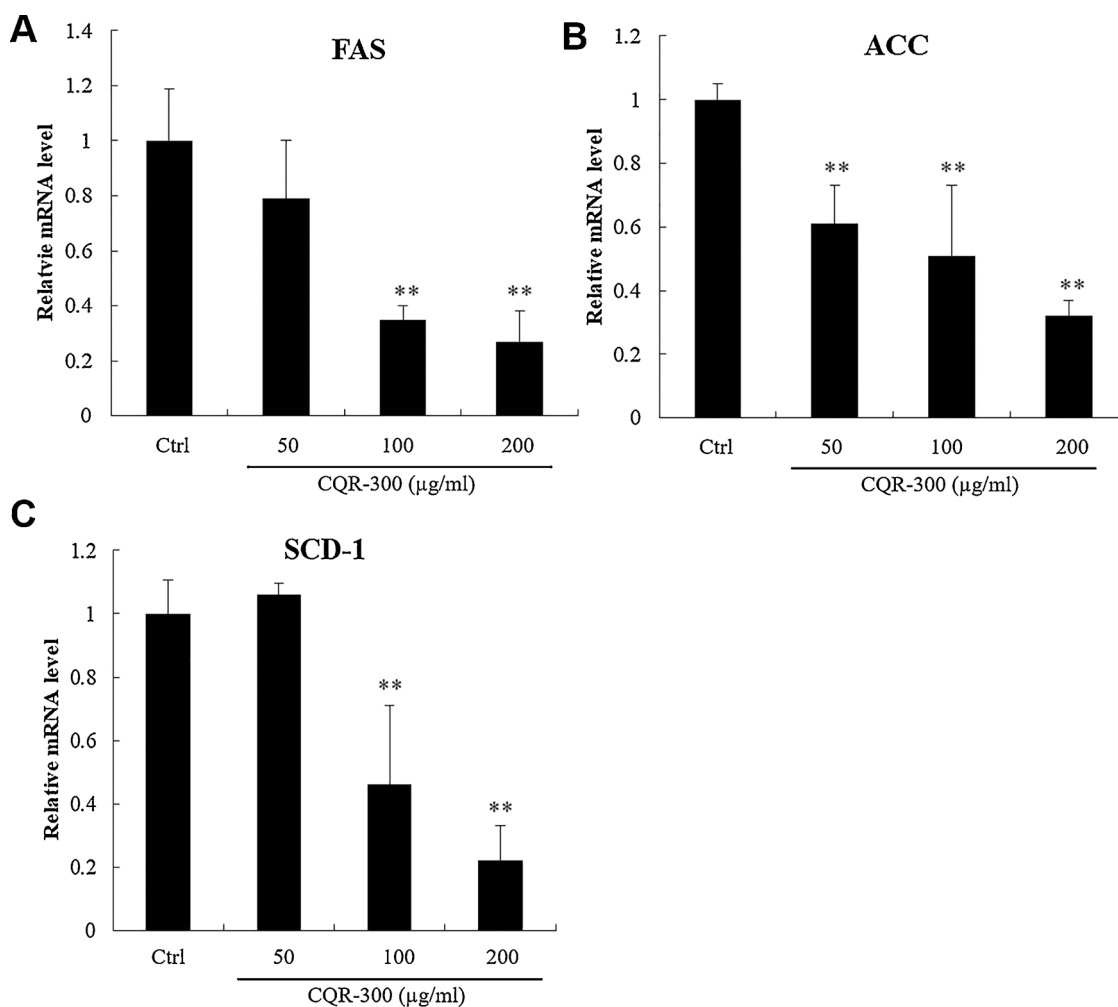


Fig. 4. Effects of CQR-300 on the expression of lipogenic genes in 3T3-L1 cells. Adipocytes were incubated with CQR-300 and mRNA expression levels of lipogenic related genes were evaluated by quantitative real-time PCR. Data are presented as means \pm SE. ** $p < 0.01$ compared to the control.

are shown in Table 1. The mRNA level was expressed as the ratio of signal intensity for each gene relative to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control.

2.7. Protein extraction and western blotting

Differentiated cells were washed with ice-cold PBS and lysed with radioimmunoprecipitation assay buffer (RIPA) containing protease inhibitor cocktail (Sigma-Aldrich, MO, USA). Cell lysates were centrifuged at 12,000 RPM for 20 min at 4 °C to collect supernatants. Protein concentrations were measured using Pierce's Bicinchoninic Acid Assay (BCA) protein assay kit (Thermo, Germany). Equal amount of protein for each sample was separated by 10% sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). These membranes were blocked with 5% of nonfat dried skim milk and then incubated with indicated primary antibodies at 4 °C overnight. They were subsequently incubated with anti-mouse or anti-rabbit HRP-conjugated secondary antibodies at room temperature for 1 h. Immunoreactive proteins were enhanced using a chemiluminescent ECL reagent and visualized using a Chemi-luminometer (CLINX Scientific Instrument Co., Ltd., Shanghai, China). Protein expression levels were normalized to those of β -actin. The density of protein band was quantified using Image J software (National Institutes of Health).

2.8. Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM). Statistical significance of difference in mean values between treatment groups was analyzed by Student's *t*-test and one-way analysis of variance (ANOVA) using Origin 7 software (Microcal Software, USA). Statistical significance was considered at $p < 0.05$ and $p < 0.01$.

3. Results

3.1. CQR-300 inhibits differentiation of 3T3-L1 cells

To investigate the effect of CQR-300 on lipid accumulation during differentiation, preadipocytes were differentiated into adipocytes in the presence or absence of CQR-300. Accumulated lipid droplets were stained with fat-specific oil red O dye (Fig. 1A). Quantification of ORO staining demonstrated that CQR-300 treatment significantly decreased adipocyte differentiation, resulting in a 44% decrease in fat accumulation compared to that of the control group ($p < 0.01$) (Fig. 1B). Cytotoxic effect of CQR-300 on 3T3-L1 cells was evaluated by MTT cytotoxicity assay. CQR-300 exerted no negative effect on cell viability at concentrations up to 200 µg/ml (Fig. 1C).

3.2. Inhibitory effect of CQR-300 on expression levels of adipogenic genes

To investigate how CQR-300 inhibited lipid accumulation, mRNA

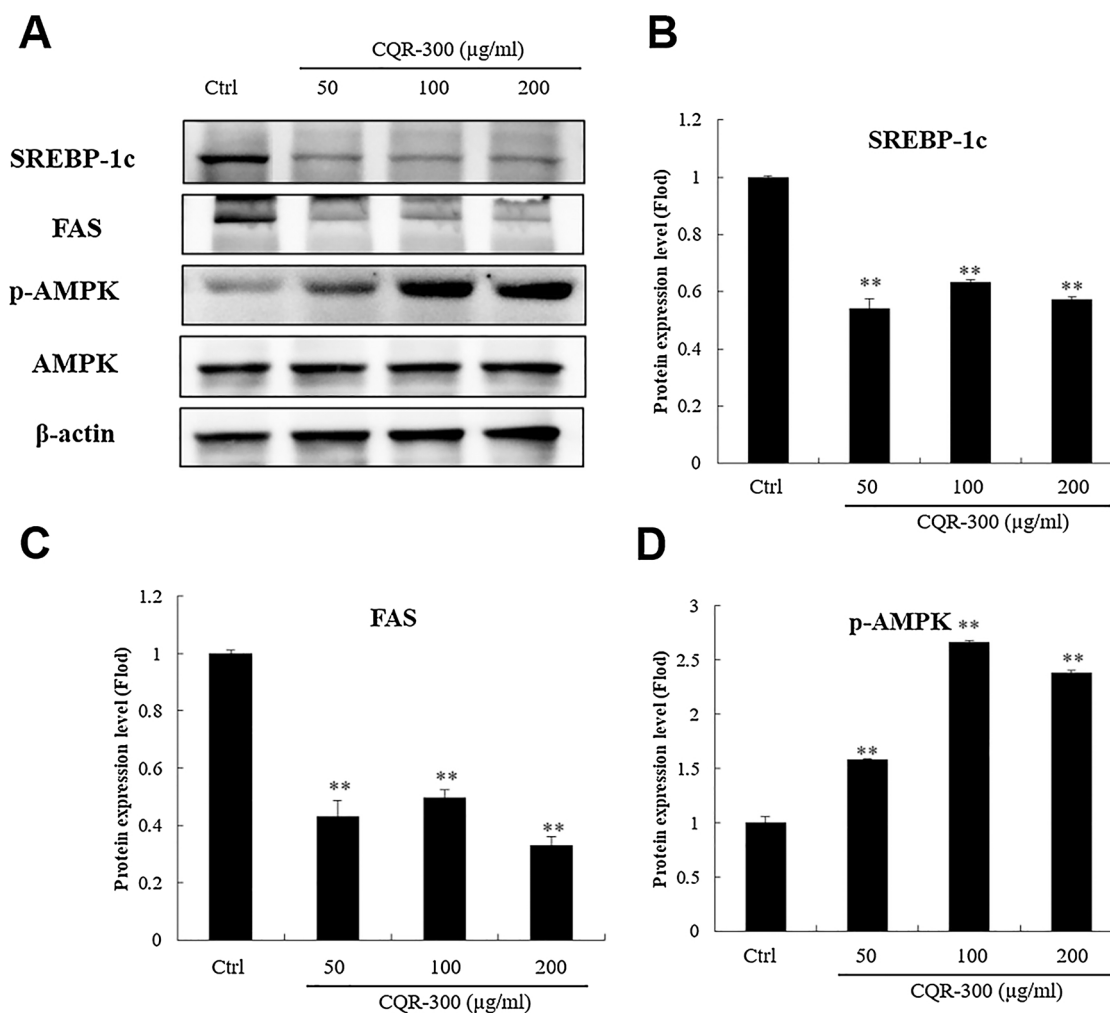


Fig. 5. Effects of CQR-300 on the expression of lipogenic proteins in 3T3-L1 cells. (A) Adipocytes treated with various concentrations of CQR-300 were subjected to Western blotting using SREBP-1c, FAS, and p-AMPK antibodies. (B) Their quantitative analysis. Data are presented as means \pm SE. ** $p < 0.01$ compared to the control.

levels of adipogenic target genes such as aP2 and LPL were examined by quantitative real-time PCR. Expression levels of aP2 and LPL were decreased after differentiated adipocytes were treated by CQR-300. At 200 $\mu\text{g/ml}$, CQR-300 significantly ($p < 0.01$) decreased expression levels of aP2 and LPL by 89 and 46%, respectively, compared to the control (Fig. 2).

3.3. Inhibitory effect of CQR-300 on expression levels of adipogenic proteins

To determine the mode of action involved in the effect of CQR-300, we measured expression levels of adipogenesis-related proteins using Western blotting (Fig. 3). As shown in Fig. 3B, expression levels of PPAR γ protein were significantly decreased in 3T3-L1 cells by 39%, 37%, and 54% relative to untreated cells after treatment with 50, 100, and 200 $\mu\text{g/ml}$ of CQR-300, respectively (all $p < 0.01$). CQR-300 at 50, 100, and 200 $\mu\text{g/ml}$ also down-regulated C/EBP α protein expression levels by 8%, 37%, and 31%, respectively compared to the control (all $p < 0.01$).

3.4. Effect of CQR-300 on expression levels of lipogenesis-associated genes

Effects of CQR-300 on expression levels of lipogenesis associated genes were examined by analyzing mRNA expression levels in 3T3-L1 cells using quantitative real-time PCR. As shown in Fig. 4, FAS, ACC, and SCD-1 mRNA expression levels were decreased by treatment with

CQR-300. FAS and ACC mRNA expression levels were reduced by 65% and 73%, respectively, after treatment with 100 $\mu\text{g/ml}$ of CQR-300. They were decreased by 54% and 78%, respectively, after treatment with 200 $\mu\text{g/ml}$ of CQR-300. CQR-300 also significantly ($p < 0.01$) decreased expression levels of ACC in a dose-dependent manner compared to the control.

3.5. Effect of CQR-300 on expression levels of lipogenesis-associated proteins

To examine how CQR-300 regulated lipid metabolism, expression levels of lipogenesis associated proteins were determined using Western blot analysis (Fig. 5). Protein expression level of SREBP-1c was significantly decreased (by 43%) after treatment with 200 $\mu\text{g/ml}$ of CQR-300. FAS protein expression level was also significantly ($p < 0.01$) decreased (by 67%) in 3T3-L1 cells treated with 200 $\mu\text{g/ml}$ of CQR-300 compared to that in untreated cells. Cells untreated by CQR-300 also showed lower p-AMPK expression levels compared to CQR-300 treated cells. However, CQR-300 at 50, 100, and 200 $\mu\text{g/ml}$ markedly induced phosphorylation levels of AMPK by 1.5%, 2.6%, and 2.4%, respectively (all $p < 0.01$). These results suggest that CQR-300 can reduce adipocyte differentiation and lipid accumulation through regulating adipogenesis/lipogenesis associated genes and proteins.

4. Discussion

Cissus quadrangularis has traditionally been used in West Asia and Africa as therapeutic medicine. It is a succulent vine belonging to the family of Vitaceae. It has been utilized in Ayurveda as an alternative medicine to treat bone fracture, digestive, eye, ear diseases, and asthma for more than a century [9,11]. Fresh stems and roots of *C. quadrangularis* have been used as food and management for various ailments [11,13]. Several studies have shown that *C. quadrangularis* has compounds such as friedelin, α -amyron, resveratrol, piceatannol, quercetin, and pallidol with various metabolic and pharmacological properties [14,15,16,17,18,19]. Although earlier researchers have reported that it can be used in the management of weight loss and metabolic syndrome [9,11,20,27], its anti-obesity effects and mechanisms are poorly understood. In the present study, we examined anti-obesity effects of CQR-300 and its potential mechanisms *in vitro*.

Obesity is caused by an imbalance of energy intake and expenditure. Excess energy will accumulate in the form of triglycerides (TG) in adipose tissue; [11]. The expansion of fat mass in adipose tissue is associated with hyperplasia (enlargement of adipocytes size) and hypertrophy (adipocytes via adipogenic differentiation of preadipocytes). One anti-obesity strategy is by inhibiting adipocyte differentiation since obesity is caused by hypertrophy and hyperplasia. 3T3-L1 preadipocytes can become mature adipocytes, resulting in the adipogenic signaling medium dependent interface (MDI-medium) through differentiation stage [8]. They are commonly used as effective tools to identify anti-obesity materials. In the present study, we found that CQR-300 treatment could inhibit lipid accumulation during differentiation of 3T3-L1 adipocytes without having cytotoxicity.

To clarify the underlying mechanisms, we measured expression levels of adipogenesis and lipogenesis related genes and proteins in 3T3-L1 adipocytes. Adipogenesis is a process in which the differentiation from preadipocytes to mature adipocytes occurs [8]. It is regulated by a number of signaling pathways and transcription factors such as PPAR γ and C/EBP α . PPAR γ and C/EBP α are known as master regulators in final stage of adipocyte differentiation. PPAR γ is known as a ligand-activated transcription factor that can mediate the expression of fat-related genes and activate adipogenesis [21]. The expression of C/EBP α can induce adipocyte differentiation and adipogenesis only in the presence of PPAR γ [4,5]. They can induce the expression of genes such as aP2 and LPL related to adipogenesis [6]. SREBP-1c regulates fatty acid synthesis and activates lipogenic transcription factors such as FAS, ACC, and SCD-1 required for lipogenesis [22]. SREBP-1c can also activate transcription of PPAR γ by stimulating the ligand for the nuclear receptor [23,24]. AMPK is a sensor of cellular energy status that regulates metabolic process from anabolizing to catabolizing lipids [25,26]. It is an important target in the treatment for metabolic diseases. Activated AMPK can suppress the lipogenesis process by down-regulating protein expression of SREBP-1c [26].

CQR-300 could operate via mechanisms of adipogenesis/lipogenesis. Of adipogenesis/lipogenesis-associated genes and proteins expressed in 3T3-L1 adipocytes, expression levels of PPAR γ and C/EBP α proteins and adipogenesis-related genes such as aP2 and LPL were significantly attenuated by CQR-300 treatment. Expression levels of lipogenesis related genes such as FAS, ACC, and SCD-1 and protein expression levels of SREBP-1c and FAS were also decreased in 3T3-L1 cells after treatment with CQR-300. Furthermore, CQR-300 up-regulated protein expression levels of p-AMPK.

In conclusion, CQR-300 could inhibit lipid accumulation in 3T3-L1 adipocytes without causing cell cytotoxicity. CQR-300 inhibited the differentiation of 3T3-L1 adipocytes by regulating adipogenesis-related genes and proteins. It could reduce the synthesis of fatty acids and triglycerides via downregulating lipogenesis related genes and proteins. These results suggest that CQR-300 could be used to prevent obesity and related diseases.

Conflict of interest statement

We have no conflicts of interest to disclose.

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