

Original Research



Effects of quercetin on cell differentiation and adipogenesis in 3T3-L1 adipocytes

Seo Young Hong ^{1*}, Ae Wha Ha ^{1,2*}, and Wookyoung Kim ^{1§}

¹Department of Food Science and Nutrition, Dankook University, Cheonan 31116, Korea

²Natural Nutraceuticals Industrialization Research Center, Dankook University, Cheonan 31116, Korea

OPEN ACCESS

Received: Dec 11, 2020

Revised: Jan 12, 2021

Accepted: Jan 20, 2021

*Corresponding Author:

Wookyoung Kim

Department of Food Science and Nutrition,
Dankook University, 119 Dandae-ro, Dongnam-
gu, Cheonan 31116, Korea.

Tel. +82-41-550-3471

Fax. +82-41-550-3471

E-mail. wkim@dankook.ac.kr

*These two authors contributed to this work
equally.

©2021 The Korean Nutrition Society and the
Korean Society of Community Nutrition
This is an Open Access article distributed
under the terms of the Creative Commons
Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0/>)
which permits unrestricted non-commercial
use, distribution, and reproduction in any
medium, provided the original work is properly
cited.

ORCID iDs

Seo Young Hong

<https://orcid.org/0000-0003-4668-0884>

Ae Wha Ha

<https://orcid.org/0000-0002-9342-5483>

Wookyoung Kim

<https://orcid.org/0000-0002-8652-5339>

Funding

This research was supported by Basic
Science Research Program through the
National Research Foundation of Korea (NRF)
funded by the Ministry of Education (NRF-
2018R1D1A1B07043918).

ABSTRACT

BACKGROUND/OBJECTIVES: Adipocytes undergo angiogenesis to receive nutrients and oxygen needed for adipocyte growth and differentiation. No study relating quercetin with angiogenesis in adipocytes exists. Therefore, this study investigated the role of quercetin on adipogenesis in 3T3-L1 cells, acting through matrix metalloproteinases (MMPs).

MATERIALS/METHODS: After proliferating preadipocytes into adipocytes, various quercetin concentrations were added to adipocytes, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to evaluate cell proliferation. Glycerol-3-phosphate dehydrogenase (GPDH) activity was investigated as an indicator of fat accumulation. The mRNA expressions of transcription factors related to adipocyte differentiation, CCAAT/enhancer-binding proteins (C/EBPs), peroxisomal proliferator-activated receptors (PPAR)- γ , and adipocyte protein 2 (aP2), were investigated. The mRNA expressions of proteins related to angiogenesis, vascular endothelial growth factor (VEGF)- α , vascular endothelial growth factor receptor (VEGFR)-2, MMP-2, and MMP-9, were investigated. Enzyme activities and concentrations of MMP-2 and MMP-9 were also measured.

RESULTS: Quercetin treatment suppressed fat accumulation and the expressions of adipocyte differentiation-related genes (C/EBP α , C/EBP β , PPAR- γ , and aP2) in a concentration-dependent manner in 3T3-L1 cells. Quercetin treatments reduced the mRNA expressions of VEGF- α , VEGFR-2, MMP-2, and MMP-9 in 3T3-L1 cells. The activities and concentrations of MMP-2 and MMP-9 were also decreased significantly as the concentration of quercetin increased.

CONCLUSIONS: The results confirm that quercetin inhibits adipose tissue differentiation and fat accumulation in 3T3-L1 cells, which could occur through inhibition of the angiogenesis process related to MMPs.

Keywords: Quercetin; adipocyte; adipogenesis; vascular endothelial growth factor

INTRODUCTION

Adipogenesis is caused by the proliferation of adipose tissue through hyperplasia and hypertrophy, which increase the number and size of adipocytes [1]. To receive the nutrients and oxygen necessary for the differentiation of adipocytes, extensive changes in extracellular matrix (ECM) remodeling have been reported [2]. Vascular endothelial growth factor (VEGF)

Conflict of Interest

The authors declare no potential conflicts of interests.

Author Contributions

Conceptualization: Kim WK; Formal analysis: Hong SY, Kim WK, Ha AW; Funding acquisition: Kim WK; Investigation: Kim WK, Ha AW, Hong SY; Methodology: Kim WK, Ha AW; Supervision: Kim WK, Ha AW; Writing - original draft: Hong SY, Ha AW; Writing - review & editing: Kim WK, Ha AW.

and matrix metalloproteinases (MMPs), angiogenic factors, have an important role in the initiation of angiogenesis [3,4]. VEGF induces endothelial cell proliferation and vascular permeability [5]. Although VEGF binds to receptors in both vascular endothelial growth factor receptor (VEGFR) 2 and VEGFR-1, VEGFR-2 is the primary signaling receptor for VEGF [6]. MMPs are proteases that break down various components in the ECM, such as collagen, laminin, and fibronectin [7]. A recent study reported that MMP promotes the binding of VEGF and its receptors in endothelial cells, consequently increasing endothelial cell differentiation [4]. These findings suggest a potential mechanism for the regulatory role of MMPs in adipogenesis and angiogenesis of adipocytes [7].

Quercetin is a flavonoid commonly present in onion peels, wine, and tea [8]. Quercetin is known to be effective in controlling anti-inflammatory, antioxidant, and immune responses [8-10]. Quercetin treatment in adipocytes activates the apoptosis pathway through AMP-activated protein kinase (AMPK) regulation [11] or inhibits the expressions of adipocyte differentiation transcription factors such as peroxisomal proliferator-activated receptors (PPAR)- γ , CCAAT/enhancer-binding protein (C/EBP) α , fatty acid-binding protein 4, and adipocyte protein 2 (aP2) [12]. On the other hand, quercetin has been reported to inhibit the metastasis of cancer cells by reducing the expression of MMP-2 and MMP-9 in cancer cells [13-16]. Thus, it can be hypothesized that if the activity of MMPs in adipocytes by quercetin can be inhibited, then the angiogenesis process is associated with adipocyte differentiation. To date, however, there have been no reports on the relationship between quercetin and angiogenesis (or MMPs activity) in adipocytes. Therefore, the purpose of this study was to elucidate the mechanism associated with the anti-obesity effects of quercetin, which include the inhibition of angiogenesis and MMP activity.

MATERIALS AND METHODS

Materials & reagents

Quercetin and all reagents were purchased from Sigma (St. Louis, MO, USA). For reagents not purchased from Sigma, the sources such as the company name, company location, and country have been specified. Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), penicillin/streptomycin (P/S), trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS) were purchased from WelGENE (WelGENE, Daegu, Korea) [17]. SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) and HiSenScript™ RH[-] RT PreMix Kit (iNtRON Biotechnology, Seongnam, Korea) were also purchased.

Cell culture

The 3T3-L1 cells (Korean Cell Line Bank, Seoul, Korea) used in this experiment were mouse-derived preadipocytes. Cells were incubated with media containing DMEM, 10% BSA, 100 U/mL penicillin/streptomycin. After 2 days, when the cells attained 70–80% confluence, the cells were washed twice with PBS and subcultured by treatment with trypsin-EDTA; the media were replaced every 2 days [17]. To differentiate cells into adipocytes, 3T3-L1 cells were cultured in a differentiation media containing DMEM, 10% FBS, P/S, 1 μ M dexamethasone (Dex), and 0.5 mM isobuthylmethylxanthine. After differentiation, adipocytes were maintained in post-differentiation media consisting of DMEM, 10% FBS, P/S, and 10 μ g/mL insulin. Quercetin was dissolved in 99.9% ethyl alcohol and diluted in post-differentiation media at concentrations of 0, 5, 10, or 20 μ M.

Cell proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Duchefa Biochemie, Haarlem, The Netherlands) was used to observe the degree of cell proliferation according to the concentration of quercetin [17]. After differentiation of adipocytes, the MTT assay was performed at 0 h before quercetin treatments and at 24, 48, and 96 h after quercetin treatments at concentrations of 0, 5, 10, or 20 μ M. Culture media were changed once every 2 days. The experiment was independently repeated 3 times.

Glycerol-3-Phosphate Dehydrogenase (GPDH) activity

A GPDH Assay Kit (Abcam, Cambridge, United Kingdom) was used to measure GPDH activity. The cells are collected and spun-down for 24 h after treatment with various concentrations of quercetin. After removing the supernatant, 200 μ L of the GPDH buffer included in the assay kit were added and the mixture homogenized. After centrifugation, the supernatant was collected and incubated at 37°C for 30 min in accordance with the assay procedure. Absorbance was measured at 450 nm with a microplate reader (Infinite 200, Groedig, Austria). The experiment was independently repeated three times.

mRNA expressions of transcription factors related to adipocyte differentiation

Total mRNA expressions of genes related to adipocyte differentiation were determined by performing real-time polymerase chain reaction (PCR) as previously described [17,18]. The reagents (Tri reagent, chloroform, and isopropanol) were purchased from Sigma. Quercetin diluted with post differentiation media at concentrations of 0, 5, 10, or 20 μ M were added to adipocytes. After 24 h, the culture solution was removed, washed, and 1 mL of Tri reagent was added. Then, the cells were collected, 200 μ L of chloroform was added, and the mixture was centrifuged. The supernatant was combined with 500 μ L of isopropanol, left at room temperature for 10 min, then centrifuged for 15 min. After washing and drying the obtained pellet, RNase-free dH₂O was added, and RNA purity and concentration were calculated at 260/280 nm using a microplate reader (Infinite 200).

To obtain cDNA, HiSenScript™ RH[−] RT PreMix Kit (iNtRON Biotechnology) and RNase-free dH₂O were added to a sample to achieve a total of 20 μ L. The reaction was carried out at 85°C for 10 min. After dispensing the cDNA sample into a strap tube, nuclease-free water, 2X SYBR green Master mix, and primers (**Table 1**) were added. Using the reverse transcription-polymerase chain reaction (RT-PCR) system (version 2.1; Applied Biosystems, Foster City, CA, USA), samples were activated with 95°C for 10 min, 95°C for 15 min, 60°C for 1 min, 95°C

Table 1. Primer sequences of transcription factors related to adipogenesis

Protein	Primer	Sequence ¹⁾
β -actin	Forward primer	5'-GTTTGAGACCTCAACACCCC-3'
	Reverse primer	5'-GTGGCCATCTCCTGCTCGAAGTC-3'
C/EBP β	Forward primer	5'-CCGGGCAGCACCACGACTTCC-3'
	Reverse primer	5'-CTTCTTGGCCTTGCTTACCTG-3'
PPAR- γ	Forward primer	5'-TTGATTCTCCAGCATTTCT-3'
	Reverse primer	5'-TGTTGTAGAGCTGGGTCTT-3'
C/EBP α	Forward primer	5'-AGACATCAGCGCTACATCG-3'
	Reverse primer	5'-TGTAGGTGCATGGTGGTCTG-3'
aP2	Forward primer	5'-CATGATCATCAGCGTAAATG-3'
	Reverse primer	5'-TCACCATCTCGTTTCTCTT-3'

C/EBP, CCAAT/enhancer-binding protein; PPAR- γ , peroxisome proliferator-activated receptor- γ ; aP2, adipocyte protein 2.

¹⁾T, thymine; A, adenine; C, cytosine; G, guanine.

Table 2. Primer sequences for transcription factors related to angiogenesis

Protein	Primer	Sequence ¹⁾
VEGF- α	Forward primer	5'-TCTCACCGGAAAGACCGAT-3'
	Reverse primer	5'-CCCAAAGTGCTCCTCGAA-3'
VEGFR-2	Forward primer	5'-GATGCAGGAACTACACGGTCA-3'
	Reverse primer	5'-GAGATCAAGGCTTCTCACC GA-3'
MMP-2	Forward primer	5'-GGCCATGCCATGGGGCTGGA-3'
	Reverse primer	5'-AGCATTAGGGCTAACATCCAAC-3'
MMP-9	Forward primer	5'-AGGCCTCTACAGAGTCTTG-3'
	Reverse primer	5'-CAGTCCAACAAGAAAGGACG-3'

VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factors receptor; MMP, matrix metalloproteinase.

¹⁾T, thymine; A, adenine; C, cytosine; G, guanine.

for 15 min, 60°C for 1 min, and 95°C for 15 min for 40 cycles, which were repeated twice. The analysis was conducted using the $\Delta\Delta\text{CT}$ method. The experiment was independently repeated three times.

Neovascularization factors and MMPs mRNA expression

The mRNA expressions of VEGFs and MMPs, which have an important role in angiogenesis, also determined by performing real-time PCR in the same way as above. Forward and reverse primers were conducted as shown in **Table 2**. The experiment was independently repeated 3 times.

Concentration and activity of MMPs

After preadipocyte differentiation, adipocytes were treated with 0, 5, 10, or 20 μM of quercetin and incubated for 24 h. Then, the culture solution was collected in a tube and centrifuged at 3,000 r/min at 4°C for 10 min. The supernatant was used for the assay of MMP concentrations and was performed by using the Mouse MMP2 ELISA Kit (LSBio, Seattle, WA, USA) and the Mouse MMP9/Gelatinase B ELISA Kit (LSBio). To measure MMP activity, the above supernatant was transferred into centrifugal filter units (Merck Millipore, Billerica, MA, USA) and concentrated at 3,500 r/min at 4°C for 30 min. MMP activity was measured using the MMP-2 colorimetric drug discovery kit and the MMP-9 colorimetric assay kit (Both from Enzo Life Science, Farmingdale, NY, USA). The experiment was independently repeated 3 times.

Statistical analysis

The experiments within this study were independently repeated three or more times, and the statistical analysis was conducted using SPSS Statistics (version 23; IBM, Armonk, NY, USA). The results are expressed as mean and standard error values. After analysis of variance, a post-test was conducted using Duncan's multiple range test to verify differences between groups, with significance accepted at the $P < 0.05$ level.

RESULTS

MTT assay

MTT assays were performed to observe the degree of proliferation of adipocytes. When quercetin was treated with 0, 5, 10, or 20 μM , there were no significant differences in cell proliferation until 24 h of incubation time, but after 48 h, cell proliferation was decreased by the 20 μM quercetin treatment (**Fig. 1**). Thus, to determine the effect of quercetin in a cell-state that does not affect cell proliferation, the following experiments were conducted with a quercetin treatment time of 24 h.

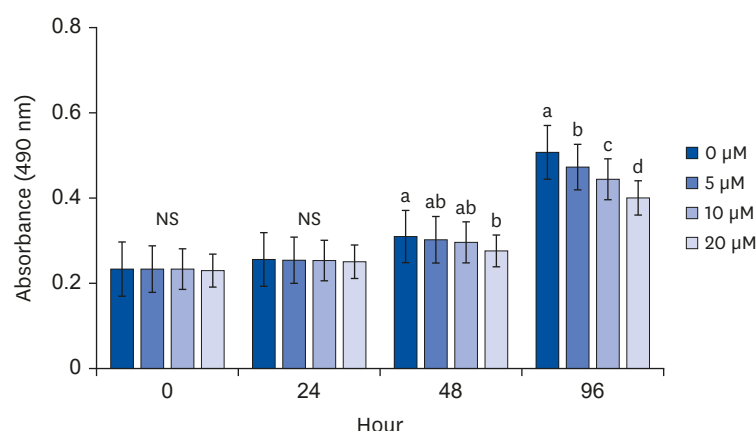


Fig. 1. Effect of quercetin on cell proliferation in 3T3-L1 cells.

After differentiation induction, as described in Methods, a cell monolayer was incubated in post-differentiation medium with 0, 5, 10, or 20 μM quercetin. Viable cell numbers were estimated by MTT assay at 0, 24, 48, and 96 h after quercetin treatments. Each bar represents the mean \pm SE. Comparisons among different concentrations of quercetin that produced significant differences ($P < 0.05$) are indicated by different letters above each bar. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

GPDH activity assay for fat accumulation

Whether lipid formation during adipocyte differentiation was inhibited by quercetin treatment was investigated (**Fig. 2**). Quercetin was treated at 0, 5, 10, or 20 μM concentrations, and GPDH activity decreased with the increase in quercetin concentration, but the only statistically significant difference was between control and 20 μM quercetin-treated samples ($P < 0.05$).

mRNA expressions of transcription factors related to adipocyte differentiation

The mRNA expressions of C/EBP α , PPAR- γ , and aP2 decreased in a quercetin concentration-dependent manner compared to that in the control treatment (0 μM quercetin). Compared to the control, the C/EBP α mRNA expression was significantly decreased to 22.4%, 84.3%, and 94.6% levels under 5, 10, and 20 μM quercetin treatments, respectively ($P < 0.05$) (**Fig. 3**). The C/EBP β expression was also significantly decreased by quercetin treatment, but the

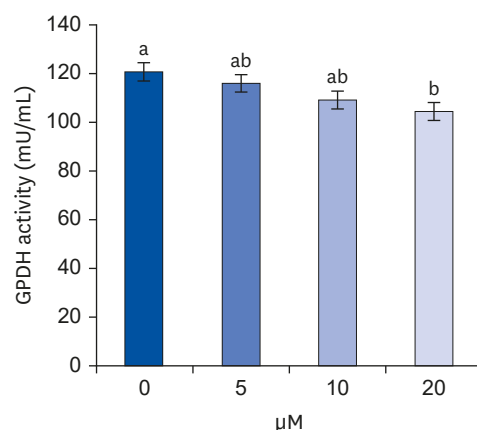


Fig. 2. Effect of quercetin on GPDH activity in 3T3-L1 cells.

After differentiation induction, the cell monolayer was incubated in post-differentiation medium with 0, 5, 10, or 20 μM quercetin for 24 h. GPDH activity was estimated by using a commercial GPDH activity assay kit. Each bar represents the mean \pm SE. Comparisons among different concentrations of quercetin that produced significant differences ($P < 0.05$) are indicated by different letters above each bar. GPDH, glycerol-3-phosphate dehydrogenase.

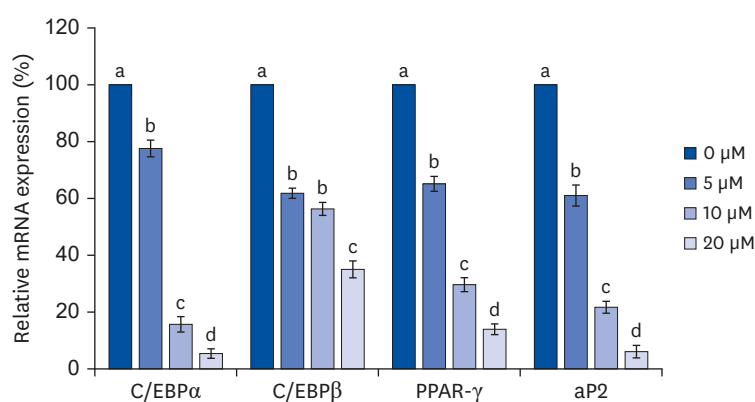


Fig. 3. Effect of quercetin on C/EBPα, C/EBPβ, PPAR-γ, and aP2 mRNA expressions in 3T3-L1 cells. After differentiation induction, the cell monolayer was incubated in post-differentiation medium with 0, 5, 10, or 20 μM quercetin for 24 h. Total RNA was isolated, and real-time PCR was performed. β-actin levels were compared, acting as the equal loading control. Each bar represents the mean ± SE. Comparisons among different concentrations of quercetin that generated significant differences ($P < 0.05$) are indicated by different letters above each bar. C/EBP, CCAAT/enhancer-binding protein; PPAR, peroxisomal proliferator-activated receptors; aP2, adipocyte protein 2; PCR, polymerase chain reaction.

decreases were not concentration-dependent. With 5 and 10 μM quercetin treatment, C/EBPβ mRNA expressions decreased by 38.3% and 43.6%, respectively, compared to the control group, but there was no significant difference between the decreases in those two treatment groups. Treatment with 20 μM quercetin significantly reduced C/EBPβ mRNA expression (65.1% reduction) compared to the control group ($P < 0.05$). Compared to the control group, the mRNA expressions of PPAR-γ following 5, 10, and 20 μM quercetin treatments were significantly reduced by 34.7%, 70.5%, and 86.1%, respectively, indicating quercetin dose-dependency ($P < 0.05$). The mRNA expression of aP2 was significantly reduced from that in the control group in a quercetin dose-dependent manner (39.1%, 78.2%, and 93.9%; $P < 0.05$).

mRNA expressions of vascular remodeling factors related to adipogenesis

The mRNA expressions of VEGF-α and VEGFR-2 were significantly reduced in a quercetin dose-dependent manner (**Fig. 4**). When quercetin was treated at 0, 5, 10, and 20 μM concentrations for 24 h, the mRNA expressions of VEGF-α were significantly reduced by 34.9%, 70.7%, and 89.5%, respectively, compared to the control ($P < 0.05$), while the mRNA expression of VEGFR-2 was decreased by 30.2%, 64.6%, and 89%, respectively ($P < 0.05$).

MMP-2 and MMP-9 mRNA expressions in the quercetin-treated groups were also decreased in a quercetin concentration-dependent manner (MMP-2: 57.9%, 80.9%, and 90.1%; MMP-9: 38.7%, 77.8%, and 91.8%) compared to the control treatment (0 μM quercetin; $P < 0.05$) (**Fig. 5**).

Enzyme activity and concentrations of MMP-2 and MMP-9

The enzyme activity of MMP-2 and MMP-9 were decreased significantly as the concentration of quercetin increased ($P < 0.05$) (**Fig. 6**). Following the treatment by 5 μM quercetin, the enzyme activities of both MMP-2 and MMP-9 decreased significantly compared to the control. At all quercetin concentrations, the MMP-2 concentration decreased to a greater extent than that of MMP-9.

The concentration of MMP-2 decreased according to the concentration of quercetin treatment, and statistical significance of the decrease was observed following the 10 μM

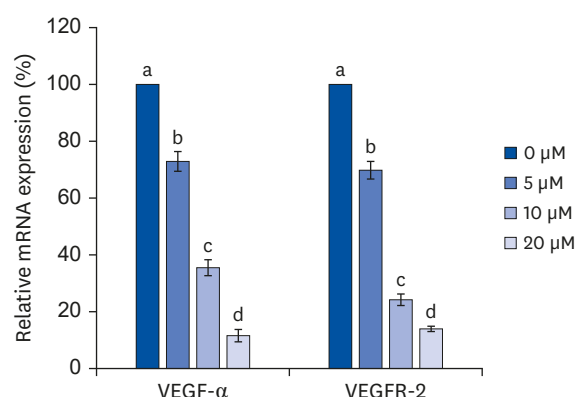


Fig. 4. Effect of quercetin on VEGF- α and VEGFR-2 mRNA expressions in 3T3-L1 cells. After differentiation induction, the cell monolayer was incubated in post-differentiation medium with 0, 5, 10, or 20 μ M quercetin for 24 h. Total RNA was isolated, and real-time PCR was performed. β -actin levels were compared, acting as the equal loading control. Each bar represents the mean \pm SE. Comparisons among different concentrations of quercetin that produced significant differences ($P < 0.05$) are indicated by different letters above each bar. VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; PCR, polymerase chain reaction.

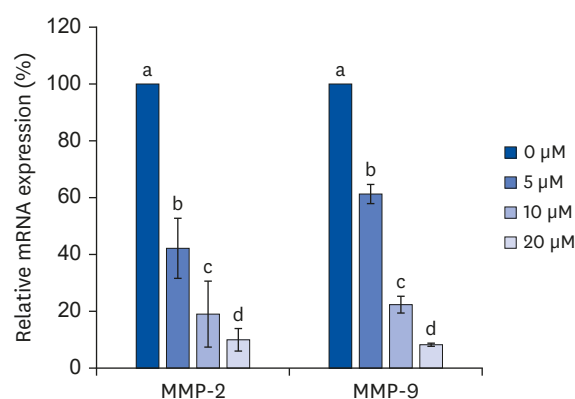


Fig. 5. Effect of quercetin on MMP-2 and MMP-9 mRNA expressions in 3T3-L1 cells. After differentiation induction, the cell monolayer was incubated in post-differentiation medium with 0, 5, 10, or 20 μ M quercetin for 24 h. Total RNA was isolated, and real-time PCR was performed. β -actin levels were compared, acting as the equal loading control. Each bar represents the mean \pm SE. Comparisons among different concentrations of quercetin that resulted in significant differences ($P < 0.05$) are indicated by different letters above each bar. MMP, matrix metalloproteinase; PCR, polymerase chain reaction.

quercetin treatment. Following 20 μ M quercetin treatment, the concentration of MMP-2 decreased the most compared to that in the control group. In the case of MMP-9, quercetin treatment at 10 μ M ($P < 0.05$) and 20 μ M ($P < 0.05$) significantly decreased the MMP-9 concentration compared to that after the 5 μ M quercetin treatment. There was no significant difference between the decreases following the 10 and 20 μ M quercetin treatments (**Fig. 7**).

DISCUSSION

Adipose tissue is highly vascularized, and each adipocyte is nourished by an extensive capillary network [1]. The development of obesity is associated with extensive adipose tissue modifications associated with adipogenesis and angiogenesis through ECM remodeling

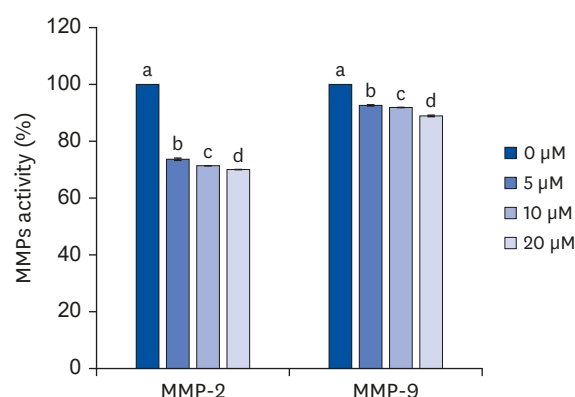


Fig. 6. Effect of quercetin on activity of MMP-2 and MMP-9 in 3T3-L1 cells.

After differentiation induction, the cell monolayer was incubated in post-differentiation medium with 0, 5, 10, or 20 μM quercetin for 24 h. The MMP-2 and MMP-9 activity levels were estimated by colorimetric assay. Each bar represents the mean ± SE. Comparisons among different concentrations of quercetin that produced significant differences ($P < 0.05$) are indicated by different letters above each bar. MMP, matrix metalloproteinase; PCR, polymerase chain reaction.

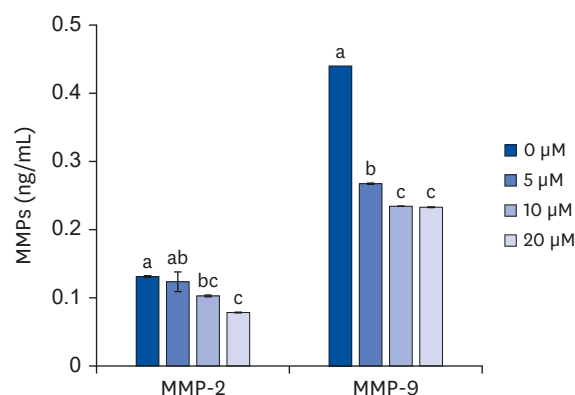


Fig. 7. Effect of quercetin on concentrations of MMP-2 and MMP-9 in 3T3-L1 cells.

After differentiation induction, as described in Methods, the cell monolayer was incubated in post-differentiation medium with 0, 5, 10, or 20 μM quercetin for 24 h. The MMP-2 and MMP-9 concentrations were estimated by using ELISA kits. Each bar represents the mean ± SE. Comparisons among different concentrations of quercetin that resulted in significant differences ($P < 0.05$) are indicated by different letters above each bar. MMP, matrix metalloproteinase; PCR, polymerase chain reaction.

[2,3]. Degradation of the ECM is reported to be the first step in the angiogenic process and MMPs, especially MMP-2 and -9 have important roles in this process [4-6]. Few studies are available regarding quercetin's role in regulating the angiogenic processes and the degradation of the ECM, especially during adipocyte differentiation [13-16]. Therefore, this study aimed to investigate whether quercetin inhibits fat accumulation in 3T3-L1 adipocytes and determine if its mechanism is related to inhibition of VEGF or MMP activity.

In this study, there was no significant difference in adipocyte viability at all quercetin concentrations after 24-h treatments, compared to the control group. Thus, in this study, to avoid affecting adipocyte survival, quercetin was treated to adipocytes for 24 h. Previous *in vitro* studies have been performed with various quercetin doses (10 to 500 μM) [12-18]. Harwood *et al.* [19] reported that a 34 μM quercetin treatment was the minimum dose that produced and experimental effect without showing toxicity in mouse lymphoma L5178Y cells. In addition, a 10 μM or more quercetin treatment showed statistically significant reductions

in fat accumulation in mature adipocytes [20]. Based on previous research, the maximum treatment concentration of quercetin used in this study was set at 20 μM . In this study, 20 μM quercetin treatment and 48-h incubation and all quercetin concentrations after 96-h incubation, produced decreased adipocyte viability compared to that of the control group, but it does not indicate adipocyte death. This indicates that quercetin exerts an anti-obesity effect by inhibiting adipocyte proliferation in preadipocytes and by reducing the number of adipocytes in mature adipose tissue [21].

In this study, to observe the effect of quercetin treatment on the accumulation of fat, the enzyme activity level of GPDH was determined. GPDH activity was significantly decreased, compared to the control group, with the 20 μM quercetin treatment ($P < 0.05$). Triglyceride synthesizes via an ester reaction between activated fatty acids and glycerol-3-phosphate. In adipose tissue and muscles, glycerol-3-phosphate is generated by the action of GPDH from glucose (as a form of dihydroxyacetone phosphate). In several studies, GPDH activity and its expression levels have been used as indicators of adipocyte differentiation or fat accumulation in cells [17,20-23].

In this study, quercetin reduced the expressions of transcription factors (C/EBP α , C/EBP β , PPAR- γ , and aP2) specifically expressed in adipocyte differentiation, thereby inhibiting fat production in adipocytes. PPAR- γ regulates adipocyte differentiation with co-expressions of members of the C/EBP family [12,15]. C/EBP α expression is induced at later stages and is active in mature adipocytes, while C/EBP β is expressed in the early stage of adipocyte differentiation [24]. The gene aP2 is overexpressed in mature adipocytes; aP2 acts like adipokine regulating metabolism in the late stage of differentiation [12]. Previous studies have shown that quercetin treatment reduced the production of triglycerides in adipocytes by suppressing the expression of C/EBP α , C/EBP β , PPAR- γ , and aP2 [11,12,22].

It has been suggested that adipogenesis (formation of adipocytes) is related to angiogenesis (formation of new blood vessels) [2-6]. Many studies reported that activation of MMPs results in the up-regulation of VEGFR-2 expression, activated VEGF- α by increasing the binding of VEGFR-2 to VEGF- α , thereby enhancing the proliferative responses in endothelial cells [4,5,13] and 3T3-L1 adipocytes [12,25-29]. Therefore, angiogenesis inhibitors are thought to be useful indicators when treating obesity and obesity-related diseases. However, there have been no reports on the relationships between quercetin and MMPs, VEGFs, or angiogenesis in 3T3-L1 adipocytes.

In this study, as the quercetin treatment concentration increased, the concentrations of MMPs decreased, and MMP enzyme activity was inhibited. Based on the RT-PCR assay results, the mRNA expressions of MMPs were inhibited in a quercetin concentration-dependent manner. During the differentiation of adipocytes, the synthesis and release of MMP-2 and MMP-9 are continuously at high levels, and adipocyte differentiation is activated [30]. In this study, the concentrations of MMPs were lowered by quercetin treatment, indicating that the synthesis and release of MMPs in adipocytes were inhibited; thus, MMPs enzyme activities and mRNA expressions of MMPs were decreased, suggesting that both adipocyte differentiation and angiogenesis were inhibited. This result is supported by additional results of this study that showed the inhibition of fat accumulation, resulting in decreased gene expressions of C/EBP α , C/EBP β , PPAR- γ , and aP2, as well as gene expressions of VEGF- α and VEGFR-2, which are vascular endothelial growth factors, by quercetin treatment. Therefore, the inhibition of MMPs by quercetin could be a regulator of obesity,

suggesting that quercetin may contribute to adipogenesis and angiogenesis during the development of obesity.

A typical dietary intake of quercetin is about 20 to 1,000 mg/day, and humans who consume enough fruits and vegetables may consume up to 250 mg of quercetin daily [31]. In several human or rodent studies, a quercetin intake of 20–50 mg/day resulted in a quercetin plasma concentration of 0.1 to 2 μM [19,32,33]. Several clinical trials have reported that 150–500 mg/day of high purity quercetin can be consumed for a long period (up to 3 mon) without side effects [31,34,35]. In a study of a 500 mg quercetin intake for 6 day, the plasma concentrations of quercetin as aglycone and nonmethylated quercetin conjugates were 15 and 450 $\mu\text{g/L}$, respectively [35]. Based on studies of quercetin's bioavailability [31–35], the effective quercetin dose used in this study was assumed to be above 5 μM , which can be obtained by consuming 100–150 mg of quercetin daily. Quercetin has a long half-life in the body (11 to 28 h), so if taken repeatedly, it can accumulate in the body and produce a cumulative effect [36]. Thus, the maximum quercetin dose of 20 μM used in this study is considered a level that can be safely consumed when repeatedly ingesting foods rich in quercetin.

In conclusion, quercetin decreased fat accumulation in 3T3-L1 adipocytes by inactivating mRNA expressions of C/EBP α , C/EBP β , PPAR- γ , and aP2. In addition, quercetin reduced the mRNA expressions of VEGF- α and VEGFR-2, and the mRNA expressions, activity, and concentrations of MMP-2 and MMP-9 in adipocytes. The results of this study indicate that quercetin inhibits adipogenesis and angiogenesis in 3T3-L1 cells, and its effects appear to be achieved via expression downregulation of MMPs (MMP-2 and MMP-9).

REFERENCES

1. Lijnen HR. Angiogenesis and obesity. *Cardiovasc Res* 2008;78:286–93.
[PUBMED](#) | [CROSSREF](#)
2. Cao Y. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest* 2007;117:2362–8.
[PUBMED](#) | [CROSSREF](#)
3. Ali AT, Hochfeld WE, Myburgh R, Pepper MS. Adipocyte and adipogenesis. *Eur J Cell Biol* 2013;92:229–36.
[PUBMED](#) | [CROSSREF](#)
4. Almalki SG, Llamas Valle Y, Agrawal DK. MMP-2 and MMP-14 silencing inhibits VEGFR2 cleavage and induces the differentiation of porcine adipose-derived mesenchymal stem cells to endothelial cells. *Stem Cells Transl Med* 2017;6:1385–98.
[PUBMED](#) | [CROSSREF](#)
5. Bagchi M, Kim LA, Boucher J, Walshe TE, Kahn CR, D'Amore PA. Vascular endothelial growth factor is important for brown adipose tissue development and maintenance. *FASEB J* 2013;27:3257–71.
[PUBMED](#) | [CROSSREF](#)
6. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;9:669–76.
[PUBMED](#) | [CROSSREF](#)
7. Berg G, Barchuk M, Miksztowicz V. Behavior of metalloproteinases in adipose tissue, liver and arterial wall: an update of extracellular matrix remodeling. *Cells* 2019;8:158.
[PUBMED](#) | [CROSSREF](#)
8. Carullo G, Cappello AR, Frattaruolo L, Badolato M, Armentano B, Aiello F. Quercetin and derivatives: useful tools in inflammation and pain management. *Future Med Chem* 2017;9:79–93.
[PUBMED](#) | [CROSSREF](#)
9. Chen S, Jiang H, Wu X, Fang J. Therapeutic effects of quercetin on inflammation, obesity, and type 2 diabetes. *Mediators Inflamm* 2016;2016:9340637.
[PUBMED](#) | [CROSSREF](#)
10. Zhao Y, Chen B, Shen J, Wan L, Zhu Y, Yi T, Xiao Z. The beneficial effects of quercetin, curcumin, and resveratrol in obesity. *Oxid Med Cell Longev* 2017;2017:1459497.
[PUBMED](#) | [CROSSREF](#)

11. Ahn J, Lee H, Kim S, Park J, Ha T. The anti-obesity effect of quercetin is mediated by the AMPK and MAPK signaling pathways. *Biochem Biophys Res Commun* 2008;373:545-9.
[PUBMED](#) | [CROSSREF](#)
12. Bae CR, Park YK, Cha YS. Quercetin-rich onion peel extract suppresses adipogenesis by down-regulating adipogenic transcription factors and gene expression in 3T3-L1 adipocytes. *J Sci Food Agric* 2014;94:2655-60.
[PUBMED](#) | [CROSSREF](#)
13. Lu J, Wang Z, Li S, Xin Q, Yuan M, Li H, Song X, Gao H, Pervaiz N, Sun X, et al. Quercetin inhibits the migration and invasion of hcc1m3 cells by suppressing the expression of p-Akt1, matrix metalloproteinase (MMP) MMP-2, and MMP-9. *Med Sci Monit* 2018;24:2583-9.
[PUBMED](#) | [CROSSREF](#)
14. Ren KW, Li YH, Wu G, Ren JZ, Lu HB, Li ZM, Han XW. Quercetin nanoparticles display antitumor activity via proliferation inhibition and apoptosis induction in liver cancer cells. *Int J Oncol* 2017;50:1299-311.
[PUBMED](#) | [CROSSREF](#)
15. Chuang CH, Yeh CL, Yeh SL, Lin ES, Wang LY, Wang YH. Quercetin metabolites inhibit MMP-2 expression in A549 lung cancer cells by PPAR- γ associated mechanisms. *J Nutr Biochem* 2016;33:45-53.
[PUBMED](#) | [CROSSREF](#)
16. Zhao J, Fang Z, Zha Z, Sun Q, Wang H, Sun M, Qiao B. Quercetin inhibits cell viability, migration and invasion by regulating miR-16/HOXA10 axis in oral cancer. *Eur J Pharmacol* 2019;847:11-8.
[PUBMED](#) | [CROSSREF](#)
17. Kim WK, Kang NE, Kim MH, Ha AW. Peanut sprout ethanol extract inhibits the adipocyte proliferation, differentiation, and matrix metalloproteinases activities in mouse fibroblast 3T3-L1 preadipocytes. *Nutr Res Pract* 2013;7:160-5.
[PUBMED](#) | [CROSSREF](#)
18. Kim HL, Ha AW, Kim WK. Effect of saccharin on inflammation in 3T3-L1 adipocytes and the related mechanism. *Nutr Res Pract* 2020;14:109-16.
[PUBMED](#) | [CROSSREF](#)
19. Harwood M, Danielewska-Nikiel B, Borzelleca JF, Flamm GW, Williams GM, Lines TC. A critical review of the data related to the safety of quercetin and lack of evidence of *in vivo* toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem Toxicol* 2007;45:2179-205.
[PUBMED](#) | [CROSSREF](#)
20. Eseberri I, Miranda J, Lasa A, Churruga I, Portillo MP. Doses of quercetin in the range of serum concentrations exert delipidating effects in 3T3-L1 preadipocytes by acting on different stages of adipogenesis, but not in mature adipocytes. *Oxid Med Cell Longev* 2015;2015:480943.
[PUBMED](#) | [CROSSREF](#)
21. Lee CW, Seo JY, Lee J, Choi JW, Cho S, Bae JY, Sohng JK, Kim SO, Kim J, Park YI. 3-O-Glucosylation of quercetin enhances inhibitory effects on the adipocyte differentiation and lipogenesis. *Biomed Pharmacother* 2017;95:589-98.
[PUBMED](#) | [CROSSREF](#)
22. Yang JY, Della-Fera MA, Rayalam S, Ambati S, Hartzell DL, Park HJ, Baile CA. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. *Life Sci* 2008;82:1032-9.
[PUBMED](#) | [CROSSREF](#)
23. Lee MS, Kim Y. Effects of isorhamnetin on adipocyte mitochondrial biogenesis and AMPK activation. *Molecules* 2018;23:1853.
[PUBMED](#) | [CROSSREF](#)
24. Lefterova MI, Zhang Y, Steger DJ, Schupp M, Schug J, Cristancho A, Feng D, Zhuo D, Stoeckert CJ Jr, Liu XS, Lazar MA. PPAR γ and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. *Genes Dev* 2008;22:2941-52.
[PUBMED](#) | [CROSSREF](#)
25. Kawamura T, Murakami K, Bujo H, Unoki H, Jiang M, Nakayama T, Saito Y. Matrix metalloproteinase-3 enhances the free fatty acids-induced VEGF expression in adipocytes through toll-like receptor 2. *Exp Biol Med (Maywood)* 2008;233:1213-21.
[PUBMED](#) | [CROSSREF](#)
26. Park BY, Lee H, Woo S, Yoon M, Kim J, Hong Y, Lee HS, Park EK, Hahm JC, Kim JW, et al. Reduction of adipose tissue mass by the angiogenesis inhibitor ALS-L1023 from *Melissa officinalis*. *PLoS One* 2015;10:e0141612.
[PUBMED](#) | [CROSSREF](#)
27. Chavey C, Mari B, Montheuol MN, Bonnafous S, Anglard P, Van Obberghen E, Tartare-Deckert S. Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. *J Biol Chem* 2003;278:11888-96.
[PUBMED](#) | [CROSSREF](#)

28. Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR. Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. *Diabetes* 2002;51:1093-101.
[PUBMED](#) | [CROSSREF](#)
29. Pratheeshkumar P, Budhraj A, Son YO, Wang X, Zhang Z, Ding S, Wang L, Hitron A, Lee JC, Xu M, et al. Quercetin inhibits angiogenesis mediated human prostate tumor growth by targeting VEGFR-2 regulated AKT/mTOR/P70S6K signaling pathways. *PLoS One* 2012;7:e47516.
[PUBMED](#) | [CROSSREF](#)
30. Bouloumié A, Sengenès C, Portolan G, Galitzky J, Lafontan M. Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. *Diabetes* 2001;50:2080-6.
[PUBMED](#) | [CROSSREF](#)
31. Andres S, Pevny S, Ziegenhagen R, Bakhiya N, Schäfer B, Hirsch-Ernst KI, Lampen A. Safety aspects of the use of quercetin as a dietary supplement. *Mol Nutr Food Res* 2018;62:1700447.
[PUBMED](#) | [CROSSREF](#)
32. Conquer JA, Maiani G, Azzini E, Raguzzini A, Holub BJ. Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. *J Nutr* 1998;128:593-7.
[PUBMED](#) | [CROSSREF](#)
33. Carbonaro M, Grant G. Absorption of quercetin and rutin in rat small intestine. *Ann Nutr Metab* 2005;49:178-82.
[PUBMED](#) | [CROSSREF](#)
34. Egert S, Bosy-Westphal A, Seiberl J, Kürbitz C, Settler U, Plachta-Danielzik S, Wagner AE, Frank J, Schrezenmeir J, Rimbach G, et al. Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-blinded, placebo-controlled cross-over study. *Br J Nutr* 2009;102:1065-74.
[PUBMED](#) | [CROSSREF](#)
35. Shi Y, Williamson G. Quercetin lowers plasma uric acid in pre-hyperuricaemic males: a randomised, double-blinded, placebo-controlled, cross-over trial. *Br J Nutr* 2016;115:800-6.
[PUBMED](#) | [CROSSREF](#)
36. Williamson G, Manach C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* 2005;81:243S-255S.
[PUBMED](#) | [CROSSREF](#)