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Original Research Article

Quercetin promotes MC3T3-E1 cell growth via PI3K/Akt signaling pathway

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Abstract

Purpose: *To investigate the effect of quercetin on mouse osteoblast MC3T3-E1 cell growth and the molecular mechanisms involved.*
Methods: *The effect of quercetin on MC3T3-E1 cells growth, cell cycle distribution, bone-related genes and PI3K/Akt signaling pathway were determined in vitro using MTT assay, flow cytometry, qPCR and western blot, respectively.*
Results: *Qercetin promoted MC3T3-E1 cell growth, as revealed from MTT assay. Flow cytometry showed that quercetin administration resulted in accumulation of cells in the S phase. In addition, quercetin up-regulated mRNA expression levels of osteopontin, ALP, osteoprotegerin, osteocalcin and RunX-2, and increased phosphorylation of AKT.*
Conclusion: *Quercetin enhances the growth of MC3T3-E1 cells via a mechanism involving accumulation of cells in S phase, increasing mRNA expression levels of bone-associated genes, and increasing the phosphorylation of AKT.*

Keywords: *Quercetin, MC3T3-E1 cells, AKT phosphorylation, Osteopontin, Osteoprotegerin, Osteocalcin, RunX-2*

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INTRODUCTION

Quercetin is a flavonoid compound present in a variety of fruits and vegetables such as grapefruit, mandarin oranges, and onions [1]. It is a free radical scavenger and a chelator of metal ions. Studies have revealed that quercetin exerts cytoprotective through its anti-inflammatory and antioxidant properties [2-4]. In addition, it possesses anti-cancer and anti-allergy activities [2]. Quercetin enhances the growth and development of osteoblasts through positive interaction with them, and it suppresses the bone-resorption activity of osteoclasts [5-8].

Skeletal defects requiring bone-graft procedures are one of the most serious challenges confronting millions of patients annually worldwide [9]. It has been estimated that 2.2 million bone-grafting surgeries are carried out annually worldwide [10]. Osteopenia is often associated with increased incidence of bone fracture, delayed healing of bone fractures, and poor quality of life of diabetic patients [11,12]. Therefore, the search for effective drugs which

can control the development of diabetic osteopenia is of great significance for patients with diabetic osteopenia.

This study was aimed at investigating the putative beneficial influence of quercetin in a mouse cell osteoblast MC3T3-E1 model of diabetic osteopenia.

EXPERIMENTAL

MC3T3-E1 cell culture

Mouse MC3T3-E1 cells were obtained from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). They were cultured routinely 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA), penicillin (100 U/mL) and streptomycin (100 µg/mL) in a humidified atmosphere containing 5 % CO_2.

Cell proliferation assay

Cell proliferation was determined using MTT assay. The MC3T3-E1 cells were cultured in 6-well plates to 80 % confluence, and the old medium was replaced with a medium containing quercetin at concentrations of 0.4, 0.6, and 0.8 mmol/L, followed by incubation for 48 h. The negative control consisted of cell cultures without quercetin. Thereafter, 20 µL of 0.5 mg/mL MTT (Sigma-Aldrich) was added to each well and the wells were incubated 37 °C for 4 h. Then, the medium was removed and the resultant formazan crystals were solubilized in 150 µL of dimethylsulfoxide (Sigma-Aldrich), and absorbance was read at 490 nm in a SpectraMax 360 pc microplate reader (Molecular Devices, CA, USA).

Flow cytometric analysis of changes in cell cycle

The MC3T3-E1 cells were harvested with tryptic digestion and centrifuged for 6 min at 1200 rpm. The cell pellet was rinsed twice with 3 mL of PBS, centrifuged and fixed for 2 h in ice-cooled 70 % ethanol at 4 °C. The fixed cells were recovered by centrifugation and the cells were re-suspended in 3 mL of PBS, sieved through a 400-mesh net, and centrifuged for 3 min at 3000 rpm. Thereafter, the cells were stained with 1 mL of 100 µg/mL propidium iodide (Sigma, MO, USA) at 4 °C for 30 min in the dark. The distribution of cells in the cell cycle phases was measured using a flow cytometer (CyFlow® Cube, Görlitz, Germany) at excitation and emission wavelengths of 488 nm and 630 nm, respectively.

Quantitative real-time PCR (qPCR)

Total RNA was isolated from the MC3T3-E1 cells with TRIzol reagent (Invitrogen). The concentration and the purity of RNA were determined by measuring the absorbance at 260/280 nm using a spectrophotometer (NANO-Drop). The isolated RNA (2 µg) was reverse-transcribed to cDNA with revertAid kit (Fermentas, USA) using commercial first-strand cDNA synthesis kit. SYBR green chemistry was used to perform quantitative determination of osteopontin, ALP, osteoprotegerin, osteocalcin and RunX-2 and β-actin transcript levels following an optimized protocol. The primer sequence of the genes of interest are indicated in Table 1.

Table 1: Primer sequences for real-time PCR analysis of gene expression*

Gene name	Primer sequence
OC	CCTTCATGTCCAAGCAGGA
	GGCGGTCTTCAAGCCATAC
OPN	CCTCCCGGTGAAAGTGAC
	CTGTGGCGCAAGGAGATT
OPG	GTTCCTGCACAGCTTCACAA
	AAACAGCCCAGTGACCATTC
ALP	TATGTCTGGAACCGCACTGAAC
	CACTAGCAAGAAGAAGCCTTTGG
Runx2	ATCCAGCCACCTTCACTTACACC
	GGGACCATTGGGAACTGATAGG
β-actin	GGGTCAGAAGGATTCCTATG
	GGTCTCAAACATGATCTGGG

*For each gene, the first and second sequences refer to forward and reverse

Western blot assay

At various time points, cells were scraped and disrupted with lysis buffer to obtain cell lysates. Equal amounts of proteins in lysate samples were subjected to 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. After incubating the membrane with specific primary antibodies and horseradish peroxidase-conjugated secondary antibodies, signals were detected using chemiluminescent substrate (KPL Guildford, UK), and the blot intensities were quantified using BandScan software (Glyko, CA).

Statistical analysis

Data are presented as mean ± SD. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Dunnett's t-test. GraphPad Prism software

(GraphPad Software Inc., La Jolla, CA) was used to analyze experimental data. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Administration of quercetin for 2 weeks significantly increased MC3T3-E1 cell growth. The increase was significant at quercetin doses of 0.40, 0.68 and 0.80 mmol/L, when compared to the control (Table 2).

Table 2: Effect of quercetin on MC3T3-E1 cell growth

Dose (mmol/L)	Degree of proliferation (%)
0	-
0.4	8.35
0.6	11.06
0.8	15.38

The results of cell cycle distribution revealed that in MC3T3-E1 cell lines, 48 h incubation with quercetin resulted in accumulation of cells in the +S phase, with concomitant reduction of percentage of cells in the G0/G1 phase (Table 3). The percentage of MC3T3-E1 cells accumulated in S phase after treatment with quercetin increased from 27.2 % (control) to 42.8 %.

Table 3: Effect of quercetin on MC3T3-E1 cell cycle distribution

Dose (mmol/L)	G0/G1	S	G2/M	S+G2
0	68.2	27.2	4.6	31.8
0.4	60.3	35.5	4.2	34.8
0.6	56.8	38.4	4.8	38.2
0.8	52.3	42.8	4.9	42.7

The mRNA expression levels of osteopontin, ALP, osteoprotegerin, osteocalcin and RunX-2 in the control and quercetin-treated groups were investigated. As shown in Table 4, administration of quercetin at different doses significantly and dose-dependently increased the mRNA levels of all the five genes, relative to the control ($p < 0.05$).

Phosphorylated Akt (p-Akt) and Akt protein levels were determined by western blot. There were no significant differences in protein expression of Akt between the quercetin group and the control group (Figure I). However, p-Akt expression was significantly increased in quercetin-treatment group (Figure 1). The relative intensity of p-Akt/total Akt was dose-dependently and significantly increased in the quercetin-MC3T3-E1 cells, when compared to the control group.

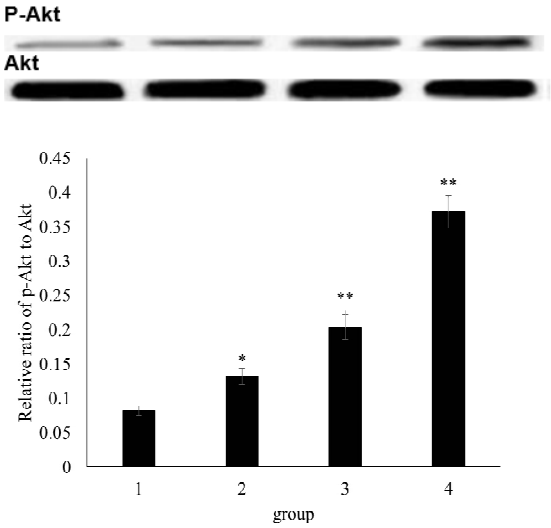

Figure 1: Effect of quercetin on p-Akt and Akt protein levels in MC3T3-E1 cells

DISCUSSION

The present study investigated the effect of quercetin on mouse MC3T3-E1 cell growth. It was found that quercetin promoted mouse MC3T3-E1 cell growth. Each phase of cell cycle has checkpoints that can promote cell cycle arrest, enabling activation of repair mechanisms and fixing the damage. The results obtained in this study showed that in mouse MC3T3-E1 cell lines, quercetin induced cell cycle arrest in S phase. Thus, the S phase arrest appears to be a possible mechanism for the growth promotion induced by quercetin. Cell population increased in S phase but decreased in G1 phase. This indicates that all phases of cell cycle were advanced, thereby shortening the proliferation cycle.

Table 4: Effect of quercetin on the expression of bone-related genes in MC3T3-E1 cells

Dose (mmol/L)	Osteopontin	ALP	Osteoprotegerin	Osteocalcin	RunX-2
0	1.592±0.132	1.242±0.107	1.426±0.122	1.173±0.105	0.932±0.083
0.4	2.036±0.183**	1.638±0.142*	1.936±0.145**	1.895±0.149**	1.216±0.113*
0.6	2.572±0.218**	2.316±0.199**	2.495±0.211**	2.553±0.206**	1.941±0.162**
0.8	3.158±0.273**	2.814±0.238**	2.963±0.242**	3.472±0.294**	2.605±0.182**

In this study, the quercetin treatment of mouse MC3T3-E1 cells was associated with bone-related genes. Quercetin treatment was also shown to increase the expression levels of osteopontin, ALP, osteoprotegerin, osteocalcin and RunX-2 mRNA in mouse MC3T3-E1 cells, indicating that these molecules are crucial for osteoblast proliferation and differentiation. Quercetin treatment was also shown to increase the phosphorylation of Akt. Thus, it is possible that quercetin treatment promotes mouse MC3T3-E1 cells growth via the PI3K/Akt signal pathway. These findings indicate the beneficial effect of quercetin on diabetes-induced osteopenia, and indicate the feasibility of developing quercetin as a potential therapeutic drug for osteopenia.

CONCLUSION

The findings of this study indicate that quercetin exerts a beneficial effect on osteopenia. Thus, the results raise the possibility of developing quercetin as a potential therapeutic drug for osteopenia disease.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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