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Effects of quercetin, a natural phenolic compound, in the differentiation of human mesenchymal stem cells (MSC) into adipocytes and osteoblasts

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Abstract

Natural phenols may have beneficial properties against oxidative stress, which is associated with aging and major chronic aging-related diseases, such as loss of bone mineral mass (osteoporosis) and diabetes. The main aim of this study was to analyze the effect of quercetin, a major nutraceutical compound present in the "Mediterranean diet", on mesenchymal stem-cell (MSC) differentiation. Such cells were induced to differentiate into osteoblasts or adipocytes in the presence of two quercetin concentrations (0.1 and 10 μM). Several physiological parameters and the expression of osteoblastogenesis and adipogenesis marker genes were monitored. Quercetin (10 μM) inhibited cell proliferation, alkaline phosphatase (ALPL) activity and mineralization, down-regulating the expression of *ALPL*, collagen type I alpha 1 (*COL1A1*) and osteocalcin [bone gamma-carboxyglutamate protein (*BGLAP*)] osteoblastogenesis-related genes in MSC differentiating into osteoblasts. Moreover, in these cultures, CCAAT/enhancer-binding protein alpha (*CEBPA*) and peroxisome proliferator-activated receptor gamma 2 (*PPARG2*) adipogenic genes were induced, and cells differentiated into adipocytes were observed. Quercetin did not affect proliferation, but increased adipogenesis, mainly at 10-μM concentration in MSC induced to differentiate to adipocytes. β- and γ-catenin (plakoglobin) nuclear levels were reduced and increased, respectively, in quercetin-treated cultures. This suggests that the effect of high concentration of quercetin on MSC osteoblastic and adipogenic differentiation is mediated *via* Wnt/β-catenin inhibition. In conclusion, quercetin supplementation inhibited osteoblastic differentiation and promoted adipogenesis at the highest tested concentration. Such possible adverse effects of high quercetin concentrations should be taken into account in nutraceutical or pharmaceutical strategies using such flavonol.

Keywords: Quercetin; Adipogenesis; Osteoblastogenesis; Mesenchymal stem cells; Beta-catenin; Gamma-catenin

1. Introduction

Phenols are secondary metabolites of plants, characterized by the presence of multiple C_6H_5OH aromatic units. They are important diet components, being present in fruits, vegetables and beverages. A great body of literature has highlighted their potential against several pathological conditions [1]. Phenols can be classified into three chemical classes: phenolic acids, flavonoids and other nonflavonoid polyphenols [2]. Flavonoids comprise the largest class of polyphenols, including different subclasses: flavonols, flavones, flavonones, flavanonols, flavanols and isoflavones [3]. Flavonols are present in human diets as both glycosides and aglycone forms.

Quercetin accounts for about 13.82-mg flavonol intake/day [4], thus being one of the most abundant flavonoids in western diets. It is mainly present as quercetin glycosides, being widely distributed in vegetable sources. They include apples, berries, onions, grapes, tea, tomatoes and red wine, as we have described [5], as well as some medicinal plants like the perforate St John's-wort (*Hypericum perforatum*) and maidenhair tree (*Gingko biloba*) [6,7]. Quercetin shows high bioavailability [6], and it may range 0.5 to 1.6 μM in human plasma [8], albeit such concentration is usually <1 μM [9]. Such flavonol has been linked to improved antioxidant activities and high scavenging potential of free radicals [10]. Indeed, it is able to exert a variety of biological activities, often related to its antioxidant nature [11]. Quercetin consumption has been related to a reduced risk of cancer and cardiovascular diseases [6] and its *in vitro* health-beneficial properties suggest that this phenolic compound could be used as a preventive nutraceutical compound [6].

Some effects are obtained at high concentrations of at least 10-μM quercetin. Yet, they can be easily reached in plasma by means of additional food supplements. But long-term quercetin studies have not been usually carried out, clinical trials are scarce and underlying mechanisms of action are partially unknown. Therefore, further research is needed to better understand quercetin properties and molecular mechanism in living cells, in order to ascertain possible undesirable side-effects of high concentrations of such flavonol [9].

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On the other hand, osteoporosis is a serious disease that afflicts many millions of people worldwide, being characterized by loss of both bone mineral mass and strength, leading to fragility fractures and even death. It arises from misbalancing metabolism in aging humans (mainly, postmenopausal women) showing excessive osteoclastic bone resorption over osteoblastic bone formation [12]. It has been proposed that an increased differentiation of mesenchymal cells into adipocyte-like ones, instead of bone-like cells, could be the main cause of decreased osteoblastic activity in this metabolic disease [12]. This hypothesis is supported by the progressive accumulation of adipose tissue within bone marrow of aged bones [13].

Osteoporosis is a multifactorial disease, involving nutrition, lifestyle and genetic factors. Besides, recent evidence indicates that oxidative stress is strongly implicated in the biological mechanisms and in pathogenesis of the age-dependent decline of bone mass and strength. Furthermore, estrogen loss may enhance aging effects on bone, by decreasing oxidative-stress defense [14]. Thus, highly antioxidant phytochemicals have been proposed in order to minimize age-related bone mass loss, enhancing osteoblastogenesis and reducing adipogenesis [15]. Quercetin is among them, but there are contradictory reports on its bone effects. Thus, protection against bone mass loss has been observed in rodents by some authors [16], but others have found that such flavonol impairs viability, differentiation and mineralization in rat osteoblasts [17]. It is important to note that quercetin may inhibit the Wnt/β-catenin signaling pathway (Wnt being derived from "wingless-related integration site") [18,19]. Such pathway is related to cellular proliferation, and its deregulation may generate tumors, further suggesting the use of quercetin as chemotherapeutic agent [20]. Yet, another role of β-catenin pathway in physiological conditions is the regulation of mesenchymal stem cell (MSC) differentiation. Thus, while its activation is required for osteoblastic differentiation, its inhibition activates the adipogenic differentiation [21].

These cells constitute an efficient and fast *in vitro* human model to assess the potential osteoblastogenic and adipogenic effects of natural compounds, including simple ones and complex mixtures, as we have previously reported [15,22]. Yet, studies of quercetin effect on human bone are scarce. Therefore, the aim of the present study was to determine the effects of two concentrations of quercetin in MSC differentiation into osteoblasts or adipocytes. The lower tested concentration (0.1 μM) may be considered within the plasma physiological range. The higher concentration (10 μM) may be reached with quercetin nutraceutical or pharmaceutical supplements, thus justifying its use in this research. To the best of our knowledge, this is the first study of such flavonol on the differentiation of MSC derived from human bone marrow.

2. Materials and methods

2.1. MSC culture, differentiation and treatments

Bone marrow samples were obtained from three volunteer donors, two women and a man (18, 23 and 31 years old), with 22, 23.4 and 25 body mass index (BMI), respectively, recruited by the Bone-Marrow Transplant Program of the Hematology Service at the Reina Sofía University-Hospital (Córdoba, Spain). All bone marrow donors gave written informed consent. MSC were directly isolated from samples and characterized, following our previously published procedure [22]. Cells were cultured in sterile plastic flask from Nunc (Kamstrupvej, Denmark) in Minimum Essential-Medium Alpha (MEM α) Eagle, with Earle's Balanced Salt-Solution (EBSS; without L-glutamine, deoxyribonucleosides or ribonucleosides) from Lonza (Basel, Switzerland), supplemented with 10% fetal bovine serum (FBS) from Gibco - Life Technologies - Thermo Fisher Scientific (Waltham, MA, USA), 2-mM UltraGlutamine (Lonza), 1-ng basic fibroblast-growth factor (bFGF)/ml and antibiotics (100-U ampicillin and 0.1-mg streptomycin/ml) from Sigma–Aldrich (Saint Louis, MO, USA) in 5% CO_2 humidified atmosphere at 37 °C. The medium was changed every 3 to 4 days, until cell confluence in monolayer (about 10 to 14 days after seeding). Cells were harvested by trypsinization [0.05% trypsin in 0.02% disodium salt of ethylenediaminetetraacetic acid (Na_2-EDTA) solution from Lonza]. Then, cell cultures were seeded in 6-, 12- and 24-well plates (Nunc) at a concentration of 500 cells/cm^2 (determined in a hemocytometer), using the same culture medium. Near-confluent cells were induced to differentiate into osteoblasts or adipocytes.

MSC differentiation into osteoblasts was induced by adding 10-nM dexamethasone, 10-mM β-glycerophosphate and 0.2-mM ascorbic acid into the culture medium. Differentiation into adipocytes was induced by 500-nM dexamethasone, 0.5-mM isobutylmethylxanthine and 50-μM indomethacin (all from Sigma–Aldrich). Experimental design included treatment of MSC induced to differentiate into osteoblasts or adipocytes with two quercetin concentrations (0.1 and 10 μM) (Sigma–Aldrich). Nontreated MSC cultures (one induced to differentiate into osteoblasts and other induced into adipocytes) were supplemented with the quercetin solvent (ethanol) and used as controls.

2.2. Dimethylthiazolyl-diphenyltetrazolium bromide assay

Cell proliferation was determined using 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) from Sigma–Aldrich. MSCs were seeded in P96 plates (7000 cells/well) in growth medium without bFGF. After 24-h incubation, cells were induced to differentiate into osteoblasts or adipocytes, in the presence or absence of 0.1- or 10-μM quercetin. In addition, some cells were also grown with such quercetin concentrations, but without differentiation inducers (controls). Culture medium was removed at days 1, 3 and 7, and wells were supplemented with 1-mg MTT/ml in Dulbecco's modified Eagle's medium (DMEM) from Sigma–Aldrich, without FBS and phenol red. Cell cultures were incubated at 37°C for 2 h. Medium was then removed, and formazan crystals were dissolved in isopropanol. Solution absorbance was measured at 570 nm (650 nm absorbance was used as reference), with a PowerWave XS microplate spectrophotometer from BioTek Instruments (Winooski, VT, USA). Results represent the mean of three independent experiments.

2.3. Reactive oxygen-species quantification

Intracellular reactive-oxygen species (ROS) were determined using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) probe from Molecular Probes (Bleiswijk, The Netherlands). Cellular ROS oxidizes such chemical into 2′,7′-dichlorofluorescein (DCF), being highly fluorescent. Thus, MSC were seeded in P96 plates (7000 cells/well) in growing medium without bFGF. After 24-h incubation, the following treatments were carried out in the presence (0.1 or 10 μM) or absence of quercetin: cultures induced to differentiate into osteoblasts (a) or adipocytes (b); and cultures not induced to differentiate (c; controls). Tissue culture medium was removed at days 1, 3 and 7, and cells were washed with MEM α without FBS. Such medium supplemented with 5-μM H2DCF-DA was then added to the wells. After incubation at 37°C for 30 min, cells were washed with Hanks' Balanced Salt Solution (HBSS) without phenol red (Sigma–Aldrich). Then, HBSS supplemented with 2% FBS was added to wells. Fluorescence was immediately quantified at 485-nm excitation and 535 emission with an Infinite 2000 microplate reader from Tecan (Mannedorf, Switzerland). Results represent the mean of three independent experiments.

2.4. Alkaline phosphatase activity

Alkaline phosphatase (ALPL) activity was determined in cells induced to differentiate into osteoblasts. Cells were washed twice with 500-μl saline solution. A total of 200-μl *p*-nitrophenyl phosphate (substrate of alkaline phosphatase) from Sigma–Aldrich were added to cells, gently mixed and incubated at 37 °C for 15 min. After that, the reaction was stopped by adding 50 μl of 3-M NaOH and measured at 405 nm using a PowerWave XS microplate spectrophotometer. Absorbance values were normalized using genomic DNA isolated from cell cultures. Relative DNA amount of each sample was determined by quantitative real-time PCR (qRT-PCR) using SYBR Green I Master in a LightCycler 480 System from Roche Diagnostics (Mannheim, Germany), using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene primers (Table 1).

2.5. Histochemical stains

Lipid accumulation was determined by oil-red staining. In short, cells were fixed and washed with isopropanol (60% in water) and stained for 15 to 20 min with a mixture of 8.2 ml of 0.3% oil red (w/v in isopropanol) and 6.8 ml of distilled water. After that, cells were washed twice in distilled water, stained with hematoxylin and photographed in a light microscope. Stain was eluted with 100% isopropanol for 10 min, and its concentration was indirectly assessed by measuring the absorbance of the elution at 510 nm in a PowerWave XS microplate spectrophotometer. Extracellular-matrix mineralization in MSC induced to differentiate into osteoblasts was tested by Alizarin-red staining at day 21. In short, cells were fixed and stained for 10 min with 1% Alizarin red (w/v in water), with pH 4.2 adjusted with ammonium hydroxide. Then, they were rinsed with water and visualized. Stain was eluted with 10% acetic acid and neutralized with 10% ammonium hydroxide (all chemicals from Sigma–Aldrich). Absorbance was measured at 405 nm using a PowerWave XS microplate spectrophotometer.