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

Antonio Casado-Díazb, Jaouad Antera, Gabriel Doradoc, José Manuel Quesada-Gómezb,⁎

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 1 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 (CEBPα) and peroxisome proliferator-activated receptor gamma 2 (PPARγ2) 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.

© 2016 Elsevier Inc. All rights reserved.

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 C₆H₄OH 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, flavanones, 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 (Ginkgo 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].

http://dx.doi.org/10.1016/j.jnutbio.2016.03.005
0955-2863/© 2016 Elsevier Inc. All rights reserved.
On the other hand, osteoporosis is a serious disease that affects 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 imbalanced 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 Sofia 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 (Kastrupvej, 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-μg basic fibroblast-growth factor (bFGF)/ml and antibiotics (100-μL ampicillin and 0.1-μg streptomycin/ml) from Sigma–Aldrich (Saint Louis, MO, USA) in 5% CO2 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 (Na2–EDTA) solution from Lonza). Then, cell cultures were seeded in 6-, 12- and 24-well plates (Nunc) at a concentration of 500 cells/cm² (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 insulin and 50 μM indomethacin (all from Sigma–Aldrich). Experimental design consisted 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. Dimethylfurfural-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 96-well 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-μg 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 (Winsoski, 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, Netherlands). Cellular ROS oxidizes such chemical into 2′,7′-dichlorofluorescein (DCF), being highly fluorescent. Thus, MSC were seeded in 96-well 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 Hank's 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 (Männedorf, 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-mL 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 1 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, dehydrated 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.
2.6. Total RNA isolation and quantitative real-time PCR

Total RNA was isolated at days 7 and 14 of differentiation induction, using the easy-spin RNA Extraction Kit from iNtRON Biotechnology (Gyeonggi-do, South Korea), according to the manufacturer’s instructions. Samples were immediately treated with DNase I (Sigma-Aldrich), and their RNA concentrations were assessed at 260 nm with an ND-1000 spectrophotometer from NanoDrop - Thermo Fisher Scientific (Waltham, MA, USA). Its quality (integrity) was checked on agarose-gel electrophoresis (AGE). cDNA was obtained using the iScript cDNA Synthesis Kit, from Bio-Rad (Hercules, CA, USA), using 1 μg of RNA as template.

The expression of eight different genes (five osteogenic and three adipogenic) was studied. Amplification by qRT-PCR was carried out in a 10-μl reaction mixture that contained 1-μl cDNA, 1 μmol of each oligonucleotide primer (Table 1) and SYBR Green I Master from Roche Diagnostics, using the genes encoding polymerase (RNA) II (DNA directed) polypeptide A (POLR2A) and ribosomal protein L13a (RPL13A) as housekeeping controls. In short, cDNA was denatured at 95 °C for 10 min and amplified in 40 cycles as follows: 95 °C for 10 s (denaturation), 65 °C for 15 s (annealing) and 72°C for 15 s (polymerization) in a LightCycler 480 System from the same manufacturer. Relative gene-expression levels were calculated using the BestKeeper software Version 1.0.[23]

2.7. Nuclear protein extraction and western blot analyses

Cells were lysed with a buffer containing 10 mM-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM-KCl, 0.1 mM-Na2-EDTA, 0.1 mM-dithiothreitol (DTT), 0.5 mM-phenylmethylsulfonyl fluoride (PMSF), 70 μg-Protease-Inhibitor Cocktail/ml and 0.5% octylphenoxypolyethoxyethanol(IGEPAL CA-630), pH 7.9 (all chemicals from Sigma-Aldrich). Lysed cells were centrifuged at 13,000 rpm for 3 min. Pellets containing the nuclear extracts were incubated with another lysis buffer containing 20 mM-HEPES, 10 mM-KCl, 0.1 mM-EDTA, 0.1 mM-disodium salt of ethylene glycol tetraacetic acid (Na2-EGTA), 1 mM-diiodothreitol (DTT), 0.5 mM-phenylmethylsulfonyl fluoride (PMSF), 70 μg-Protease-Inhibitor Cocktail/ml and 0.5% octylphenoxypolyethoxyethanol (ICGEPAL CA-630), pH 7.9 (all chemicals from Sigma-Aldrich). Cells were centrifuged at 13,000 rpm for 3 min. Pellets containing the nuclear extracts were incubated with another lysis buffer containing 20 mM-HEPES, 0.4 mM-NaCl, 1 mM-Na2-EDTA, 1 mM-Na2-EGTA, 1 mM-DTT, 1 mM-PMSF and 46-μg Protease Inhibitor Cocktail/ml, pH 7.9 from the same manufacturer. Peptide concentration was determined by the Bradford Protein Assay from Bio-Rad.

Osteoblastic marker
Alkaline phosphatase from liver/bone/kidney (ALPL)
Runt-related transcription factor 2 (RUNX2)
Collagen, type 1, alpha 1 (COL1A1)
Osterix (SP7)
Osteocalcin (BGLAP)
Adipogenic marker
Peroxisome proliferator-activated receptor gamma 2 (PPARG2)
Lipoprotein lipase (LPL)
Fatty acid binding protein 4, adipocyte (FABP4)
CCAAT/enhancer binding protein (CEBP), alpha (CEBPα)
Cyclin D1
Cyclin D1 (CND1)

Membranes were further incubated at room temperature for 1 h with antirabbit secondary antibody (1:3000) conjugated to horseradish peroxidase (HRP) from Cell Signaling Technology. Bands were revealed by chemiluminescence, using the Amersham ECL Prime Western Blotting Detection Reagent from General Electric (GE) Healthcare (Little Chalfont, UK) and visualized with an ImageQuant LAS 4000 from the same manufacturer.

2.8. Statistical analyses

Statistical analyses were performed with SPSS Version 15.0 from IBM (Armonk, NY, USA). Means were compared using one-way analysis of variance (ANOVA). The post hoc comparison was made by Tukey’s method, and the results were considered significant when P<.05.

3. Results

3.1. Proliferation of undifferentiated and induced MSC into osteoblasts or adipocytes

MSC not induced to differentiate increased proliferation until day 3. No changes in proliferation were observed at day 7, in relation to previous days. The same pattern was observed for cultures induced to differentiate into osteoblasts, albeit with a higher proliferation rate. On the contrary and interestingly, cellular proliferation was blocked from the beginning of adipogenic induction (Fig. 1). In addition, cultures induced to differentiate into osteoblasts showed a significant proliferation reduction at 24 h of 0.1-μM quercetin (low; QL) treatment. No significant proliferation change was observed for the other concentration/times (Fig. 2A). On the other hand, 10-μM quercetin (high; QH) reduced the number of undifferentiated cells at day 7, in relation to untreated ones (Fig. 2A). Cellular proliferation was reduced at all tested times for cultures induced into osteoblasts, whereas the ones induced into adipocytes were negatively affected only at 24 h of treatment (Fig. 2A).

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′–&gt;3′) (forward above; reverse below)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Housekeeping</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>TGATGACATCAAGAAGGTTGAAAG</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>TCTTGGACGGCCATGTCAGCAT</td>
<td></td>
</tr>
<tr>
<td>Polymerase (RNA) II (DNA directed) polypeptide A (POLR2A)</td>
<td>TTTTGTGAGCCTAGTCAACTGC</td>
<td>125</td>
</tr>
<tr>
<td>Ribosomal protein L13a (RPL13A)</td>
<td>CCACCTGGTCACCGTCAACCTTC</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>CTGGTACTTCCAGGCAACCTTC</td>
<td></td>
</tr>
<tr>
<td>Osteoblastic marker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase from liver/bone/kidney (ALPL)</td>
<td>CCAACGGTGGTAAAAAGTCTCATC</td>
<td>175</td>
</tr>
<tr>
<td></td>
<td>TGGGGATTGCTTGTCTACCTGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGGTGATCCCTCGACGCTTAC</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>ACTGTTCTGAGAAGGTTTGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGCGTGGCAACGCACTTCTTC</td>
<td>263</td>
</tr>
<tr>
<td></td>
<td>AGCCGCAAGGTTCAACCTTC</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>AGCCGGCGCAGTCTCCAAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCCGACAAGCTCAAGCAGTCA</td>
<td>258</td>
</tr>
<tr>
<td></td>
<td>GCTGACAGAACCTTCTCAACATC</td>
<td></td>
</tr>
<tr>
<td>Adipogenic marker</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor gamma 2 (PPARG2)</td>
<td>GCAATTCCTCCATAGTACCTGC</td>
<td>136</td>
</tr>
<tr>
<td>Lipoprotein lipase (LPL)</td>
<td>GGGCCTCTTGGCTTCTCACCATAC</td>
<td></td>
</tr>
<tr>
<td>Fatty acid binding protein 4, adipocyte (FABP4)</td>
<td>GCCTTCTGTGATGATCAGT</td>
<td>113</td>
</tr>
<tr>
<td>CCAAT/enhancer binding protein (CEBP), alpha (CEBPα)</td>
<td>TACTGGTTTGCTTCTAG</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>TGGGGTTGCTTCTTCTTCTCA</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>CTGGTTCTTCTCATCTTCTCA</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Oligonucleotide primer pair sequences
3.2. Intracellular ROS production

The putative quercetin effect on cellular ROS was measured at days 1, 3 and 7 after osteoblastic or osteogenic differentiation. QL reduced ROS only in cultures induced to differentiate into osteoblasts at day 3, without differences for the other tested times and cultures (undifferentiated and induced into osteoblasts or adipocytes) (Fig. 2B). On the contrary, QH reduced ROS for all times and cultures, except the ones induced into osteoblasts at day 7 (Fig. 2B).

3.3. Alkaline–Phosphatase activity and extracellular matrix mineralization

ALPL activity during osteoblastogenesis was significantly inhibited at days 7 and 14 of differentiation when cells were exposed to QH (Fig. 3A). The activity of this enzyme was not affected by QL, even at day 14 of treatment. On the other hand, QH reduced extracellular matrix mineralization at day 21 for MSC induced into osteoblasts, as revealed by Alizarin-red staining (Fig. 3B).

3.4. Osteoblastogenesis gene marker expression

Expression of runt-related transcription factor 2 (RUNX2) gene (associated to the osteoblastogenesis induction) was down-regulated at day 7 of treatment only with QL, not changing at day 14. However, expression of osterix (SP7) gene (encoding another transcription factor associated to the first stages of osteoblastogenesis) was not affected by the quercetin treatment. Expression of COL1A1 and ALPL genes (two early osteoblastic-markers involved in the formation of the extracellular matrix) showed a clear down-regulation at day 7 of treatment with QH. Both genes were not affected by QH at day 14, but COL1A1 decreased his expression with QL. On the other hand, BGLAP (late-stage osteoblastogenesis marker) mRNA (cDNA) levels decreased with QH at days 7 and 14 of treatment (Fig. 4).

3.5. Morphological changes during adipogenic differentiation

MSC in adipogenic medium acquired morphological features characteristic of adipocytes (e.g., intracellular accumulation of lipid droplets) during the differentiation process, which were visualized by oil-red staining (Fig. 5A). Furthermore, quercetin promoted accumulation of lipid droplets in adipocytes at day 14, suggesting that it enhanced adipocyte differentiation at both tested concentrations (Fig. 5).

3.6. Gene expression during adipogenesis differentiation

Expression of the adipogenesis marker genes peroxisome proliferator-activated receptor gamma 2 (PPARG2), fatty acid-binding protein 4 (FABP4) and lipoprotein lipase (LPL) were measured. Expression of PPARG2 was significantly up-regulated at days 7 and 14, but only when cells were treated with the higher quercetin concentration. FABP4 gene expression was up-regulated only in adipocytes treated with QH at day 7. QL induced FABP4 expression at day 14. Cultures treated with QH also showed an increase of such gene expression, albeit not being statistically significant. On the other hand, LPL (early adipogenesis-marker) mRNA levels increased only by QH at day 7 of treatment. Both tested quercetin concentrations down-regulated LPL at day 14 of treatment (Fig. 6).

3.7. Quercetin effects on beta and gamma catenin during MSC differentiation

Putative effects of quercetin on nuclear β- and γ-catenin levels at first differentiation stages (in which cells are committed towards a particular cell type), were studied by western blotting. Thus, such protein was measured in noninduced MSC, as well as induced to differentiate into osteoblasts or adipocytes. Quercetin reduced β-catenin levels in all such cultures at both studied times, mostly when using high concentration (Fig. 7A). Interestingly, QL had a lower effect on undifferentiated MSC (Fig. 7A). Also interesting was the increase of γ-catenin levels in the presence of quercetin, in parallel with β-catenin reduction (Fig. 7A). Expression of cyclin D1 gene (CCND1), required for G1/S cell-cycle transition, and partially regulated by β-catenin, was also quantified. Such gene expression was much lower in cultures induced into adipocytes than in the other two (Fig. 7B). Quercetin did not affect CCND1 expression of all three cultures at day 3, but QH repressed it at day 7 in undifferentiated MSC and the ones induced into osteoblasts (Fig. 7B).

3.8. Adipogenic markers in nondifferentiating cultures and induced to differentiate into osteoblasts

QH significantly reduced cellular proliferation and nuclear β-catenin levels in both undifferentiated MSC, as well as the ones induced to differentiate into osteoblasts. Thus, adipogenic markers were analyzed in the first differentiation stages. Expression of genes encoding PPARG2 and CEBPA adipogenic transcription factors was quantified at day 3 and 7. PPARG2 expression was not detected in nondifferentiating cultures, but QH up-regulated CEBPA in these cells at day 3, albeit not showing at day 7 (Fig. 8A). Both genes were up-regulated with very high significance by QH in cultures induced into osteoblasts, mostly at day 7 (Fig. 8A). Interestingly, this suggests an increase in adipogenic differentiation for such cultures. Indeed, oil-red O staining confirmed such results, showing that QH generated adipocytes with cytoplasmic lipid droplets in cultures induced into osteoblasts at day 7 (Fig. 8B). Such effect was not observed in nondifferentiating cells (in the absence or presence of quercetin), or the ones induced into osteoblasts (in the absence or presence of QL) (Fig. 8B).

4. Discussion

The use of naturally derived phenolic substances as nutraceuticals takes into account their putative beneficial effects in the prevention of pathological conditions like osteoporosis, diabetes and cancers [10,15,24]. In this sense, quercetin may be considered a candidate nutraceutical molecule. However, to propose the use of natural molecules as dietary supplements or nutraceuticals, their biological activity must be carefully assessed [9,25]. Thus, we have evaluated the
effect of two quercetin concentrations on MSC differentiation, including a physiological dose (QL) and 100 times higher but attainable in serum via food supplementation (QH).

It is generally accepted that quercetin has a positive effect on bone metabolism, protecting against bone mass loss in ovariectomized diabetic rats [16]. Thus, it has been proposed for bone-grafting food supplementation (QH). Moreover, quercetin weakly inhibits bone loss in ovariectomized mice, suggesting that this phenolic compound is not a potent inhibitor of osteoclastogenesis. This way, quercetin may either favor or reduce the osteoblastic differentiation, depending on the preosteoblast environment. Concentrations of 25- to 100-μM quercetin favored osteoblastic differentiation in bone marrow MSC. Yet, some relevant osteoblastogenesis genes like RUNX2, osteoprotegerin (OPG), BGLAP and SP7 were down-regulated [33].

On the other hand, there are contradictory results on the putative quercetin influence on osteoblasts. Thus, some authors have described an osteoblastogenesis-activating effect [31–34], whereas others have reported opposite findings [35]. Our results show that quercetin consistently inhibits proliferation of MSC differentiating into osteoblast, mainly at the high concentration tested, which is in agreement with previous reports observed in rat calvarial osteoblast-like (ROB) cells. In such report, it was suggested that this flavonoid did not induce apoptosis but caused arrest of cellular cycle at G1 phase [17]. Our results also show that ALPL activity was markedly inhibited by QH, which is in agreement with such other authors under similar experimental conditions (day 7 with 10-μM quercetin supplementation).

Concentrations of 10-μM quercetin reduced MC3T3-E1 mouse-preosteoblastic-cell viability. Besides, they reduced migration of such cells and protected against hydrogen peroxide (H2O2)-induced oxidative stress. Yet, they promoted viability loss in the presence of tumor-necrosis factor alpha (TNFα) [35]. Thus, interestingly, quercetin may either favor or reduce the osteoblastic differentiation, depending on the preosteoblast environment. Concentrations of 25- to 100-μM quercetin favored mice osteoblastogenesis in bone marrow MSC. Yet, some relevant osteoblastogenesis genes like RUNX2, osteoprotegerin (OPG), BGLAP and SP7 were down-regulated [33].

In contrast, Prouillet et al. [32] showed a positive effect of a wide range (1 to 50 μM) of quercetin supplementations on the alkaline-phosphatase activity in the MC-63 human-osteoblastic cell line, not affecting cell viability. However, it was observed after only 24 to 48 h of culture, concluding that it could have been due to the requirement of an extracellular-regulated kinase (ERK) activation for ALPL induction. Contrary to our results, it has been shown that 2- to 10-μM quercetin favored osteoblastic differentiation of human adipose tissue stromal cells (hADSC), increasing the extracellular matrix mineralization and up-regulating osteoblastic genes [31]. Yet, while quercetin was present in the tissue-culture medium all the time through the differentiation process in our experiments, the other authors exposed the cells to the flavonoid only 3 days before or during the differentiation [31]. This suggests that quercetin exposure
such authors found that the induction of RANKL-induced NF-κB activation on osteoclast precursors. Yet, mineralization. This is in agreement with reported quercetin inhibitory effect, reducing viability, expression of osteoblastic genes and differentiation, since it is antagonist not only of the NF-κB pathway, but also of the Smad one [such name is a portmanteau of "small body-size" (SMA) protein and "Drosophila melanogaster" mothers against decapentaplegic" (MAD) protein], induced by the transforming growth factor beta (TGFβ) and bone morphogenetic-protein 2 (BMP2), which activate osteoblastogenesis [28]. Thus, our results and those from other authors suggest that the modulating quercetin effects on bone formation are dose-dependent (besides time-dependent). This way, although such flavonol inhibits osteoblastogenesis, high concentrations may also inhibit osteoblastogenesis.

On the other hand, quercetin did not affect cell proliferation in cultures induced into adipocytes, even at the high concentration tested. Besides, adipocyte lipid droplets were increased by quercetin treatment at day 14. These results are in agreement with the fact that PPARG2, LPL and FABP4 gene expressions were up-regulated at day 7; thus, 10-μM quercetin favored MSC adipogenic differentiation. Although cultures treated with 10-μM quercetin showed a higher PPARG2 expression at day 14, LPL (early marker of adipogenic differentiation) was down-regulated in relation to untreated cultures induced to differentiate into adipocytes. This fact, together with the higher content of lipid droplets at such time indicates that cultures treated with QH reach a higher differentiation than untreated ones, therefore suggesting a slowing down of fatty-acid inclusions into lipid droplets via LPL.

Yet, other authors have found that 10- to 100-μM quercetin exhibited a dose-dependent antiadipogenic effect in 3 T3-L1 mouse cells, also activating apoptosis in mature adipocytes [36,37]. The differential results may stem from the different experimental models used, including adipogenic induction timing. Thus, 3 T3-L1 cells were induced for 2 days in adipogenic medium containing 0.25-μM dexamethasone, 0.25-mM 3-isobutyl-1-methylxanthine (IBMX) and 1-μg insulin/ml. Dexamethasone and IBMX were removed on the third day, while insulin was kept for 2 more days. Such induction treatment is quite different than the one that we have used with human MSC, maintaining inducers, together with quercetin, in the tissue culture medium during all the differentiation process. Indeed, sometimes quercetin treatment by other authors was only applied for 48 h during differentiation, and cells were treated after being differentiated in the case of apoptosis [37].

Adipogenesis inhibition has also been reported in 3 T3-L1 cells exposed to onion (Allium cepa) peel extracts (containing 68 to 91 μM quercetin), albeit with up-regulated FABP4 gene expression. The retroperitoneal and visceral fat was lower, with smaller PPARG gene expression, in rats fed with fat-rich diets supplemented with such extracts than in control ones without supplementation [38]. Yet, different results were obtained with 3 T3-F442 A preadipocytes, which increased their adipocyte differentiation in the presence of a goatweed (Scoparia dulcis) extract called SDF7, which is rich in quercetin [39].

On the other hand, it has been found that PPARG2 gene expression already reduced by TNFα can be induced by quercetin in human adipocytes [40]. In addition, 1- to 10-μM quercetin did not affect conditions may have a differential effect on osteoblastic differentiation and that continuous high concentrations may mainly modulate last stages, as in our case. Indeed, we have observed that 10-μM quercetin reduced cell proliferation during the first days of osteoblastic differentiation. This is further supported by the fact that 4 days of treatment with such quercetin concentration also reduced hADSC proliferation [31].

Quercetin may favor or not the induction of osteoblastogenic genes during mice adipose-tissue stromal-cell differentiation, depending on concentrations and exposure timing [34], which further highlights the relevance of such factors. Our results showed that while the lower (0.1 μM) quercetin concentration did not have a marked effect on osteoblastic differentiation, the higher one (10 μM) had a negative effect, reducing viability, expression of osteoblastic genes and mineralization. This is in agreement with reported quercetin inhibition of RANKL-induced NF-κB activation on osteoclast precursors. Yet, such authors found that ≥10-μM quercetin had a negative effect on osteoblastogenesis, since it is antagonist not only of the NF-κB pathway, but also of the Smad one [such name is a portmanteau of the Caenorhabditis elegans "small body-size" (SMA) protein and Drosophila melanogaster "mothers against decapentaplegic" (MAD) protein], induced by the transforming growth factor beta (TGFβ) and bone morphogenetic-protein 2 (BMP2), which activate osteoblastogenesis [28]. Thus, our results and those from other authors suggest that the modulating quercetin effects on bone formation are dose-dependent (besides time-dependent). This way, although such flavonol inhibits osteoblastogenesis, high concentrations may also inhibit osteoblastogenesis.

Fig. 3. Quercetin effects on ALPL activity and mineralization. Results are shown at days 7 and 14, in the presence of quercetin, from the beginning of differentiation into osteoblasts (A). Effects of quercetin on mineralization in MSC induced to differentiate into osteoblast at day 21 (B). Cells were stained with Alizarin red and the stain eluted and spectrophotometrically quantified. *Significance level (P<0.05) versus Co. C: Undifferentiated MSC. Co, QL and QH: MSC induced to differentiate into osteoblast treated with 0, 0.1 and 10-μM quercetin, respectively.
Fig. 4. MSC osteoblastogenic differentiation. Changes in relative expression levels of RUNX2, SP7, ALPL, COL1A1 and BGLAP (osteocalcin) in MSC induced to differentiate into osteoblasts. mRNA levels were measured by qRT-PCR at day 7 and 14 of treatment. Ordinates show the relative quantity of mRNA of studied genes. See legend of Fig. 3.
Fig. 5. Effects of quercetin on adipogenic MSC differentiation. MSC induced to differentiate into adipocytes were treated with 0.1- and 10-μM quercetin since start of differentiation. Staining images of cells after 14 days of differentiation (200×) (A). Oil-red stain quantification by spectrophotometry (B). *Significance level (P<.05) versus Ca. C: Undifferentiated MSC. Ca, QL and QH: MSC induced to differentiate into adipocytes treated with 0, 0.1 and 10-μM quercetin, respectively.

Fig. 6. MSC adipogenic differentiation. Changes on relative mRNA levels of PPARG2, LPL and FABP4 genes in MSC differentiating into adipocytes in presence or absence of quercetin at day 7 and 14. mRNA levels were measured by qRT-PCR. Data are normalized with respect to undifferentiated MSC values. See legend of Fig. 5.
cellular viability and proliferation of human AML-I preadipocyte cell line. Yet, 100-μM quercetin reduced both, albeit inducing adipocyte differentiation, favoring fat droplet accumulation and PPARG expression [41]. Such authors suggested that differentiation into adipocytes may be independent of mitotic clonal expansion in such human cell line, contrary to what has been observed in mouse cell lines, like 3 T3-L1. Thus, our results, similar to the ones obtained by Morikawa et al. [41], suggest that quercetin may have a differential effect, depending on the precursor–cell differentiation requiring (mouse) or not (human) mitotic clonal expansion.

Adipogenesis induction by high quercetin-concentrations is related to its effect up-regulating PPARG expression. As reported by other authors, that is not due to an agonistic effect of quercetin on such receptor [42]. A possible mechanism explaining this effect might be based on phenolic compounds acting as prooxidants at high concentrations [43]. Thus, quercetin may exhibit redox activities, including oxidoreductive activation, being subject to redox-cycling and intracellularly generating reactive oxygen species (ROS) [44]. Therefore, the possible quercetin prooxidant effects may increase adipogenesis through the PPARG pathway [45], while inhibiting the osteoblast differentiation at the same time. Yet, our results clearly show that QH reduces ROS, thus being antioxidant. Consequently, the antiproliferative and adipogenic effects of 10-μM quercetin are not associated to an increase in oxidative stress in our experiments.

The other potential mechanism by which quercetin may affect MSC proliferation and differentiation is via Wnt pathway, through β-catenin. The latter is inhibited by quercetin concentrations in the μM range [18,19]. Cytoplasmic β-catenin may be phosphorylated by a proteic complex including glycogen-synthase kinase 3 beta (GSK3β). Phosphorylated β-catenin is ubiquitinated and quickly degraded in proteasomes. Yet, phosphorylation and thus degradation are inhibited when Wnt proteins bind Frizzled membrane receptors and low-density lipoprotein receptor-related protein 5 or 6 (LRP5 or LRP6). The subsequent β-catenin increase in cytoplasm promotes its translocation into the nucleus. There, it binds T-cell transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF-1), producing a complex which induces transcription of Wnt pathway target genes. They include the ones involved in cell-cycle regulation, proliferation and differentiation, being a fundamental pathway to maintain stem cell activity [46].

Interestingly, we have found that QH reduces nuclear β-catenin levels in undifferentiated MSC, as well as the ones induced to differentiate into osteoblasts. Such effect may be associated to the reduced proliferation observed in such cells. This is further supported by cyclin D1 (which is involved in G1/S cell-cycle transition) down-regulation in them, being regulated by Wnt/β-catenin pathway in stem cells [47]. Cell cycle arrest takes place from the first day of adipogenic induction, as shown by our results on cell proliferation and cyclin D1 gene expression, as previously described [48]. Therefore, quercetin treatments did not significantly affect proliferation in cultures differing into adipocytes, mostly after the first 24 h.

Besides proliferation, β-catenin Wnt pathway regulates MSC osteoblastic and adipogenic differentiation. It is well known that activation of such pathway promotes osteoblastic differentiation, while its inhibition leads to adipogenesis [21]. Therefore, the lower amount of nuclear β-catenin caused by QH in cultures induced to differentiate into osteoblasts suggests that such quercetin concentration is responsible for the antosteoblastic effect observed, at least in part via inhibition of such pathway. Besides, such cell cultures showed both osteoblastogenesis reduction (as demonstrated by decreased extracellular matrix mineralization), as well as adipogenesis increase (as revealed by lipid droplets inside cells). This way, expression of genes encoding two of the main transcription factors inducing adipogenesis (PPARG2 and CEBPA) increased very significantly. This is in agreement with the fact that both are repressed by Wnt pathway during osteoblastogenesis [49].

Although nuclear β-catenin levels also significantly diminished with QH treatment in undifferentiated cell cultures, no adipocytes were observed in such cells. QH induced CEBPA expression in them at day 3, but not at day 7. On the other hand, PPARG2 expression was not detected on evaluated times. Activation of such factor is required for adipogenesis [50]. Lack of PPARG2 gene expression in undifferentiated
cells treated with QH was probably due to absence of differentiation inducers like dexamethasone in medium [51], being present in osteogenic medium. In addition, quercetin inhibitory effect on β-catenin in cultures induced to differentiate into adipocytes may explain in part the observed enhanced and accelerated adipogenic differentiation process.

Interestingly, our results show that, contrary to what happens with β-catenin, quercetin treatment, mostly at 10 μM, increased concentration of nuclear γ-catenin, both in undifferentiated cells and differentiating ones into osteoblasts and adipocytes. On the other hand, γ-catenin is mainly found in desmosomes and intermediate junctions. It may interact and compete with β-catenin at multiple cellular levels, inhibiting Wnt/β-catenin pathway through TCF/LEF transcription factors [52,53]. In addition, it has been found that an increase of nuclear γ-catenin in precursor cardiac cells favors adipocytic differentiation by inhibiting Wnt/β-catenin pathway. This may represent in part the mechanism underlying arrhythmogenic right ventricular cardiomyopathy (ARVC), which is a disease of desmosome proteins characterized by

Fig. 8. Quercetin effects on adipogenic markers. Results correspond to uninduced MSC and induced to differentiate into osteoblasts. PPARG2 and CEBPA gene expression were quantified at day 3 and 7 of osteoblastic differentiation (A). Pictures of cultures stained with oil-red O were taken with optical microscope (200×) at day 7. Arrows show cells differentiated into adipocytes with lipid droplets (B).
fibroblastogenesis in the myocardium [54]. All that suggests that \(\beta\)-catenin and inhibit Wnt/\(\beta\)-catenin pathway, as reported by others [54].

In conclusion, high quercetin concentrations may inhibit osteoblastogenesis and enhance adipogenesis in MSC as a consequence of Wnt/\(\beta\)-catenin pathway inhibition, thus, favoring bone mass loss in such circumstances. But, taking into account other studies on osteoclasts, it seems that physiological doses of this phenolic compound may protect or promote bone formation by avoiding osteoclast formation, without inducing MSC differentiation into adipocytes. Our results suggest that, although physiological quercetin concentrations may be beneficial, therapeutic strategies increasing such levels should take into account their possible effects on both undifferentiated MSC, as well as differentiating ones into osteoblasts and adipocytes.

Acknowledgements

Supported by “Intensification of Research Activity”; “Grupo PAIDI” CTS-413 of “Junta de Andalucía”; P06-FQM-0151, PI-0200/2009 and PI-0335-2012 of “Junta de Andalucía”; and RETIC EF (Spain). We thank Sebastián Demyda for his enrichment comments.

References

[15] Junta de Andalucía; and RETICEF (Spain). We thank Sebastián Demyda for his enrichment comments.


[18] Junta de Andalucía; and RETICEF (Spain). We thank Sebastián Demyda for his enrichment comments.


