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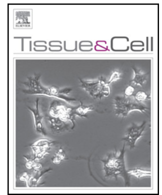

Strontium ranelate increases osteoblast activity

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ABSTRACT

Strontium ranelate (SR) is the first generation of a new class of medication for osteoporosis, which is capable of inducing bone formation and, to a certain extent, inhibiting bone resorption. The aim of this study was to evaluate the *in vitro* effects of SR on osteoblastic cell cultures. MC3TE-E1 cells were seeded in 24-well plates at a density of 2×10^4 cells/well and exposed to SR at 0.05, 0.1, and 0.5 mM. The following parameters were assayed: 1) Cell proliferation by hemocytometer counting after 24, 48 and 72 h, 2) Cell viability by MTT assay after 24, 48 and 72 h, 3) Type I Collagen and Osteopontin (OPN) quantification by Western Blotting, ELISA, and Real Time PCR after 48 h, 3) Immunolocalization of fibronectin (FN) by epifluorescence, and 4) matrix mineralization by Alizarin Red staining after 14 days. After 24, 48 and 72 h, the cell proliferation and viability were not affected by SR at 0.05 and 0.1 mM ($p > 0.05$). However, cell cultures exposed to SR at 0.5 mM exhibited a decrease in both cell proliferation and cell viability in all time points assayed ($p < 0.05$). High levels of protein and mRNA for Type I Collagen and OPN were detected in cultures exposed to SR, particularly at 0.5 mM ($p < 0.05$). SR allowed the expression of FN in osteoblastic cell cultures as observed by epifluorescence analysis. The mineralized bone-like nodule formation was affected in a concentration-dependent manner by SR, with large bone-like nodules being detected in osteoblastic cell cultures exposed to SR at 0.5 mM. In conclusion, these results suggest that SR can accelerate acquisition of the osteoblastic phenotype, which explains, at least in part, the rebalancing of bone turnover in favor of bone formation.

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1. Introduction

Bone remodeling is an active and dynamic process based on the correct balance between bone resorption by osteoclasts and bone deposition by osteoblasts (Rucci, 2008). In the long term, this biological phenomenon is maintained and orchestrated by a complex system, which includes hormones, growth factors and physical activity (Bernabei et al., 2014). In pathological conditions such as osteoporosis, rheumatoid arthritis and metastatic bone diseases, some pharmacological agents are used to decrease bone resorption and/or to stimulate bone mass augmentation (Baron et al., 2011). Among these drugs, bisphosphonates, denosumab, hormones (Chen and Sambrook, 2011) and, more recently, strontium ranelate (SR) have been extensively used as pharmacological treatments in order to inhibit massive bone loss (Reginster et al., 2015).

SR is an anti-osteoporosis drug reported to reduce vertebral and non-vertebral fracture risk in postmenopausal women (Meunier et al., 2004; Reginster et al., 2005; Seeman et al., 2006). SR exhibits a dual mechanism of action, affecting both osteoblast and osteoclast activities. These effects are mediated, partially, by the calcium-sensing receptor (CaSR) present in bone cells, which can be activated by divalent ions such as calcium and strontium (Coulombe et al., 2004; Caverzasio, 2008). Thus, once strontium binds to CaSR, the levels of inositol 1,4,5-triphosphate increase, resulting in bone metabolism activation (Chattopadhyay et al., 2007; Caudrillier et al., 2010). Although some biological mechanisms underlying the actions of SR on bone tissue have been elucidated, its role on the enhancement of bone mass is not fully understood. Therefore, considering the importance of SR as a therapeutic agent in bone disorders (Wei et al., 2014; Zhang et al., 2014) this *in vitro* study aimed to analyze the effects of this drug on

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osteoblastic cells, in terms of organic matrix secretion and mineralization.

2. Material and methods

2.1. Cell culture

Pre-osteoblastic MC3T3-E1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM/F-12 medium (LGC Biotechnology, São Paulo, SP, Brazil) supplemented with 10% (v/v) bovine fetal serum (LGC Biotechnology), 100 U/ml penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, MO). Once the cultures reached confluence, the cells were detached using a solution containing 1 mM ethylenediamine tetraacetic acid (EDTA) (Gibco/Invitrogen Life Technologies, Grand Island, NY) and 2.5 mg/mL trypsin (Gibco); then the cells were cultured in 96-well polystyrene plates or 21.5 cm^2 petri dishes at a cell density 110 cell/mm^2. To induce osteogenic differentiation, the cells were cultured in osteogenic medium containing DMEM/F-12 (LGC Biotechnology) supplemented with 10% (v/v) fetal bovine serum (LGC Biotechnology), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma), 10 mM of β-glycerophosphate (Sigma) and 50 μg/mL of ascorbic acid (Gibco). During the culture period, cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 3 days.

2.2. Drug supplementation

The cells were seeded in 96-well polystyrene plates or 21.5 cm^2 petri dishes and were allowed a period of 24 h for adherence. The culture medium was then removed and replaced by the culture medium containing SR (Sigma) at 0, 0.05, 0.1, and 0.5 mM (Time 0 h). The culture medium containing the drug was replaced every 3 days. The cells were cultured under standard cell cultivation for up to 14 days, as described above.

2.3. Cell proliferation

Cell proliferation was assayed by direct cell counting. Briefly, after 24, 48, and 72 h, the cells were enzymatically detached with 1 mM EDTA (Gibco) and 0.25% trypsin solution (Gibco). The cells were then counted using a hemocytometer (Hausser Scientific, Horsham, PA). Cell proliferation was expressed as number of cells $\times 10^4$.

2.4. MTT assay

Cell viability was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyl tetrazolium bromide (MTT; Sigma) assay after 24, 48, and 72 h. Briefly, cells were incubated with 10% of MTT (5 mg/mL) in culture medium at 37 °C for 4 h. The MTT solution was then aspirated from the well and 200 μL of Dimethyl Sulfoxide (Sigma) was added to each well. The plates were agitated on a plate shaker for 5 min, and 150 μL of this solution were transferred to a new 96-well plate. The optical density was read at 570–650 nm on the plate reader (Epoch; Bio-Tek, Winooski, VT) and data were expressed as absorbance.

2.5. Real time PCR

Total RNA was extracted from cell cultures using RNeasy Mini Kit (QIAGEN, Valencia, CA). The RNA was quantified by NanoVue spectrophotometer (GE Healthcare Life Sciences, Piscataway, NJ) and stored at −80 °C. Aliquots of 1 μg of total RNA from each sample were used for reverse transcription reactions with the Superscript III First Strand cDNA Synthesis kit (Life Technologies), according to the manufacturer's instructions. The primer sets were as follows: Type I Collagen, F 5′-ACAAGGTGACAGAGGCATAAAGG-3′ and R 5′-GCCTGCAGGACCTGAAGCT-3′, Osteopontin (OPN), F 5′-TGCTTGGGTTTGCAGTCTTCT-3′ and R 5′-CCAAACAGGCAAAAGCAAATC-3′, and for internal gene reference *GAPDH*, F 5′-TGGCCTCCAAGGAGTAAGAAAC-3′ and R 5′-TGGAAATTGTGAGGGAGATGCT-3′. Real Time PCR was performed using a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA) with SYBR Green as detection dye and 40 ng of cDNA. Cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The quantification data were analyzed with the SDS System Software (Applied Biosystems) and the relative expression levels were calculated according to the Comparative Ct method, as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

2.6. Western blotting

The cell cultures were harvested and homogenized in lysis buffer (50 mM, Tris-HCL pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitors. After centrifugation at 15000*g* for 15 min at 4 °C, the supernatants were collected and protein concentration was measured by BCA assay (Pierce, Rockford, IL). Protein extracts were separated in a 10% sodium dodecylsulfate-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Hybond; GE Healthcare), and probed for 1 h with the primary polyclonal antibodies anti-Type I Collagen (1:1000, Abcam, La Jolla, CA) and anti-Osteopontin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBST + 5% of skimmed milk. GAPDH primary polyclonal antibody was used as an endogenous control (1:1000; Santa Cruz Biotechnology). After incubation with goat anti-rabbit secondary antibody (1:5000; Sigma) for 1 h, the reaction was revealed with chemiluminescent detection reagents (Pierce) and visualized using a Digital Imaging equipment (ImageQuant LAS 4000, GE Healthcare). Optical density measurements were performed with NIH Image 1.37 (National Institutes of Health, Bethesda, MD) for scanned membranes. OD values were calculated as a percentage density normalized to the control.

2.7. Quantification of type I collagen by ELISA

Quantification of Type I Collagen was also evaluated by means of ELISA. Briefly, after 24, 48, and 72 h, the culture medium was collected and centrifuged at 336*g* for 10 min, and the resulting supernatant was collected, aliquoted and stored at −80 °C. Type I Collagen quantification was carried out using Mouse Collagen Type I Kit (Wuxi Donglin Sci&Tech Development, Wuxi/Jiangsu Province, China), according to the manufacturer's instructions. The values were expressed as pg/cell.

2.8. Indirect immunofluorescence and mineralized bone-like nodule formation

After 14 days, cultures were fixed with 4% formaldehyde in PBS buffer, pH 7.2, for 1 h at room temperature. The samples were then washed in the same buffer, and stained with 2% Alizarin red (Sigma), pH 4.2, for 10 min at room temperature. After washing, the samples were permeabilized with 0.5% Triton X-100 in PBS for 10 min, followed by blocking with 5% skimmed milk in PBS at room temperature for 30 min. Primary monoclonal antibody to fibronectin (anti-FN 1:300, Dako, Cytomation, Glostrup, Copenhagen, Denmark) was used. The control staining reaction was performed using PBS as a substitute for the primary antibody. The secondary antibody used was Alexa Fluor 488 (green fluorescence)-conjugated goat anti-mouse (1:200, Molecular Probes). After washing, the preparations were mounted using

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