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Effects of risedronate on osteoblastic cell cultures

Maurilio Malavasi[a], Ricardo Louro[a], Marcelo Barbosa Barros[a], Lucas Novaes Teixeira[b], Daiane Cristina Peruzzo[b], Júlio César Joly[a], Elizabeth Ferreira Martinez[b], Marcelo Henrique Napimoga[a,*]

[a] Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, Campinas, SP, Brazil
[b] Department of Oral Pathology, São Leopoldo Mandic Institute and Research Center, Campinas, SP, Brazil

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ABSTRACT

Objective: Bisphosphonates (BPs) have been widely used in the treatment of bone disorders due to their ability to modulate bone turnover. The biological mechanisms through BFs exert their effects on osteoclasts are well established. However, the role of BFs on the osteoblasts is controversial. The present study aimed to evaluate the effects of risedronate on osteoblastic cells.
Design: MC3TE-E1 cells were exposed to risedronate at 0, 10^{-8}, 10^{-6}, 10^{-4}, and 10^{-3} M. 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 quantification by ELISA after 24, 48 and 72 h, (3) alkaline phosphatase activity after 7 and 10 days and (4) matrix mineralization after 14 days.
Results: After 24 h, risedronate did not affect both cell proliferation and viability ($p > 0.05$). However, after 48 and 72 h, a decrease in cell proliferation and viability was detected in osteoblastic cultures exposed to risedronate at 10^{-4} and 10^{-3} M ($p < 0.05$). After 48 and 72 h, Type I Collagen synthesis was stimulated by risedronate at 10^{-4} M ($p < 0.05$). High levels of ALP activity were detected in cultures exposed to risedronate at 10^{-4} M after 7 and 10 days ($p < 0.05$). After 14 day, high calcium content was observed in cultures exposed to risedronate at 10^{-4} M ($p > 0.05$).
Conclusion: These results indicated that risedronate can promote osteoblast differentiation.

1. Introduction

Bisphosphonates (BPs) are synthetic analogues of pyrophosphate commonly used for the treatment of bone pathologies such as osteoporosis, Paget's disease, metastasis to the bone, and inflammation-related bone loss (Glatt, Pataki, Evans, Hornby, & Green, 2004; Mhaskar et al., 2012; Rodan & Martin, 2000). The clinical efficacy of BPs is mainly based on two key properties: their capacity to bind strongly to hydroxyapatite crystals of bone and their inhibitory effects on osteoclast precursors and mature osteoclasts (Tassone et al., 2003).

The BPs are classified as nitrogen-containing and non-nitrogen containing BPs. Nitrogen-containing BPs block the mevalonate pathway of cholesterol synthesis via inhibition of the enzyme farnesyl diphosphate synthase and, consequently, avoid the prenylation of small GTPases, which are essential for the osteoclast activities (Reszka & Rodan, 2004). On the other hand, once incorporated by the osteoclasts, the non-nitrogen-containing BPs are metabolized into adenosine triphosphate (ATP) analogues that inhibit the osteoclast function and induce osteoclast apoptosis (Frith, Mönkkönen, Auriola, Mönkkönen, & Rogers, 2001).

Despite their benefits, BPs are related to a potentially severe adverse drug reaction: osteonecrosis of the jaw (ONJ), which was first described in 2003 (Marx, 2003). The incidence of ONJ in patients under oral bisphosphonates therapy for osteoporosis has been calculated at 1.04–69 cases per 100,000 patient/years (Etminan, Aminzadeh, Matthew, & Brophy, 2008; Khan et al., 2011; Tennis et al., 2012; Ulmner et al., 2014), while the incidence in patients under intravenous bisphosphonate therapy varies from 0 to 90 cases per 100,000 patient/years (Devogelaer et al., 2007; Grbic et al., 2008).

The ONJ is marked by the development of jawbone necrosis, which manifest clinically as sites of exposed necrotic bone through mucosal or facial skin (Khosla et al., 2007; Ruggiero et al., 2009; Sambrook, Olver, & Goss, 2006). In some cases, ONJ can also present clinically without soft tissue perforation, with affected patients exhibiting unexplained painful symptoms, tooth mobility

* Corresponding author at: Laboratory of Immunology and Molecular Biology, São Leopoldo Mandic Institute and Research Center, R. José Rocha Junqueira, 13, Campinas, SP 13045-755, Brazil.
E-mail addresses: napimogamh@yahoo.com, marcelo.napimoga@gmail.com (M.H. Napimoga).

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or tooth loss, intraoral or extraoral fistulae, sinusitis, or mandibular facture (Fedele et al., 2010). Irrespectively of the ONJ variant, both types can show large areas of necrosis, secondary infection, and severe pain (Bagan, Hens-Aumente, Leopoldo-Rodado, Poveda-Roda, & Bagan, 2012), which may have a negative impact on the quality of life of affected patients (Miksad et al., 2011).

Risedronate is a nitrogen-containing BP that has been used for the prevention and treatment of postmenopausal (Recker & Barger-Lux, 2005; Välimäki et al., 2007) and corticosteroid-induced osteoporosis (Dougherty, 2002; Reid et al., 2009). The augmentation in bone density detected in patients under risedronate therapy may be attributed, at least in part, to its ability to reduce the number, viability and activity of osteoclasts, as well as the levels of pro-osteoclastogenic cytokines (D'Amelio et al., 2008).

Recently, a great interest has emerged regarding the possible mechanisms which BPs may affect other bone cells besides osteoclasts, such as osteoblasts and osteocytes (Plotkin et al., 2008). Indeed, some BPs can regulate osteoblast functions, although with varying or contradictory effects, depending on the concentration and type of BPs as well as the experimental models used (Maruotti, Corrado, Neve, & Cantatore, 2012). In this context, the aim of the present in vitro study was to evaluate the effects of risedronate on osteogenic cell cultures, in terms of cell proliferation, Type I Collagen synthesis and extracellular matrix (ECM) mineralization.

2. Material and methods

2.1. Cell culture

Pre-osteoblastic MC3T3-E1 cells (ATTC, Manassas, VA) were cultured in α-MEM medium (Nutricell, Campinas, Brazil) supplemented with 10% (v/v) fetal bovine serum (Cultilab, Campinas, SP, Brazil), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, St. Louis, Missouri) in 75 cm^2 flasks (Corning Incorporated, Costar, Corning, New York, NY) at 37 °C in an atmosphere with 100% humidity and 5% CO_2. Once they reached confluence, the cells were harvested using 1 mM ethylenediamine tetraacetic acid (EDTA) (Gibco) and 2.5 mg/mL trypsin solution (Gibco); then the cells were cultured on 96-well polystyrene plates at a cell density 110 cell/mm^2. To induce osteogenic differentiation, the cells were culture in osteogenic medium containing α-MEM medium (Nutricell) supplemented with 10% (v/v) fetal bovine serum (Cultilab), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma), 10 mM of β-glycerophosphate (Sigma) and 50 μg/mL of ascorbic acid (Gibco/Invitrogen Life Technologies, Grand Island, NY). 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 and were let to adhere for 24 h. Then, the culture medium was removed and replaced by the culture medium containing risedronate (Risedronate sodium, Sigma) at 0, 10^{-8}, 10^{-6}, 10^{-4} and 10^{-3} M. 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 2.5 mg/mL trypsin solution (Gibco). The cells were then counted using a hemocytometer (Hausser Scientific, Horsham, PA). Cell proliferation was expressed as number of cells × 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, the cells were incubated with 1.2 mM MTT 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. Then, 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 nm on the plate reader (Epoch; Bio-Tek, Winooski, VT) and data were expressed as absorbance.

2.5. Quantification of Type I Collagen

The quantification of Type I Collagen was evaluated by means of ELISA. Briefly, after 24, 48, and 72 h, the culture medium from the wells used for the proliferation assay was collected and centrifuged at 336*g* for 10 min, and the resulting supernatant was collected, aliquoted and stored at −80 °C. The 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 normalized by the total number of cells obtained from proliferation assay and expressed as pg/cell.

2.6. Alkaline phosphatase (ALP) activity

ALP activity was evaluated by the release of thymolphthalein from thymolphthalein monophosphate using a commercial kit (Labtest Diagnostica SA, Lagoa Santa, MG, Brazil) at 4, 7 and 10 days. Briefly, the culture medium was removed and the cultures were washed with phosphate-buffered saline (Gibco) at 37 °C and filled with 1 mL of 3.5 mM sodium lauryl sulfate (Sigma). After 30 min, the cultures were homogenized by pipetting to promote cell lysis. In a test tube were added 50 μL of thymolphthalein monophosphate mixed with 0.5 mL of 0.3 M diethanolamine buffer, pH 10.1, and left for 2 min at 37 °C. Then, 50 μL of the cell lysates obtained from each well were added in the test tube. After, 10 min incubation at 37 °C, 2 mL of 0.09 mmol/mL Na_2CO_3 and 0.25 mmol/mL NaOH was added to allow color development. After 30 min, 200 μL of each tube were place in 96-well plate and the absorbance was read at 590 nm in a spectrophotometer (Epoch; Bio-Tek). The values were normalized by total protein content, which was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts) in the same cell lysate used to quantify ALP activity. Data were expressed as μmol thymolphthalein/h/mg of protein.

2.7. Matrix mineralization

Mineralized matrix formation was assayed by Alizarin Red S (Sigma). At day 14, the cultures were fixed in 1.33 M formalin at room temperature (RT) for 2 h. The cultures were then dehydrated through a graded series of alcohol (30%, 50%, 70%, and 100%) and stained with 0.02 g/mL Alizarin Red S (Sigma), pH 4.2, for 10 min. The calcium content was carried out using a colorimetric method (Gregory, Gunn, Peister, & Prockop, 2004). Briefly, 280 μL of 1.73 M acetic acid was added to each well stained with Alizarin Red S, and the plate was incubated at RT for 30 min under shaking. Then, this solution was transferred to a polypropylene tube, heated at 85 °C for 10 min, and transferred to ice for 5 min. The samples were then

centrifuged at 20,000*g* for 15 min and 100 μL of the supernatants were transferred to a 96-well plate. Then, 40 μL of 0.7 M ammonium hydroxide was added and the plate was read at 405 nm in a spectrophotometer (Epoch; Bio-Tek, Winooski, VT). The values were normalized by total protein content, which was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), and calibrated by the control group. Data were expressed as percentage.

2.8. Statistical analysis

The experiments were carried out in triplicate. The data were submitted either to ANOVA One-Way or ANOVA Two-Way, followed by Tukey's test. The level of significance was established at $P \leq 0.05$.

3. Results

3.1. Cell proliferation

After 24 h, cell proliferation was similar among the concentrations ($p > 0.05$). However, after 48 h, the exposure to risedronate at 10^{-4} M and 10^{-3} M resulted in a reduction in cell proliferation ($p < 0.05$). No differences were detected among the other experimental groups ($p > 0.05$). After 72 h, risedronate caused a decrease in cell proliferation, particularly at 10^{-4} M and 10^{-3} M ($p < 0.05$) (Fig. 1A).

3.2. MTT assay

Risedronate did not affect cell viability after 24 h of exposure ($p > 0.05$). Nevertheless, after 48 and 72 h, the osteogenic cultures exposed to risedronate at 10^{-4} M and 10^{-3} M exhibited a decrease in cell viability ($p < 0.05$). No differences were detected among the other experimental groups ($p > 0.05$). (Fig. 1B).

3.3. Quantification of Type I Collagen

The exposure to risedronate after 24 h did not affect the Type I Collagen synthesis by osteoblastic cells ($p > 0.05$). However, high levels of Type I Collagen were observed in osteogenic cultures exposed to risedronate at 10^{-4} M for 48 and 72 h ($p < 0.05$) (Fig. 2).

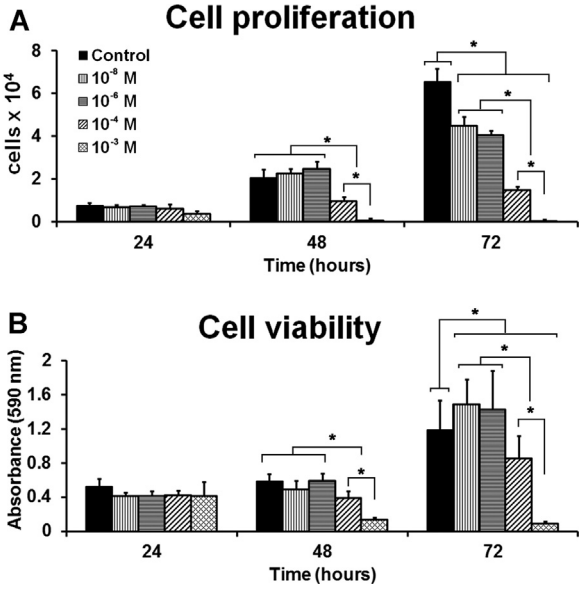

Fig. 1. Osteoblastic cell cultures exposed to risedronate. (A) Cell proliferation was expressed as number of cells × 10^4. (B) Cell viability was expressed as absorbance. Data were reported as mean ± SD. *$p < 0.05$.

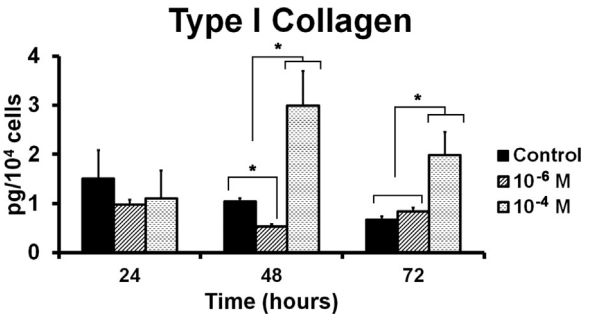

Fig. 2. Type I Collagen quantification in osteoblastic cell cultures exposed to risedronate. Data were reported as mean ± SD and expressed as pg/10^4 cells. *$p < 0.05$.

3.4. ALP activity

Risedronate affected ALP activity in MC3T3-E1 cultures after 7 and 10 days of treatment ($p < 0.05$). After 7 days, high levels of ALP activity were detected in cultures exposed to risedronate at 10^{-4} M compared to the other groups ($p < 0.05$). No differences were observed between the control and the 10^{-6} M groups ($p > 0.05$). After 10 days, high ALP activity was observed in cultures exposed to the both concentrations of risedronate compared to the control group ($p < 0.05$). However, cultures exposed to 10^{-4} M exhibited elevated ALP activity levels in comparison to the 10^{-6} M group ($p < 0.05$) (Fig. 3)

3.5. Matrix mineralization

Calcium content measured by the extraction of Alizarin Red S from mineralized matrix was affected by risedronate treatment at day 14 ($p < 0.05$). The osteogenic cultures exposed to risedronate at 10^{-4} M exhibited high calcium content in comparison to the other experimental groups ($p < 0.05$). No differences were detected between the control and the 10^{-6} M groups ($p > 0.05$) (Fig. 4).

4. Discussion

BPs have been widely used in the treatment of several bone pathologies due to their ability to modulate bone turnover (Russell, 2011). Although the underlying mechanisms through BPs exert their effects on osteoclasts are well-known in the literature (Kwak et al., 2009), the role of BPs on the other bone cells, particularly on the osteoblasts, is still a matter of debate (Roelofs, Thompson, Ebetino, Rogers, & Coxon, 2010). The present study aimed to

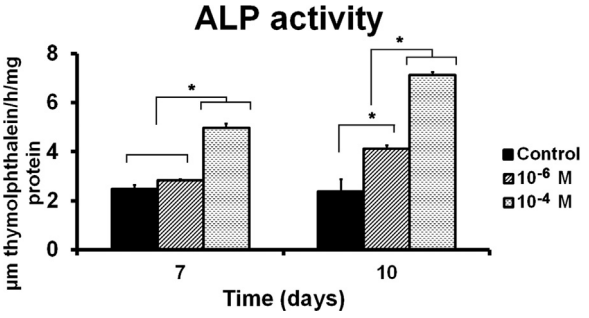

Fig. 3. ALP activity in osteoblastic cell cultures exposed to risedronate. Data were reported as mean ± SD and expressed as μmol thymolphthalein/h/mg of protein. *$p < 0.05$.

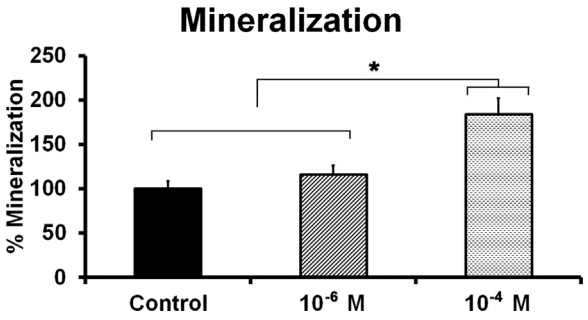

Fig. 4. Calcium quantification by Alizarin Red Stain extraction in osteoblastic cell cultures exposed to risedronate after 14 days. Data were reported as mean ± SD and expressed as percentage. $^{*}p < 0.05$.

evaluate the effects of risedronate on osteoblastic cells. The results showed this BP promoted Type I Collagen synthesis and ALP activity as well as ECM mineralization in MC3T3-E1 cell cultures. Taken together, these findings indicated that risedronate can positively modulate osteoblast differentiation.

Cell viability and proliferation were negatively affected in a concentration-dependent manner by risedronate. The osteoblastic cultures exposed to high concentration (10^{-3} and 10^{-4} M) of risedronate exhibited a reduction in both cell viability and proliferation. Garcia-Moreno et al. (1998) demonstrated that the alendronate can inhibit osteoblast proliferation at concentrations ranging from 10^{-1} M to 10^{-4} M. This effect may be attributed to the cytotoxicity of the drug at high concentrations and/or the reduction of the expression of genes encoding cell cycle regulator proteins. Indeed, Koch, Yekta, Merkel, Ziebart, and Smeets (2010) showed that zoledronate (5×10^{-5} M) decreased cyclin D1 expression in osteoblastic cell cultures. On the other hand, the cell proliferation inhibition by risedronate may be associated with the increase in cell differentiation; a finding described for others BPs (Reinholz et al., 2000). Thus, in this context, the analysis of extracellular matrix deposition and mineralization could provide essential clues about the effects of risedronate on osteoblast metabolism and function.

The main function of osteoblastic cells is to synthesize extracellular collagenous and non-collagenous proteins as well as mediators of bone mineralization (Clarke, 2008; Garnero, 2015; Lees, 2003). In our study, osteoblastic cells exposed to risedronate exhibited higher levels of Type I Collagen, particularly at the 10^{-4} M concentration. Romanello et al. (2014) demonstrated that risedronate enhances the expression of the growth factor progranulin in osteoblastic cell cultures, which in turn stimulates the synthesis of proteins associated with several biological processes. This mechanism may be responsible, at least in part, for the effects of risedronate on osteoblastic cells. Similarly, a differential gene expression has been described in osteoblastic cells exposed to risedronate, including genes encoding ECM proteins (Wang & Stern, 2011). These authors showed that the low (10^{-8} M), but not the high concentration (10^{-4} M) of risedronate upregulated many genes that control osteoblast differentiation (Wang & Stern, 2011). In the present study, even the high concentration of risedronate (10^{-4} M) was able to stimulate the synthesis of Type I Collagen. This finding may be associated with the period of exposure to risedronate used in our work, which was longer than the previous study described.

The ALP expression is an indicator of early osteoblast differentiation (Ilmer, Karow, Geissler, Jochum, & Neth, 2009). In the present study, the cultures exposed to risedronate exhibited high levels of ALP activity. A previous work showed that risedronate at lower concentrations (10^{-8} M and 10^{-9} M) can promote ALP activity (Casado-Díaz, Santiago-Mora, Dorado, & Quesada-Gómez, 2013). Interestingly, in our study, MC3T3-E1 cell cultures exposed to risedronate at 10^{-6} M and 10^{-4} M also exhibited high ALP activity. Jeong et al. (2013) demonstrated that high concentration of risedronate can stimulate the expression of key osteoblastic markers as well as potentiate the activity of transcription factors associated with osteogenesis in C2C12 cell cultures. Considering that, one explanation for the higher levels of ALP activity detected in MC3T3-E1 cells exposed to risedronate at 10^{-6} M and 10^{-4} M could be attributed to the same mechanism described in the C2C12 cell line.

During the collagen matrix mineralization, the presence of inorganic phosphate in the extracellular milieu is crucial for the nucleation and growth of the hydroxyapatite crystals (Bellows, Heersche, & Aubin, 1992). The supplying of inorganic phosphate is provided by ALP (Sugawara, Suzuki, Koshikawa, Ando, & Iida, 2002). In the present study, the higher levels of ALP activity in MC3T3-E1 cultures exposed to risedronate reflected directly in high calcium content, as demonstrated by the Alizarin Red S. It has been reported a controversial effect of BPs on extracellular matrix mineralization. As discussed for ALP activity, the lower concentrations ranging from 10^{-9} to 10^{-6} M usually promote matrix mineralization (Casado-Díaz et al., 2013; Giuliani et al., 1998; Kim, Kim, Abbas, & Yoon, 2009; Pan et al., 2004), while deleterious effects are described for concentrations higher than 10^{-5} M (Idris, Rojas, Greig, Van't Hof, & Ralston, 2008; Orriss, Key, Colston, & Arnett, 2009).Besides the concentrations, the structural differences among the BPs are also responsible for their distinct biological and pharmacological activities (Russell, Watts, Ebetino, & Rogers, 2008). Thus, the higher calcium content detected in MC3T3-E1 cultures exposed to risedronate at 10^{-6} M and 10^{-4} M could be related to the intrinsic characteristics of this cell line as well as to the chemical structural features of risedronate. Further studies are necessary to elucidate the molecular mechanisms associated with the mineralization in osteoblasts exposed to high concentrations of risedronate.

Our results highlighted a potential anabolic effect of risedronate on osteoblastic cells. This property is not only useful for the treatment of bone diseases, but for any situation where bone augmentation is required. Indeed, some studies have been demonstrated that the BPs-coated dental implants exhibited an improvement in its stability and fixation in bone (Abtahi, Tengvall, & Aspenberg, 2012; Abtahi, Agholme, Sandberg, & Aspenberg, 2013). In this context, the adsorption and/or incorporation of risedronate to implant surfaces and/or biomaterials could promote bone growth mainly through its dual effect on the bone cells, i.e., osteoclast inhibition and osteoblast activation.

Despite these interesting findings, our study has some limitations, such as the use of only one cell type. Although MC3T3-E1 cell line is a good model for studying *in vitro* osteoblast differentiation (Oya et al., 2010; Quarles, Yohay, Lever, Caton, & Wenstrup, 1992; Wang, de Boer, & de Groot, 2008), the use of other cell lines would be interesting to verify the effects of risedronate on cells at different stages of maturation, i.e., undifferentiated cells and mature osteoblast/osteocytes. Moreover, molecular analysis could be contributed to clarify which signaling pathways are activated in osteoblasts exposed to risedronate.

In conclusion, our data showed that risedronate can modulate positively osteoblast differentiation, as demonstrated by the high levels of Type I Collagen, ALP activity, and matrix mineralization. These findings may explain, at least partially, the enhancement in bone mineral density and the reduction of the risk of bone fracture detected in patients under risedronate therapy. Additionally, due to its anabolic effects on osteoblasts, risedronate should be considered as potential molecule to be incorporated on the surface of dental implants.

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