Effects of zoledronic acid on odontoblast-like cells

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ABSTRACT

The aim of the study was to evaluate the effects of a highly potent bisphosphonate, zoledronic acid (ZOL), on cultured odontoblast-like cells MDPC-23. The cells (1.5×10^4 cells/cm^2) were seeded for 48 h in wells of 24-well dished. Then, the plain culture medium (DMEM) was replaced by fresh medium without fetal bovine serum. After 24 h, ZOL (1 or 5 μM) was added to the medium and maintained in contact with the cells for 24 h. After this period, the succinic dehydrogenase (SDH) enzyme production (cell viability – MTT assay), total protein (TP) production, alkaline phosphatase (ALP) activity, and gene expression (qPCR) of collagen type I (Col-I) and ALP were evaluated. Cell morphology was assessed by SEM. Five μM ZOL caused a significant decrease in SDH production. Both ZOL concentrations caused a dose-dependent significant decrease in TP production and ALP activity. ZOL also produced discret morphological alterations in the MDPC-23 cells. Regarding gene expression, 1 μM ZOL caused a significant increase in Col-I expression. Although 5 μM ZOL did not affect Col-I expression, it caused a significant alteration in ALP expression (ANOVA and Tukey's test, $p < 0.05$). ZOL presented a dose-dependent cytotoxic effect on the odontoblast-like cells, suggesting that under clinical conditions the release of this drug from dentin could cause damage to the pulpo-dentin complex.

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1. Introduction

Bisphosphonates are a class of synthetic analogs of pyrophosphate, which have been widely used in treatment of diseases with intense bone activity resorption, such as osteoporosis, Paget's disease and some bone tumours, such as multiple myeloma, and bone metastases of breast and prostate tumours.[1,2]

These drugs are physiological modulators of bone resorption and calcification with high affinity for hydroxyapatite crystals, thus remaining adhered to the mineralized tissues of body.[3,4] In the same way as the bone tissue, dentin is characterized as a partially mineralized connective tissue with great hydroxyapatite content, and recent studies have been suggested that bisphosphonates can also adhere to this dental tissue.[5] However, to date, little is known about how bisphosphonates adhere to the dental tissues, the mechanisms by which this adherence occurs or the conditions under which these drugs are released to the pulp.

In vivo studies have demonstrated that treatment with bisphosphonates during the formation of teeth was associated with the occurrence of amelogenesis imperfecta and formation of a disorganized dentin tissue.[6,7] Sakai et al.[6] reported that bisphosphonates can adhere to dentin, promoting a complete or intermittent inhibition of dentinogenesis.

It has been described that bisphosphonates can be released from mineralized tissues during bone resorption or remodelling.[8] It is known that remodelling processes of mineralized tissues do not occur in the adult dentition. However, pulpal injuries caused by events, such as trauma and chronic inflammatory processes, could activate odontoclast

differentiation and induce a resorptive process in dentin, ultimately causing the release of these drugs to interact with the pulp tissue.[5] Another hypothesis of the action of bisphosphonates on the pulp tissue would be their cytotoxicity at the moment of infusion. However, it is suggested that, the limited drug concentration at the moment of infusion would not be sufficient to produce a cytotoxic effects to the pulp cells.[5]

Recent studies have shown that bisphosphonates are cytotoxic to different cell types.[5,9–11] Therefore, the release of this drug to the pulp tissue could promote cytotoxic effects to the pulp cells, reducing the reparative capacity of this tissue. In mammalian teeth, odontoblasts are organized in a monolayer that underlies the coronal and root dentin, and thus these peripheral pulp cells would be the first to get in contact with bisphosphonates released from dentin.[12,13] Therefore, the aim of this study was to evaluate the effects of zoledronic acid (ZOL), a highly potent, heterocyclic nitrogen-containing bisphosphonate, on odontoblast-like MDPC-23 cells by evaluating succinic dehydrogenase (SDH) enzyme production (cell viability – MTT assay), total protein (TP) production, alkaline phosphatase (ALP) activity, reverse transcriptase polymerase chain reaction (qPCR) for collagen type I (Col-I) and ALP, and morphology (scanning electron microscopy – SEM).

2. Material and methods

2.1. MDPC-23 cell culture

The odontoblast-like cells MDPC-23 used in this study were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich Corp., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and containing 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L glutamine (Gibco) in an humidified incubator with 5% CO_2 and 95% air at 37 °C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). The MDPC-23 cells in DMEM containing 10% FBS were sub-cultured at every 2 days until an adequate number of cells were obtained for the study, and then plated (1.5×10^4 cells/cm^2) onto sterile 24-well plates (Costar Corp., Cambridge, MA, USA), which were maintained in the humidified incubator with 5% CO_2 and 95% air at 37 °C for 48 h. Three groups were then established: one control group, which received no treatment, and two experimental groups, which were treated with ZOL at concentrations of 1 μM and 5 μM.

2.2. ZOL on MDPC-23 cell culture

Zoledronic acid (ZOL) was selected for investigation in the present study because it is a high potent bisphosphonate and, according to recent studies,[14,15] is one of the most frequently prescribed bisphosphonates. In addition, several workers reported that this nitrogen containing bisphosphonate may cause intense cytotoxic effects in different types of cell cultures, including pulp cells.[10,11,16]

After the 48-incubation period, the culture medium was aspirated and replaced by fresh FBS-free DMEM containing ZOL (Zometa 4 mg; Novartis Biociências S.A., São Paulo, SP, Brazil) at concentrations of either 1 μM or 5 μM, according to the group. These concentrations were chosen based on a study by Scheper et al.[17] who showed that ZOL can be found at these concentrations in the alveolar bone and saliva of patients under treatment with this drug. The culture medium with the drug remained in contact with the cells in the incubator with 5% CO_2 and 95% air at 37 °C for 24 h.

2.3. Analysis of cell viability (SDH production – MTT assay)

Cell viability was evaluated using the methyltetrazolium (MTT) assay.[18–20] This method determines the activity of SDH enzyme, which is a measure of cellular (mitochondrial) respiration, and can be considered as the metabolic rate of cells. After 24 h of incubation of the cells in contact with DMEM alone (control group) or containing the two ZOL concentrations (experimental groups), the culture medium was aspirated and replaced by 900 μL of fresh DMEM plus 100 μL of MTT solution (5 mg/mL sterile PBS). The cells were incubated at 37 °C for 4 h. Thereafter, the culture medium with the MTT solution was aspirated and replaced by 700 μL of acidified isopropanol solution (0.04 N HCl) in each well to dissolve the violet formazan crystals resulting from the cleavage of the MTT salt ring by the SDH enzyme present in the mitochondria of viable cells, producing a homogenous bluish solution. After agitation and confirmation of the homogeneity of the solutions, three 100 μL aliquots of each well were transferred to a 96-well plate (Costar Corp.). Cell viability was evaluated by spectrophotometry as being proportional to the absorbance measured at 570 nm wavelength with an ELISA plate reader (Thermo Plate, Nanshan District, Shenzhen, Gandong, China). The values obtained from the three aliquots were averaged to provide a single value. The absorbance was expressed in numerical values, which were subjected to statistical analysis to determine the effect of ZOL on the mitochondrial activity of the cells.

2.4. Total protein expression

Total protein expression was evaluated as previously described.[20] After 24 h of incubation of the cells in contact with DMEM alone (control group) or containing the two ZOL concentrations (experimental groups), the culture medium with ZOL was aspirated and the cells were washed three times with 1 mL PBS at 37 °C. An amount of 1 mL of 0.1% sodium lauryl sulphate (Sigma Aldrich Corp., St. Louis, MO, USA) were added to each well and maintained for 40 min at room temperature to produce cell lysis. The samples were homogenized and 1 mL from each well was transferred to properly labelled Falcon tubes (Corning Incorporated, Corning, NY, USA). One millilitre of distilled water was added to the blank tube. Next, 1 mL of Lowry reagent solution (Sigma Aldrich Corp.) was added to all tubes, which were agitated for 10 s in a tube agitator (Phoenix AP 56, Araraquara, SP, Brazil). After 20 min at room temperature, 500 μL of Folin-Ciocalteau's phenol reagent solution (Sigma Aldrich Corp.) were added to each tube followed by 10 s agitation. Thirty min later, three 100 μL aliquots of each tube were transferred to a 96-well plate and the absorbance of the test and blank tubes was measured

at 655 nm wavelength with the ELISA plate reader (Thermo Plate). Total protein production was calculated from a standard curve created using known protein concentrations.

2.5. ALP activity

Analysis of ALP activity was performed using the colorimetric endpoint assay (ALP Kit; Labtest Diagnóstico S.A., Lagoa Santa, MG, Brazil) employed in previous studies.[20] This test uses a thymolphthalein monophosphate substrate, which is a phosphoric acid ester substrate. ALP hydrolyzes the thymolphthalein monophosphate substrate, releasing thymolphthalein. Therefore, it is possible to measure directly the product of hydrolysis, altering the pH. The altered pH interrupts the enzymatic activity and provides bluish colour to the solution, which is characteristic of the reaction. The intensity of the resulting colour is directly proportional to the enzymatic activity and is analyzed spectrophotometrically. After 24 h incubation of the cells in contact with DMEM alone (control group) or containing the two ZOL concentrations (experimental groups), the culture medium with ZOL was aspirated and the cells were washed three times with 1 mL PBS at 37 °C. An amount of 1 mL of 0.1% sodium lauryl sulphate (Sigma Aldrich Corp.) were added to each well and maintained for 40 min at room temperature to produce cell lysis. The test was performed according to the instructions of the Kit's manufacturer. The absorbance of the samples was measured at 590 nm wavelength with a spectrophotometer (Thermo Plate). ALP activity was calculated by a standard curve using known concentrations of the enzyme.

2.6. Analysis of cell morphology by SEM

SEM analysis was used to identify possible morphological alterations caused by the addition of different concentrations of ZOL to DMEM culture medium in which the MDPC-23 cells were cultured. The following protocol used in previous studies was employed.[20,21] Sterile 13-mm-diameter cover glasses (Fisher Scientific, Pittsburgh, PA, USA) were sterilized in 70% ethanol for 24 h and placed on the bottom of the wells immediately before seeding the cells. After 24 h of incubation of the cells in contact with DMEM alone (control group) or containing the two ZOL concentrations (experimental groups), the culture medium with ZOL was aspirated and the cells were fixed in 1 mL of 2.5% glutaraldehyde in PBS for 1 h. Then, the glutaraldehyde was removed and the cells were washed with PBS and post-fixed with 1% osmium tetroxide for 1 h at room temperature. The cells that remained adhered to the glass substrate were washed with PBS and distilled water two consecutive times (5 min each) and then dehydrated in a series of increasing ethanol concentrations (30, 50 and 70%, one time for 30 min each; 95 and 100%, two times for 60 min each) and covered three times with 200 μL of 1,1,1,3,3,3-hexamethyldisilazane (HMDS; Sigma Aldrich Corp.) at 20 min intervals. The cells were mounted on metallic stubs, stored in a desiccator overnight, sputter-coated with gold, and their morphology was examined with a scanning electron microscope (JMS-T33A scanning microscope, JEOL, Tokyo, Japan).

2.7. Analysis of Col-I and ALP expression

2.7.1. RNA extraction and cDNA synthesis

The effects of ZOL on Col-I and ALP expression was evaluated after 48 h of contact of the drug with the cells by two-step real time polymerase chain reaction (qPCR), which is a sensitive and fast method to evaluate gene expression. Unlike conventional PCR, this technique needs a small number of samples, less methodological standardization and no contaminant reagents. Another advantage is that the amplification can be observed at any cycle, and no post processing of samples is required.[22]

For this test, the cell were transferred to microcentrifuge tubes to which 1 mL of trizol (Invitrogen, Carlsbad, CA, USA) was added to inhibit the action of RNAases and the cells were incubated for 5 min at room temperature. Next, 0.2 mL of chloroform was added for each 1.0 mL de trizol (Sigma Aldrich Corp., St. Louis, MO, USA) to promote release of the cytoplasmic proteins. The tubes were agitated manually for 15 s, left rest for 2–3 min at room temperature, and centrifuged at 1200 rcf (Microcentrifuge Eppendorf model 5415R, Eppendorf, Hamburg, Germany) for 15 min at 4 °C. After centrifugation, the samples presented three phases: a precipitated phase, corresponding to the organic portion (phenol, chloroform, DNA), an intermediate phase (proteins) and a more aqueous supernatant phase, corresponding to RNA (RNA and buffer).

The aqueous phase was aliquoted to a new tube, in which 0.5 mL of isopropanol (Sigma–Aldrich Corp.) was added for each 1.0 mL of trizol to promote precipitation of RNA in solution. The samples were maintained at room temperature for 10 min and then centrifuged at 12,000 rcf for 10 min at 4 °C. After this stage, formation of a precipitated fraction (pellet) was observed at the bottom of the tube. The supernatant fraction was discarded and the precipitated phase was dried by inverting the tubes onto a blotting paper sheet during 10 min. After drying, 1.0 mL of 75% ethanol (Sigma–Aldrich Corp.) was added for each 1.0 mL of trizol and the samples were agitated and centrifuged at 7500 rcf for 5 min at 4 °C. The supernatant fraction was discarded and the RNA was subjected to the same drying procedure for 30 min. Next, the RNA was resuspended in 10 μL of ultrapure water (Invitrogen) and the resulting solution was incubated at 55 °C for 10 min. Part of the obtained RNA (1.0 mL) was diluted in ultrapure water at 1:50 for quantification of RNA in an Eppendorf biophotometer (model Eppendorf RS-232C, Eppendorf, Hamburg, Germany).

cDNA was synthesized from each RNA sample for qPCR using the High Capacity cDNA Reverse Transcriptions Kit (Applied Biosystems, Foster City, CA, USA), according to the following protocol. In a microcentrifuge tube were added $10\times$ RT Buffer, $10\times$ RT Random Primers, $25\times$ dNTP Mix, reverse transcriptase and 0.5 μg of the RNA of each sample. The samples were then subjected to the following amplification cycling conditions: 25 °C (10 min), 37 °C (120 min), 85 °C (5 s) and 4 °C thereafter.

2.7.2. qPCR

After cDNA synthesis, the expression of the genes that encode for Col-I and ALP was evaluated by qPCR. For each gene,

specific primers were synthesized from the mRNA sequence (Table 1). The reactions were prepared with standard reagents for qPCR (Syber Green PCR Master Mix; Applied Biosystems) together with the primer/probe sets specific for each gene (Table 1). The fluorescence readings were performed using the Step One Plus System (Applied Biosystems) at each amplification cycle, and were analyzed subsequently using the Step One Software 2.1 (Applied Biosystems). All reactions were subjected to the same analytical conditions and were normalized by the ROX™ passive reference dye signal to correct fluctuations on reading resulting from variations of volume and evaporation during the reaction. The result, expressed in CT values, refers to the number of cycles necessary for the fluorescent signal to reach the detection threshold. The individual results expressed in CT values were recorded in worksheets, grouped according to the groups and normalized according to the expression of the selected endogenous reference gene (β-actin). Then, the RNAm concentrations of each target gene were analyzed statistically.

2.8. Statistical analysis

After analysis of data distribution (Shapiro-Wilk, $p > 0.05$) and homogeneity of variances (Levene, $p > 0.05$), cell viability (SDH production), TP production, ALP activity and Col-I and ALP expression data were independently subjected to one-way analysis of variance (*treatment*: control, 1 μM or 5 μM ZOL). Once rejected the null hypothesis of absence of differences among the groups, additional Tukey's tests were also applied for pairwise comparison. A significance level of 5% was set for all analyses.

3. Results

Data from SDH production, TP production and ALP activity are presented in Table 2. The use of 1 μM ZOL did not cause a significant ($p > 0.05$) reduction in SDH production compared with the control group. However, SDH production decreased significantly compared with the control group ($p < 0.05$) when ZOL concentration increased to 5 μM. No statistically significant difference was found between the 1 and 5 μM ZOL concentrations (Table 2).

Table 2 – Data from SDH production, TP production and ALP activity of MDPC-23 cells exposed to zoledronic acid (ZOL) at different concentrations.

Group	SDH production[a]	TP production[b]	ALP activity[b]
Control group	0.659 (0.094)[a]	284.47 (27.81)[a]	155.99 (45.11)[a]
1 μM ZOL	0.567 (0.204)[ab]	190.05 (17.73)[b]	86.60 (39.59)[b]
5 μM ZOL	0.450 (0.117)[b]	171.86 (30.03)[b]	87.02 (40.01)[b]

All values are given as mean (standard deviation), $n = 8$.
[a] Values expressed in absorbance (470 nm).
[b] Values expressed in U/L. Means followed by same letters in columns do not differ significantly (Tukey's test, $p > 0.05$).

Application of ZOL on the odontoblast-like cells caused a significant ($p < 0.05$) decrease in TP production and ALP activity (Table 2) compared with the control group. No statistically significant difference ($p > 0.05$) was found between the 1 and 5 μM ZOL concentrations (Table 2).

Col-I and ALP expression detected by qPCR are presented in Fig. 1. When the MDPC-23 cells were exposed to ZOL at 5 μM concentration, Col-I expression did not differ significantly ($p > 0.05$) from the control group in which the drug was not used. On the other hand, exposure to 1 μM ZOL stimulated the odontoblast-like cells to produce Col-I, leading to a statistically significant increase ($p < 0.05$) in its expression compared to the other groups. A statistically significant decrease ($p < 0.05$) in ALP expression was observed when the cells were exposed to 5 μM ZOL compared with the expression of this protein in the other groups (control and 1 μM ZOL).

The SEM analysis of the odontoblast-like cells MDPC-23 incubated in contact with ZOL revealed that both concentrations of the drug induced morphological alterations, especially reduction of cell size, which created large intercellular spaces and exposed the cover glass that served as substrate for cell culture. On the other hand, in the control group, the MDPC-23 cells were near confluence and had a wide cytoplasm covering the entire surface of the glass substrate (Fig. 2).
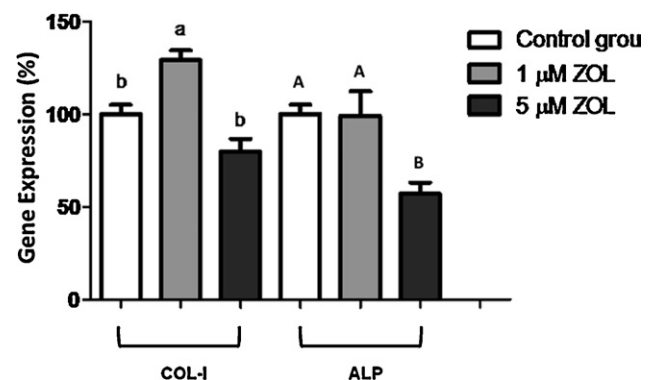

Fig. 1 – Col-I and ALP expression (qPCR) of MDPC-23 cells exposed to zoledronic acid (ZOL) at different concentrations. Columns represent means and error bars represent standard deviations, $n = 4$. Columns indicated with the same letters do not differ significantly (Tukey's test, $p > 0.05$).
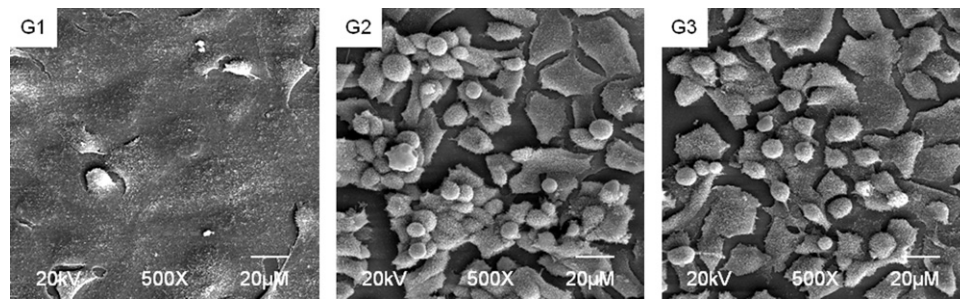
Fig. 2 – SEM micrographs showing the morphology of odontoblast-like cells MDPC-23 not treated (control group) and exposed to zoledronic acid at 1 μM or 5 μM. In the control group, cells are near confluence covering the entire surface of the glass substrate. In the experimental groups, the cells exhibited shrunken cytoplasm and loss of their normal morphology. In addition, it can be noted that cells detached from the glass substrate, leaving cell-free spaces. SEM, Original magnification 500×.

4. Discussion

Bisphosphonates have been indicated for treatment of osteopenic and osteoporotic conditions.[2] The high affinity of bisphosphonates for Ca^{2+} ions and their strong binding to hydroxyapatite promotes a rapid incorporation of these drugs to the tissues.[4] ZOL is a highly potent nitrogen-containing bisphosphonate that presents a prolonged adhesion to bone surface and effect, and has been widely used for various clinical conditions.[14]

A recent study[5] demonstrated that bisphosphonates may adhere to dentin because this mineralized dental tissue is very similar to those of bone tissue. This adhesion process may occur during odontogenesis, in children treated with these drugs during the formation and mineralization of dental tissues, as well as during physiological deposition of secondary dentin.[15]

Events that induce bone resorption or remodelling are capable of triggering the osteoclastic activity, resulting in adherence of the osteoclasts to the bone surfaces and decrease of local pH. The consequent loss of affinity between bisphosphonates and the mineralized tissue leads to drug release from the tissue.[23]

Regarding the oral cavity, some factors, such as progression of caries lesions, dental trauma and toxicity of dental materials may disorganize the odontoblast layer or even the pre-dentin, triggering and activating the action of local clasts, which starts the dentin resorption process.[13,24,25] The induction of these events in patients under bisphosphonate therapy may result in release of the drug adhered to dentin hydroxyapatite, intensifying the damages to the dentinopulpar complex. When bisphosphonates are released from dentin, the pulp odontoblasts are the first cell line exposed to these drugs because they underlies the dentin and are responsible for its formation and maintenance.[12,13]

A previous study[26] using dentin discs showed that bisphosphonates are capable to adhere to dentin, inhibiting its resorption. Other studies revealed that daily administration of bisphosphonates during periods of 7 and 14 days affected the mineralization of the dentin matrix[6] and that the infusion of some types of bisphosphonates in rats caused enamel and dentin malformations.[7] More recently, the findings of a bioassay showed that the ZOL concentrations found in the oral cavity of patients under treatment with this drug ranged from 0.4 to 5 μM.[17] Thereafter, some authors have demonstrated that this drug can be toxic to different cell types, such as osteoblasts, endothelial cells and fibroblasts.[10,11,16] This cytotoxic effect could be due to contact of high concentrations of bisphosphonates released from the mineralized tissues to the adjacent cells. A recent study[5] evaluating the cytotoxicity of ZOL to pulp cells *in vitro* showed that this drug caused a significant decrease of the viability, proliferation and TP production of these cells. These data were confirmed in the present study in which ZOL concentrations (1 μM and 5 μM) simulating those found in the alveolar bone tissue of patients under treatment with this drug,[17] caused reduction of cell viability.

In addition to the analysis of odontoblast-like cell viability, TP production and ALP activity, molecular biology experiments were also carried out in the present study, which indicated that Col-I and ALP expression can be inhibited in a dose-dependent by the action of ZOL. This inhibitory effect of ZOL could affect negatively the repair of the pulpo-dentin complex *in vivo*, as Col-I is the main component of reactionary dentin matrix, which is produced by odontoblasts that suffer aggressions[12,27] and ALP is directly involved in the mineralization of this newly formed dentin matrix.[28,29]

The present study demonstrated that ZOL at concentration of 1 μM increase Col-I expression. Similar result was also reported in previous studies that revealed an increase in the expression of this gene *in vitro* within the first days after contact of the drug with the cells.[30,31] However, Col-I expression decreased over time, suggesting that the inhibitory effect of ZOL was both dose- and time-dependent.[31]

The results of ZOL cytotoxicity to the odontoblast-like MDPC-23 cells demonstrated by the *in vitro* cellular and molecular biology protocols used in the present study were confirmed in the analysis of cell morphology by SEM. The cells incubated in contact with both ZOL concentrations presented size reduction, probably due to cytoskeletal shrinkage. This cell response pattern in contact with low toxic agents has been extensively described in the literature,[19,21] which helps establishing the effects of the tested drug. This is because

the decrease of cell viability indicated by the MTT assay might be due to a direct inhibitory effect of the drug on cell activity, which results in reversible morphological alterations, or to necrotic or apoptotic cell death, which represents an irreversible condition. In both situations, the MTT assay provides values that represent a smaller number of formazan crystals formed. However, SEM analysis of the morphology and number of cells that remained adhered to the glass substrate provides complementary data that indicate the actual effect of the drug. This way, the maintenance of the number of MDPC-23 cells and the discrete alterations in their morphology observed in present study demonstrate that in spite of presenting cytotoxic effects, ZOL did not cause direct cell death even at the higher concentration (5 μM). Perhaps, the same ZOL concentrations evaluated in the present study (1 and 5 μM) could cause more intense cytopathic effects, if maintained for a longer time in contact with the odontoblast-like cell cultures, as described by Koch et al.[31]

The effects of bisphosphonates on odontoblas-like cells could be related to the activation of different pathways, such as Mitogen-activated protein kinase (MAPK), Jun N- terminal kinase (JNK) as well as caspase pathways that regulate mitogenic activity, gene expression and apoptosis of cells.[17,32]

Further *in vitro* and *in vivo* studies are necessary to characterize the relationship between cytotoxicity and the concentration and contact time of ZOL with blast cells.

5.　Conclusions

Based on the methodology used in the present *in vitro* study and the obtained results, it may be concluded that ZOL at concentrations of 1 μM and 5 μM presented a dose-dependent cytotoxic effects to the odontoblast-like cells MDPC-23 and decreased the expression of typical dentin matrix proteins, suggesting that under clinical conditions the release of this drug from dentin may cause damage to the pulp–dentin complex.

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Competing interests

The authors declare no conflict of interests.

Ethical approval

Not required.

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