Zoledronic Acid Inhibits Human Osteoblast Activities

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Abstract

Background: Bisphosphonates are potent inhibitors of bone resorption. These kinds of drugs, which are used for the treatment of osteolytic diseases, have been associated with the occurrence of oral osteonecrosis, especially in patients over 60 years old. Current studies have demonstrated that the cytotoxic effects of bisphosphonates on osteoblasts play an important role in oral osteonecrosis development. Objective: The aim of this study was to evaluate the effect of long-term application of a highly potent bisphosphonate – zoledronic acid (ZA) – on human osteoblasts in vitro. Methods: Human osteoblasts (MG63 cell line) were seeded for 72 h in wells of 24-well plates. The Dulbecco’s modified Eagle’s medium (DMEM) was then replaced by culture medium without fetal bovine serum (FBS), and the cells were incubated for an additional 24 h, after which ZA was added to the DMEM without FBS and incubated in contact with osteoblasts for 7, 14 or 21 days. Cell viability (CV), total protein production (TPP), alkaline phosphatase (ALP) activity, mineral nodule formation (MNF), and gene expression of ALP and osteocalcin (OCN), as well as cell morphology by scanning electronic microscopy, were evaluated. Data were statistically analyzed by Kruskal-Wallis and Mann-Whitney tests, with a significance level of 5%. Results: The cytotoxic effects of ZA on osteoblasts were characterized by reduction of CV, TPP, ALP and MNF production. In addition, ZA MNF caused a decrease in gene expression of ALP and OCN, as well as intense cell morphology alterations. All these negative effects of ZA were concentration and period dependent. Conclusion: Both concentrations of ZA (1 and 5 μ M) caused cytotoxic effects to osteoblasts which reduced the production and expression of proteins that play an important role in bone matrix synthesis and mineralization.

Introduction

Bisphosphonates are synthetic drugs, derived from pyrophosphate, that have been widely used for the treatment of bone-resorptive diseases [1], which are more incident in patients over 60 years old [2, 3]. These drugs have also been recommended for adjunctive treatment of bone tumors and bone tumor metastasis [2, 4]. It is known that bisphosphonates act as bone metabolism regulators, mainly by inhibiting osteoclast activity and apoptosis, which cause decreased bone resorption [2, 4, 5].

Despite the positive effects of bisphosphonates on osteolytic diseases, these kinds of drugs can also interact...
with other cell types, like osteoblasts, fibroblasts and endothelial cells [6]. It has been reported that bisphosphonates may have cytotoxic effects on such cells, characterized by reductions in cell proliferation and viability, as well as decreased gene expression of growth factors and collagen type I [6–10].

Bisphosphonate-induced osteonecrosis has been described as an adverse side effect observed in patients subjected to long-term bisphosphonate therapy. This is mainly observed with zoledronic acid (ZA), a highly potent bisphosphonate with a high capacity for bone adhesion [11, 12]. In this situation, osteonecrosis may be caused by the intense toxic effects of this kind of drug attached to bone tissue on the surrounding osteoblasts. This negative toxic effect of ZA on bone cells is characterized by reductions in cell viability and gene expression, which, in turn, inhibit local bone healing [10, 13]. The etiology of osteonecrosis has also been related to the cytotoxic effects of bisphosphonates on oral mucosal adjacent cells, such as fibroblasts and epithelial cells, interfering with local oral mucosal regeneration [14, 15].

In fact, the elucidation of the etiopathogenesis of osteonecrosis is crucial, since the incidence of this pathological condition increases with time. Therefore, patients over 60 years old may experience life quality greatly compromised by osteonecrosis. In addition, older patients also frequently show other diseases, such as diabetes mellitus and thrombotic disorders, which can potentiate the risk of osteonecrosis development or even exacerbate this condition [2, 3, 16–19].

In the last few years, several studies have aimed to evaluate the effects of bisphosphonates on different cell types, such as osteoblasts [20, 21]. In these studies, the authors evaluated at short time intervals the viability, gene expression and mineralization capacity of these cells in an isolated way, which hampers the comparison of results. In addition, some of these studies did not use human cell lines. Therefore, the aim of the present study was to evaluate the effects of ZA applied for different periods on cultured human osteoblasts by assessing their cell viability, morphology, gene expression and mineralization capacity.

Methods

Cell Culture

A human osteoblast MG63 cell line (CRL-1427; ATCC, Manassas, Va., USA) was cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Grand Island, N.Y., USA) with 10% fetal bovine serum (FBS; GIBCO), and 100 IU/ml, 100 μg/ml and 2 mM of penicillin, streptomycin and glutamine, respectively (GIBCO).

Effects of ZA on Osteoblasts

Table 1. Distribution of groups according to ZA concentrations and evaluation periods (groups 1, 4 and 7 were used as controls for each ZA concentration used and each period of evaluation)

<table>
<thead>
<tr>
<th>Group</th>
<th>ZA concentration, μM</th>
<th>Period, days</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>7</td>
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<td>3</td>
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<td>7</td>
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<tr>
<td>8</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>21</td>
</tr>
</tbody>
</table>

The cells were incubated with 5% CO₂ at 37°C atmosphere (Thermo Plate, Fisher Scientific, Pittsburgh, Pa., USA) [22].

For the experiments, cells were seeded (3 × 10⁴ cells/cm²) in wells of 24-well plates in complete DMEM containing β-glycerol phosphate and ascorbic acid (Sigma-Aldrich, St. Louis, Mo., USA). After a 72-hour incubation period, the complete DMEM was replaced by new DMEM without FBS, and the cells were incubated for an additional 24 h. Then, two different concentrations of ZA (1 and 5 μM) were added to DMEM without FBS (table 1) and the cells were incubated in contact with these ZA solutions for 7, 14 or 21 days. During these periods, the ZA solutions were replaced every 72 h. Finally, the osteoblasts were evaluated for cell viability (CV), total protein production (TPP), alkaline phosphatase (ALP) activity, mineral nodule formation (MNF), and gene expression of AP and osteocalcin (OCN), as well as cell morphology (scanning electronic microscopy), as described below.

Cell Viability: MTT Assay

Cell viability was determined by MTT assay, which evaluates the respiratory activity of viable cells [22, 23]. For this assay, a 900-μl quantity of DMEM without FBS and a 100-μl quantity of MTT solution (5 mg/ml of MTT salt; Sigma-Aldrich) was then added to each sample (n = 8) for 20 min, followed by Folin and Ciocalteau’s Phenol Reagent (Sigma-Aldrich). After a 72-hour incubation period, the complete DMEM was replaced by new DMEM without FBS, and the cells were incubated for an additional 24 h. Then, two different concentrations of ZA (1 and 5 μM) were added to the samples (n = 8) and incubated for 4 h. Then, the MTT solution was aspirated and the formazan crystals were dissolved with 600 nl of acidified isopropanol (0.04 N of HCl). Cell viability was determined by absorbance of the purple solution obtained from crystal dissolution in a spectrophotometer at 570 nm (Thermo Plate, Nanshan District, Shenzhen, China). The protocol was performed in triplicate.

Total Protein Production

TPP was evaluated as previously described [22]. After ZA treatment for experimental periods, cells underwent lysis with 0.1% sodium lauryl sulfate (Sigma-Aldrich) for 40 min. Lowry Solution (Sigma-Aldrich) was then added to each sample (n = 8) for 20 min, followed by Folin and Ciocalteau’s Phenol Reagent (Sigma-Aldrich). After 30 min, the samples were aliquoted and protein production was determined by the solutions’ absorbance at 655 nm (Thermo Plate) [22]. Protein concentration was obtained based on a standard curve with known protein concentrations. The protocol was performed in triplicate. The TPP results from each sample were used in order to normalize ALP activity results.
ALP Activity

ALP activity was assessed (n = 8) according to an end-point kit protocol (Labtest Diagnóstico S.A., Lagoa Santa, Brazil) [24]. This assay is based on dephosphorylation of thymolphthalein by ALP.

ALP was determined based on the solution’s absorbance at 590 nm (Thermo Plate). For this test, a standard curve with known concentrations of ALP was used to determine ALP activity. The protocol was performed in triplicate.

Mineral Nodule Formation

The formation of mineral nodules characterizes the mineralization capability of osteoblasts [21]. For this evaluation, Alizarin Red stain was used, as described below.

Cells were fixed in 70% ethanol for 1 h, after which Alizarin Red solution (40 nm; pH 4.2; Sigma-Aldrich) was added to the samples (n = 8) for 20 min with shaking. The samples were washed twice with distilled water. For quantitative analysis by absorbance evaluation, nodules were solubilized with 10% cetylpyridinium chloride (Sigma-Aldrich) for 15 min with shaking. Absorbance was determined at 562 nm (Thermo Plate).

MNF of each group was determined by means of a light microscope (Olympus BX51; Olympus, Miami, USA) equipped with a digital camera (Olympus C5060; Olympus). The protocol was performed in triplicate.

Gene Expression of ALP and OCN

To complement the data on mineralization capacity of cultured osteoblasts after treatment with ZA for 7-, 14- and 21-day periods, gene expression of ALP and OCN was evaluated by real-time PCR. These genes are considered osteoblast and mineralization markers [22]. In this protocol, RNA extraction and cDNA synthesis were performed as previously described [22]. RNA isolation was performed by Trizol method, according to the manufactures’ instructions. The RNA concentration was evaluated by means of a BioPhotometer (Eppendorf, Hamburg, Germany) at a proportion of 1:49. Analysis of the RNA concentration was used to standardize the amount of RNA of each sample for cDNA synthesis. The cDNA was obtained with the use of the High Capacity cDNA Reverse Transcriptions Kit (Applied Biosystems, Foster City, Calif., USA), following the recommended protocol [25°C (10 min), 37°C (120 min), 85°C (5 s), 4°C].

Gene expression was evaluated by real-time PCR (qPCR) [22]. Specific primer sets were used for each selected gene (table 2). The reactions were prepared with standardized qPCR reagents (SYBR® Green PCR Master Mix; Applied Biosystems) associated with the primer sets for each gene. Fluorescence was determined by Step One Plus (Applied Biosystems) at each amplification cycle and analyzed by Step One Software 2.1 (Applied Biosystems). All reactions were subjected to the same conditions and normalized by ROX fluorescence to reduce artifacts caused by sample evaporation and volume differences. The results were obtained as CT values representing the numbers of amplification cycles needed to achieve detection for each sample. These results were grouped by ZA concentrations and periods of treatment, and normalized by an endogenous control gene (RPL13). The protocol was performed in triplicate.

Cell Morphology Analysis

Cell morphology was evaluated by scanning electron microscopy as in previous studies [22, 24, 26]. Sterilized round glasses (13 mm in diameter) were individually placed at the bottoms of wells of 24-well plates. The cells were then seeded and treated as described above (n = 2). After 7, 14 and 21 days, the ZA solutions were aspirated and the cells attached to the round glass substrate were rinsed with phosphate buffer. The cells were then fixed with 2.5% glutaraldehyde solution (Sigma-Aldrich) for 1 h at room temperature and postfixed with 1% osmium tetroxide for 1 h. After that, cells were treated with different concentrations of ethanol for 30 min each (30, 50 and 70%). The cells were maintained in 95 and 100% ethanol solutions for 60 min each and then treated for 20 min with 200 μl of 1,1,1,3,3,3 hexamethyldisilazane (HMDS; Sigma-Aldrich; 3 times).

The round glasses containing cells were removed from the wells, set on metallic stubs and maintained in a desiccator overnight. Before analysis, the samples were covered with gold and analyzed by scanning electronic microscopy (JEOL-JMS-T33A Scanning Electron Microscope, JEOL USA Inc., Peabody, Mass., USA).

Statistical Analysis

CV, TPP, MNF and qPCR data were analyzed by Kruskal-Wallis tests complemented by Mann-Whitney tests for comparison of different concentrations of ZA and different treatment periods. Statistical significance was set at 5%.

Results

Cell Viability: MTT Assay

The results of cell viability evaluation by MTT assay of osteoblasts treated with ZA for 7, 14 and 21 days showed that at 7 days both concentrations of ZA significantly decreased cell viability compared to that in the control group (p < 0.05; fig. 1). No difference in cell viability in the control groups at 7 and 14 days was observed (p > 0.05). In the 21-day control group, a significant increase of cell viability was observed compared to that in the 7- and 14-day periods (p < 0.05).

Both concentrations of ZA (1 and 5 μM) decreased cell viability at the 7- and 14-day periods in a concentration-dependent manner. At the 21-day period, both ZA concentrations caused an intense decrease in cell viability (p > 0.05).

Table 2. Primer sequences of each selected gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
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<tr>
<td>ALP Forward: 5'-GACAAGAAGCCCTTCACTGC-3'</td>
<td>Reverse: 5'-GAAGCGGGGCACTTGTTGTG-3'</td>
</tr>
<tr>
<td>OCN Forward: 5'-GCGCTACCTGTATCAATGG-3'</td>
<td>Reverse: 5'-TCCGCAACTCGTCACAGTC-3'</td>
</tr>
<tr>
<td>RPL13 Forward: 5'-CGCTGCTGGACCGCTCACA-3'</td>
<td>Reverse: 5'-CCTGGGTACTTCCAGCAAACCT-3'</td>
</tr>
</tbody>
</table>
Both ZA concentrations reduced cell viability in a period-dependent manner. For the 1-μM ZA solution, no statistical difference between the 7- and 14-day periods was observed (p > 0.05). However, for the 5-μM ZA solution, the cell viability reduction was significant for all treatment periods (p < 0.05). No difference was observed between the 14- and 21-day periods (p > 0.05). Besides cell viability, other cell functions were compromised by prolonged ZA treatment.

**Total Protein Production**

The TPP was evaluated by a Lowry’s modified method and demonstrated the total amount of protein production by osteoblasts treated with ZA for the different periods proposed. Regarding the control group, the TPP was only increased at 21 days after cell culture compared to that at the 7- and 14-day periods (p < 0.05). When the cells were treated with 1 μM ZA solution, a significant decrease in TPP occurred only after 21 days of treatment, compared to that after the 7- and 14-day periods (p < 0.05; fig. 2). Results of TPP were also used to normalize ALP activity data of cultured osteoblasts treated with ZA.

**ALP Activity**

ALP is an enzyme strictly related to matrix mineralization and the activity of this enzyme was evaluated by an end-point assay. Results showed that an increased ALP activity was observed only when the osteoblasts were treated for 7 days with 5 μM ZA solution (p < 0.05). At 14 days, both ZA concentrations (1 and 5 μM) significantly decreased the ALP activity compared to the control group (p < 0.05). At the 21-day period, no difference was found in ALP when the experimental and control groups were compared (p > 0.05).

Considering both ZA concentrations used in this study, no difference was observed when osteoblasts were treated with only 1 μM of ZA in all experimental periods (p > 0.05). However, treatment with 5 μM of ZA solution caused significant ALP reduction at the 14- and 21-day periods (fig. 3).

**Mineral Nodule Formation**

The mineralization capacity of cultured osteoblasts treated with ZA was also evaluated by mineral nodules formation, by Alizarin Red assay. In general, the MNF observed in this study was concentration and time dependent. In control groups, a significant MNF increase occurred with time. In those groups in which the cells were exposed to ZA, a decrease of MNF was observed compared to that in the control groups (p < 0.05). However, independent of the ZA concentration applied to the cells, the increase of nodule formation was directly related to the periods of treatment (fig. 4, 5).

Mineralization capacity and differentiation of osteoblasts were confirmed by evaluation of gene expression of ALP and OCN.
Gene Expression of ALP and OCN

Gene expression determined by real-time PCR demonstrated no different OCN expression for control groups at different periods (p > 0.05). Independent of the ZA concentration used in this study (1 or 5 μM), a significant decrease in OCN expression occurred at the 21-day period (fig. 6).

Decreased ALP gene expression was observed at the 7-day period (for the 5-μM ZA solution) and the 14-day period (for the 1-μM ZA solution), which was concentration dependent. At the 21-day period, ALP expression was almost none for both ZA concentrations evaluated, with no statistically significant difference between them (p > 0.05; fig. 7).

Cell Morphology Analysis

The evaluation of cell morphology by scanning electron microscopy is a qualitative analysis that allowed the observation of cultured osteoblasts after prolonged treatment with ZA. Culture of human osteoblasts exposed to ZA at 1- and 5-μM solutions caused intense alterations in the morphology of those cells that remained attached to the glass substrate. It was also observed that a number of cells lethally affected by the ZA added to the culture medium detached from the substrate. At 14- and 21-day periods of cell exposure to ZA solutions, the alterations in cell morphology were more evident than those observed at 7 days. In addition, more dead cells detached from the substrate, which exhibited greater cell-free areas. In control groups, numerous osteoblasts with normal morphology were attached to the glass substrate (fig. 8).

Fig. 3. ALP activity of osteoblasts (MG63) treated with ZA at 1 and 5 μM for 7, 14 and 21 days. Letters allow comparison among groups within each period of evaluation. Groups represented by the same letter are not statistically different (Mann-Whitney, p > 0.05).

Fig. 4. MNF (Alizarin Red stain) by cultured human osteoblasts (MG63) treated with ZA at 1 and 5 μM for 7, 14 and 21 days. Letters allow comparison among groups within each period of evaluation. Groups represented by the same letter are not statistically different (Mann-Whitney, p > 0.05).

Fig. 5. General view of in vitro MNF after treatment of osteoblasts with ZA at different concentrations (1 and 5 μM) for 7, 14 and 21 days. 7 days (a); 14 days (b); 21 days (c): control group (1); ZA 1 μM (2); ZA 5 μM (3).
Discussion

Therapies with bisphosphonates have been related to the occurrence of oral osteonecrosis, which is frequently found in patients over 60 years old. This fact can be associated with the increased prescription of these kinds of drugs to this group of patients, or even with other local and systemic factors that have already been related to the osteonecrosis etiopathogenesis, such as administration of drugs that: (1) inhibit local vascularization [27], (2) increase bone loss and impair bone remodeling [28, 29], and (3) increase local bacterial colonization [30], even in clinical situations in which there is local persistent trauma caused by a removable dental prosthesis [31].

Recent studies have shown that the etiopathogenesis of this condition is more frequent in patients under nitrogen-containing bisphosphonate, which present a higher potency, due to the increased capacity of adhesion to mineral tissues [11, 12]. Therefore, the authors selected ZA for this study, considering that ZA is the most potent of all nitrogen-containing bisphosphonates and has been highly related to osteonecrosis development. Considering the bisphosphonates-treated bone diseases, this study also considered the cancer patients under bisphosphonate treatment, for whom, in most cases, ZA is prescribed [1].

Several studies have evaluated the effects of bisphosphonates, including ZA, on cell cultures [6, 8, 9]. Several authors have demonstrated that the cytotoxicity of ZA plays an important role in the development of osteonecrosis.
tensive cytotoxic effect of ZA at 5 μM, which was confirmed by previous studies. The absence of differences in cell viability between control groups and osteoblasts exposed for 14 days to ZA at 10 and 100 nM, and 1 and 10 μM decreased the numbers of viable cells by 20, 30, 40 and 90%, respectively. These results were further corroborated by Walter et al. [6], who evaluated the negative side effects caused by ZA on cultured endothelial cells, fibroblasts and osteoblasts. In contrast, Im et al. [25] showed that low concentrations of bisphosphonates, such as 10^{-4} M and 10^{-12} M, can increase osteoblast proliferation. However, the bisphosphonate concentrations used by Im et al. [25] were significantly lower than those determined in serum levels, saliva, or even bone tissue of patients subjected to therapy with these kinds of drugs [8]. Consequently, analysis of the data presented in this in vitro study, as well as those presented in previous investigations, confirms that the negative side effects caused by bisphosphonates, such as ZA, depend on both the length of therapy and the concentration of the drug.

In the present study, osteoblasts exposed for 7 days to ZA solution at 5 μM also exhibited ALP activity reduction. Similar data, which were also observed in a previous study [20], reflect the osteogenic differentiation of cultured osteoblasts, despite the environmental conditions available. However, intense ALP reduction occurs with time (14- and 21-day periods), indicating that specific concentrations of ZA can inhibit osteoblast differentiation and its mineralization capacity, as previously demonstrated [9, 20, 21]. In addition, it was shown in the present study that both ZA concentrations (1 and 5 μM) inhibited MNF in a concentration-dependent manner. In control groups, the osteoblasts that were not exposed to ZA presented an increase in MNF over time, which corroborated the results of previous studies [20, 21].

As in this study, Simon et al. [9] demonstrated a reduction in Col-I expression by osteoblasts exposed to ZA at different concentrations. Regarding ALP and OCN expression relative to matrix mineralization, Im et al. [25] also observed that ZA treatment for 7, 14 and 21 days caused decreased expression of these genes. The decreased gene expression of these three proteins may impair bone formation and remodeling [9, 25]. All these negative side effects of ZA on osteoblast activities may be clinically related to the development and evolution of osteonecrosis, affecting the life-quality of patients under bisphosphonate therapy.


**Conclusions**

According to the protocols used in this in vitro study, it can be concluded that ZA caused cytotoxic effects on cultured human osteoblasts in a dose-dependent manner and a decrease of the mineralization capacity of these cells, which are directly involved in bone matrix synthesis and mineralization processes.

**Acknowledgements**

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**References**