Dental Traumatology 2006; doi: 10.1111/j.1600-9657.2005.00434.x

DENTAL TRAUMATOLOGY

Cytotoxicity evaluation of sodium alendronate on cultured human periodontal ligament fibroblasts

Correia VFP, Caldeira CL, Marques MM. Cytotoxicity evaluation of sodium alendronate on cultured human periodontal ligament fibroblasts. © Blackwell Munksgaard, 2006.

Abstract – External root resorption processes are usually associated with dental trauma, mainly avulsion and intrusion. In such cases endodontic therapy aims to prevent this process by using medications that can inhibit osteoclastic activity, such as bisphosphonates. However, these drugs must be biocompatible to the periapical tissues. The aim of this study was to analyze the cytotoxicity of a bisphosphonate (sodium alendronate) on human periodontal ligament fibroblasts (PDL cells). Cells were plated in a density of 1×10^3 cells per dish. The experimental groups were GI (control) no sodium alendronate, and GII, GIII, and GIV with sodium alendronate at the concentrations of 10^{-5}, 10^{-6}, and 10^{-7} M, respectively. The experimental times were 1, 6, 12, and 24 h (short-term) for viability and 2, 4, 6, and 8 days (long-term) for cell survival. Data in triplicate were statistically analyzed. Cultures treated with the highest alendronate concentration (GII) showed cell viability percentages significantly lower ($P < 0.01$) than those of the other groups (GI, GIII, and GIV), at 12 and 24 h. Cell growth on GII and GIII groups was similar. GII presented smaller growth than the other groups ($P < 0.05$). We concluded that sodium alendronate, on direct contact with human periodontal ligament fibroblasts, is cytotoxic in concentrations higher than of 10^{-6} M.

Vera de Fátima Padrão Correia, Celso L. Caldeira, Márcia Martins Marques

Department of Endodontics, School of Dentistry, University of São Paulo, São Paulo, Brazil

Key words: root resorption; bisphosphonates; sodium alendronate; cell culture; intracanal medication

Márcia Martins Marques, Departamento de Endodontia, Faculdade de Odontologia, Universidade de São Paulo, Av. Prof. Lineu Prestes, 2227, 005508-900 São Paulo SP, Brazil
Tel.: +55 11 30917839 ext 27
Fax: +55 11 30917839 ext 27
e-mail: mmmarques@usp.br

Accepted 15 August, 2005

Dental trauma is a common occurrence in young patients. The most common physical dental trauma is crown fracture, with favorable prognosis. However, avulsion and intrusive luxation present more severe consequences for the teeth and periodontal tissues. The most alarming consequence observed is dental root external resorption (1).

Damages, according to Trope (2), that are caused to periodontal and pulp structures can result in three different kinds of dental root resorption: superficial, inflammatory, and by substitution. Superficial resorption does not require treatment because it is a repair process that occurs after low intensity injuries to the periodontal structures. However, inflammatory and substitutive resorptions require therapy with intracanal medication in order to avoid or control osteoclast activity.

Osteoclast inhibition could improve the prognosis of tooth replantation, however, nowadays therapies for tooth avulsion are only able to delay rather than inhibit the resorption process (3). Some of these therapy modalities are based on the use of calcium hydroxide (4), association of antibiotics and corticosteroids (5), and calcitonin (6). More recently, Levin et al. (3) employed an anti-osteoclastic drug in order to inhibit the root resorption of replanted dog teeth. This drug, alendronate, which is a third generation bisphosphonate with demonstrated oste-

oclast inhibitory activity, enhanced healing and preservation of tooth mass subsequent to replantation (3).

Bisphosphonates are substances analogous to pyrophosphate and are able to reduce the bone mineral hydroxiapatite dissolution index, preventing the bone resorption process by inhibiting osteoclastic resorption activities (7). They are used as therapeutic agents on hyper resorptive bone diseases such as Paget's disease, hypercalcemia of malignancy and osteoporosis (8).

Based on the anti-osteoclastic characteristic of the sodium alendronate, the use of this drug in the therapeutic approach to dental trauma is promising. But, for this purpose sodium alendronate must be biocompatible to the periodontal tissue. Before testing this drug *in vivo*, the aim of this study was to evaluate the sodium alendronate cytotoxicity on human PDL cells in culture.

Materials and methods

The cytotoxicity of three different concentration solutions of sodium alendronate (10^{-5}, 10^{-6}, and 10^{-7} M) was measured *in vitro*. Human periodontal ligament (PDL) cells (9) were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO, USA) supplemented by 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% antibiotic–antimycotic solution (Sigma) in a 5% CO_2 atmosphere with 95% humidity. The experimental groups were: (I) control, cultures grown on fresh DMEM; (II) DMEM containing 10^{-5} M sodium alendronate; (III) DMEM containing 10^{-6} M sodium alendronate; and (IV) DMEM containing 10^{-7} M sodium alendronate. Both an immediate or short-term response and a long-term survival that measured the retention of the self-renewal capacity of the cells were analyzed.

Morphological analysis

The overall morphology of the cultures of all groups was monitored throughout the experimental time using phase microscopy. Additionally, the individual morphology of the cells, as well as the presence of both living and dead cells was analyzed. Phase photomicrographs were obtained form a phase inverted microscope TMS (Nikon Co., Tokyo, Japan).

Short-term assay (cell viability)

Cells (1×10^4) were plated on 35-mm Petri dishes. Three days later, the experimental culture medium was exchanged for the medium containing the dilutions of the testing solution (GII, GIII, GIV), while the control cultures (GI) received fresh medium. The media inside the dishes were discarded and replaced by the solutions diluted in fresh DME medium containing 10% fetal bovine serum. After 0, 6, 12, and 24 h the cells of three dishes per group were counted, and viability curves were plotted. Because cultured cells can be prepared in a uniform suspension in most cases, the provision of large numbers of replicates is often unnecessary. Usually three replicates are sufficient, and for many simple observations, e.g. cell counting, duplicates may be sufficient (10).

Long-term assay (cell survival)

Cells (1×10^3) were plated on 35 mm diameter culture dishes. After 6 h, when the cells were all attached, the culture medium was exchanged with culture medium containing the dilutions of the test solutions (GII, GIII, GIV) while the control cultures (GI) received fresh DMEM. The attached cells were harvested from the dishes 1, 2, 4, 6 and 8 days after seeding, using a 0.25% trypsin solution (Sigma). The cells in suspension were then counted and growth curves were plotted. Maintenance of cell viability was obtained by exchanging half of the culture medium with fresh DMEM every other day.

Growth and viability cell curves

Growth curves were made as described in other studies (10–12). Briefly, the cell number was determined by counting the viable cells in a haemocytometer using the Trypan blue dye exclusion assay. For each period, cells from three dishes of each group were harvested using 0.25% trypsin solution and then, these cells in suspension were counted. The number of viable cells harvested from each Petri dish was obtained by the following mathematical equation: $UC \times D \times 10^4/\#SQ$, where UC = unstained cell count (viable cells), D = the dilution of the cell suspension, and #SQ = number of squares of the hemocytometer counted. The viability percentage of the cell population of each Petri dish was obtained by applying the following mathematical equation: $UC/TC \times 100$, where UC = unstained cell count (viable cells) and TC = total cell count (stained plus unstained cells).

Statistical analysis

Each data point corresponded to the mean ± standard error of mean (SEM) of either the percentages of cell viability or the cell number from three dishes. The data were compared by ANOVA complemented by the Tukey's test. The level of significance was 5% ($P \leq 0.05$).

Results

Morphological analysis

The morphological aspects of the human PDL cells under the influence of sodium alendronate in various concentrations (10^{-5}, 10^{-6}, and 10^{-7} M) at 8 days are shown in Fig. 1.

The usual morphological aspects of the human PDL cells are depicted in Fig. 1a. These control cells were fusiform and arranged as bundles in different directions. When under the action of sodium alendronate at 10^{-5} M (Fig. 1b) the cells had their cytoskeleton disrupted and assumed a globular shape detaching from the substrate. Most cells were clumped and weakly attached to the substrate.

Figure 1c shows the culture of PDL cells under the effect of 10^{-6} M sodium alendronate solution. These cells lost their fusiform shape and assumed a triangular shape with some cytoplasmic extensions. Some cells detached from the substrate. Under the sodium alendronate at 10^{-7} M concentration the cultures of PDL cells present the morphological aspects of control cells after 8 days in contact with the substance (Fig. 1d).

Short-term assay (cytotoxicity)

The percentages of cell viability in the short-term assay are illustrated in Fig. 2. Control cells (GI) had stable cell viability around 90% in the first 24 h. PDL cells treated with sodium alendronate at 10^{-5} M concentration presented significantly smaller percentages of cell viability than control cultures after 6 h of contact with the cells. Groups III and IV presented similar cell viability percentages as control cells (GI).

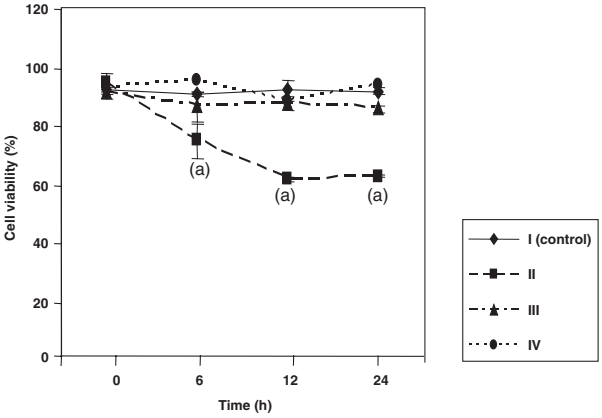

Fig. 2. Graphic representation of the viability curves of PDL cells in the short-term assay. Note the decrease of cell viability in 6 and 24 h at the GII treated with sodium alendronate at 10^{-5} M. Results are the mean ± SEM of three experiments. (a) Significantly different from control (GI).

Long-term assay

The cell growth curves are shown in Fig. 3. After 4 days in contact with different concentrations of sodium alendronate (GII, GIII, GIV) or with fresh medium (GI) the PDL cell cultures presented increased cell growth. Culture treated with sodium

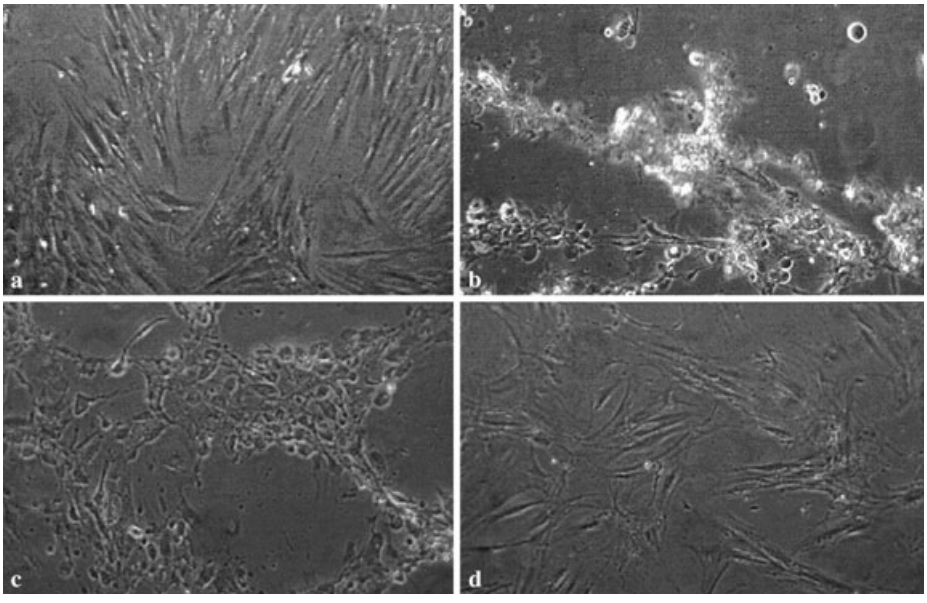

Fig. 1. Phase photomicrographs of control PDL cells (a, GI) and cells treated with sodium alendronate at 10^{-5} M (b, GII), 10^{-6} M (c, GIII) and 10^{-7} M (d, GIV). Observe the usual shape of the fibroblasts (a, d) and the alterations of cytoskeleton (b, c). Cells from GII are detached from the substrate (b).

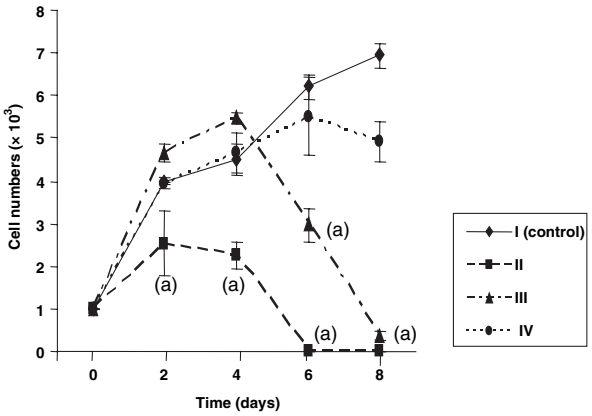

Fig. 3. Graphic representation of the cell growth curves of PDL cells in the long-term assay. Note that the cultures of GI and GIV present progressive cell growth. Cultures treated with sodium alendronate at 10^{-5} M (GII) present decrease in cell growth after 2 days in culture and reach total cell death in 6 days. Cultures treated with sodium alendronate at 10^{-6} M (GIII) present decrease in cell growth after 4 days in culture and reach total cell death in 8 days. Results are the mean ± SEM of three experiments. (a) Significantly different from control (GI).

alendronate at 10^{-5} M concentration (GII) presented diminishing in growth reaching total cell death at the sixth day. Before that the cell numbers of this group were significantly smaller than the other groups from the second to the eighth day.

Cultures treated with sodium alendronate at 10^{-6} M concentration (GIII) also presented a decrease in cell numbers after 4 days in culture, reaching total cell death at the 8th day.

Cultures treated with sodium alendronate at 10^{-7} M concentration (GIV) and control cells (G I) presented progressive cell growth reaching confluence after 6 days in culture.

Discussion

Faced with more severe dental traumatic injuries, such as intrusion and avulsion, there is an inflammatory response that causes damaged tissue resorption. This resorption process is directly associated to PDL inflammatory response that exposes dentin and promotes a direct communication with the pulp through dentinal tubules. Damage to the PDL, whether because of mechanical trauma, dehydration or others, affects the viability of the PDL cells, worsens the prognosis for teeth replantation. In replacement resorption, the resorbing cells may be stimulated by parathyroid hormone (PTH), which mediates root resorption in the same way as it mediates normal bone remodeling. The root is progressively resorbed and the alveolar bone fuses with the dental hard tissues. This may occur because of a mistake by clastic cells, which are unable to distinguish between cementum, dentin and bone once the organic coating of the root is removed (13). There is currently no effective treatment for dento-alveolar ankylosis and replacement resorption.

Inflammatory resorption treatment is related to inflammatory reaction control and the removal of the maintaining agent of this process. With knowledge about the processes that culminate and hold back resorption, it is possible to determine more adequate clinical conduct associated with the use of medications that are able to reach damaged areas and block or even inhibit the resorption process. According to Vanderas (14), although numerous substances have been proposed for such cases the ideal intracanal medication has not been found yet. However, the substance is not the only factor that needs to be accounted for, the correct canal preparation is also important, using instruments and chemical substances that promote an increase in root dentin permeability and allow a means of medication diffusion so as to reach the external root surface.

To accomplish an endodontic therapeutic success it is first necessary to control possible contamination through the use of medication that maintains the sanitation of the region obtained during chemical-surgery preparation. Second, the medication used must be biocompatible and effective on the resorption control process action.

The selection of drugs used in the intracanal medication phase, in cases of avulsion, has presented many difficulties in that the resorption processes are extremely challenging to control. Faced with this, the need to develop methodologies that are able to evaluate the cytotoxicity of substances and drugs used during endodontic therapy becomes clear.

The objective of the present study was to analyze the sodium alendronate cytotoxicity *in vitro* using cell cultures originated from human periodontal ligament fibroblasts (PDL cells). Cytotoxicity tests using cells in culture are fundamental for understanding the biological behavior of dental materials as there are considerable limitations to methods and the interpretation of results involving cell cultures from other animals (15).

Among bisphosphonates, sodium alendronate is one of the most potent osteoclastic activity inhibitors. Where bone metabolism is higher, in regions of elevated physiologic activity, bisphosphonates concentrate themselves in a selective form and after being absorbed, they link to exposed bone hydroxiapatite in regions of resorption. The mechanism of osteoclast inhibition has been attributed to a decrease in osteoclastic activity with minimal effects on recruitment, the interference of receptors on the osteoclasts for specific bone matrix proteins,

promoting the production of osteoclast inhibitor by osteoblast, which reduces the lifespan and/or the number of differentiated osteoclasts, and the obstruction of resorption by interfering in the ruffled border of the osteoclast (16).

In our experiment, we used three different sodium alendronate concentrations: 10^{-5}, 10^{-6}, and 10^{-7} M. These concentrations were based on the results observed in a pilot study where concentrations higher than 10^{-5} M showed extreme cytotoxicity. Although the results obtained by Kum et al. (17) demonstrated that osteoclast formation was significantly inhibited at alendronate concentration of 10^{-5} M. These results, as well as the results of our experiments, revealed cytotoxicity directly proportional to the sodium alendronate concentration. Both results confirm those of earlier research carried out by Sommercorn et al. (18), who showed that a concentration level of 10^{-5} M is cytotoxic to osteoblasts.

Other studies are in agreement with our results. Evaluations carried out by Liewhr et al. (13) which analyzed osteoclastic viability got a significant decrease in dentin resorption using 10^{-5} and 10^{-6} M concentrations of two bisphosphonates, while Garcia-Moreno et al. (19), found that for alendronate concentrations of 10^{-4} M or higher no viable cell was seen on osteoblastic cultures.

Although cytotoxic to PDL cells in culture, the sodium alendronate and other bisphosphonates, as observed in a number of studies in the literature (3, 8, 19–21), have demonstrated beneficial effects on osteoclastic resorption protection caused by periodontites or on mucoperiostal flap procedures. Additionally, considering the binding kinetics of this drug for hydroxyapatite, including a half-life of 10 years in humans, topically applied, dentin bound alendronate could exert a long-term effect on the inhibition or retardation of root resorption even if ankylosis is present.

Based on our results and supported by results of other research analysis, we suggest that the local administration of sodium alendronate solution at a concentration of 10^{-7} M, that is non-cytotoxic to PDL cells and is still effective on osteoclastic inhibition (19, 22), should be used for treating traumatized teeth. This substance applied through the root canal of avulsed and replanted teeth, using it as a reservoir (23), or through the immersion of the dental root for a period of time (3) may be an effective preventive treatment for substitutive dental root resorption. However, new *in vitro* research should be carried out before further studies using animal as well as human subjects for the treatment and control of dental root substitutive resorption is begun. These studies could analyze different concentrations and application forms of sodium alendronate, as well as examine the cytotoxicy of dentin-bound alendronate toward PDL cells.

References

1. Andreasen JO. Etiology and pathogenesis of traumatic dental injuries. A clinical study of 1,298 cases. Scand J Dent Res 1970;78:329–42.
2. Trope M. Root resorption of dental traumatic origin: classification based on etiology. Pract Periodont Aesthet Dent 1998;10:515–22.
3. Levin L, Bryson EC, Caplan D, Trope M. Effect of topical alendronate on root resorption of dried replanted dog teeth. Dent Traumatol. 2001;17:120–6.
4. Tronstad L. Root resorption – etiology, terminology and clinical manifestations. Endod Dent Traumatol 1988;4:241–52 (review).
5. Pierce A, Lindskog S. The effect of an antibiotic/corticosteroid paste on inflammatory root resorption in vivo. Oral Surg Oral Med Oral Pathol 1987;64:216–20.
6. Pierce A, Lindskog S. Coated pits and vesicles in the osteoclast. J Submicrosc Cytol Pathol 1988;20:161–7.
7. Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD. Bisphosphonate Action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. J Clin Invest 1991;88:2095–105.
8. Yaffe A, Iztkovich M, Earon Y, Alt I, Lilov R, Binderman I. Local delivery of an amino bisphosphonate prevents the resorptive phase of alveolar bone following mucoperiosteal flap surgery in rats. J Periodontol 1997;68:884–9.
9. Sant'Ana ACP, Marques MM, Barroso E, Passanezi E. Culture and characterization of human derived periodontal ligament cells. Rev Fac Odontol Bauru 2002;10:134–40.
10. Freshney TF. Culture of animal cells: a manual of basic technique, 4th edn. New York: Wiley; 2000.
11. Scelza MF, Daniel RL, Santos EM, Jaeger MM. Cytotoxic effects of 10% citric acid and EDTA-T used as root canal irrigants: an *in vitro* analysis. J Endod 2001;27:741–3.
12. de Azevedo CL, Marques MM, Bombana AC. Cytotoxic effects of cyanocrylates used as retrograde filling materials: an *in vivo* analysis. Pesqui Odontol Bras 2003;17: 113–18.
13. Liewehr FR, Craft DW, Primack PD, Kulild JC, Turgeon DK, Sutherland DE et al. Effect of bisphosphonates and gallium on dentin resorption in vitro. Endod Dent Traumatol 1995;11:20–6.
14. Vanderas AP. Effects of intracanal medications on inflammatory resorption or occurrence of ankylosis in mature traumatized teeth: a review. Endod Dent Traumatol 1993;6:175–184.
15. Schwalz G. Use of cell cultures for toxicity testing of dental materials: advantages and limitations. J Dent 1994;22:S6–S11.
16. Zimolo Z, Wesolowski G, Rodan GA. Acid extrusion is induced by osteoclast attachment to bone: inhibition by alendronate and calcitonina. J Clin Invest 1995;96:2277–83.
17. Kum KY, Park JH, Yoo YJ, Choi BK, Lee HJ, Lee SJ. The inhibitory effect of alendronate and taurine on osteoclast differentiation mediated by *Porphyromanas gingivalis* sonicates *in vitro*. J Endod 2003;29:28–30.
18. Sommercorn LM, Di Fiore PM, Dixit SN, Koerber A, Lingen MW, Veis A. Effect of alendronate on immature human dental root explants. J Endod 2000;26:133–7.
19. Garcia Moreno C, Serrano S, Nacher M, Diez A, Marinoso ML, Carbonell J et al. Effect of alendronate on cultured normal human osteoblasts. Bone 1998;22:233–9.
20. Weinreb M, Quartuccio H, Seedor JG, Aufdemorte TB, Brunsvold M, Chaves E et al. Histomorphometrical analysis

of the effects of the bisphosphonate alendronate on bone loss caused by experimental periodontitis in monkeys. J Periodont Res 1994;29:35–40.
21. Reddy MS, Weatherford TW, Anne Smith C, West BD, Jeffcoat MK, Jacks TM. Alendronate treatment of naturally occurring periodontites in beagle dogs. J Periodontol 1995;66:211–17.
22. Meraw S, Reeve CM. Qualitative analysis of peripheral peri-implant bone and influence of alendronate sodium on early bone regeneration. J Periodontol 1999;70:1228–33.
23. Moreira MS, Katayama E, Bombana AC, Marques MM. Cytotoxicity analysis of alendronate on cultured endothelial cells and subcutaneous tissue. A pilot study. Dent Traumatol 2005;21:329–35.