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Cytotoxicity analysis of alendronate on cultured endothelial cells and subcutaneous tissue. A pilot study

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Abstract – The use of alendronate, a bisphosphonate which is able to inhibit bone resorption, in order to prevent dental root resorption after tooth replantation would be of clinical relevance. However, this drug must be biocompatible to the periapical tissues. The aim of this study was to analyze the effect of an alendronate paste in polyethyleneglycol (2 g ml^{-1}) on endothelial cells in culture (*in vitro*) and on rat subcutaneous tissue (*in vivo*). For the *in vitro* study the paste was applied on round glass coverslips that were immersed into confluent cell cultures (clone Cips). The cell viability percentages of these cultures were obtained 0, 6 and 12 h after contact with the substance. As control, cultures that received plain coverslips were used. This analysis was carried out in triplicate using the Trypan blue dye exclusion assay. For the *in vivo* study the paste was introduced into polyethylene tubes that were placed into the rat subcutaneous tissue. The rats were killed 7 and 14 days later; then, the tissue sections stained with hematoxylin–eosin were analyzed. *In vitro*, the alendronate caused a significant decrease in the cell viability in 6 h ($P < 0.05$) and 12 h ($P < 0.01$), when compared with the control cultures. *In vivo* the tissue response was exuberant and similar at the two experimental times. There was a necrosis in a comprehensive area in contact with the open end of the tube. Presence of micro-abscesses and intense inflammatory infiltrate in the hypoderm permeating the muscle fibers and fat lobules were observed. In conclusion, the alendronate paste in polyethylene glycol as used showed to be highly cytotoxic *in vitro* as well as *in vivo*.

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Dental avulsion is a traumatic lesion with serious complications for the periodontal and pulp tissues (1). The prognosis of the replanted teeth depends on several factors that can jeopardize the success of this therapy (1–3). Depending on storage conditions of the avulsed tooth the replantation can culminate with root resorption (1–4). In fact, areas of the root surface that lack vital periodontal ligament (PDL) cells attack osteoclasts preventing the PDL regeneration. The osteoclast inhibition could improve the prognosis of tooth replantation; however, the present-day therapies for tooth avulsion only delay rather than inhibit the resorption process (4). Some of these therapy modalities are based on the use of calcium hydroxide (5), association of antibiotics and corticosteroids (6) and calcitonin (7). More recently, Levin et al. (4) employed an anti-osteoclastic drug in order to inhibit the root resorption of replanted dogteeth. This drug, the alendronate, which is a third generation bisphosphonate with demonstrated

osteoclast inhibitory activity, enhanced healing and preservation of tooth mass subsequent to replantation.

Although alendronate presents promising properties for dental avulsion treatment, recent researches have shown that in high doses this drug may cause oral avascular bone necrosis because of antiangiogenic effect in patient on chemotherapy for malignancies (8–9). Nevertheless, before indicating this drug for clinical use it is mandatory to test the biocompatibility of alendronate in a concentration for topical use. Thus, the aim of this study was to analyze the cytotoxicity of alendronate on cultured endothelial cells (*in vitro*), the target cells for the side effect of alendronate, as well as in rat subcutaneous tissue (*in vivo*).

Material and methods

The cytotoxic effects of alendronate were tested *in vitro* using an endothelial cell line and *in vivo* at the rat subcutaneous tissue. The Ethical Committee of the School of Dentistry, University of Sao Paulo approved this study.

In vivo assay (cytotoxicity)

The cytotoxicity of a paste of alendronate ($2\ g\ ml^{-1}$, 0.6×10^{-5} M) in polyethylene glycol 400 (PEG) was measured *in vitro*. This formulation of alendronate (Fórmula & Ação, São Paulo, Brazil) was prepared in order to obtain a paste with a density appropriated for filling the root canal.

Cell culture

Endothelial cell line derived from rabbit's aorta (kindly provided by Dr Helena Nader, Federal University of Sao Paulo-UNIFESP) (10) were grown at 37°C in Ham's F-12 medium (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.

Experimental groups

The experimental groups were: (i) control: cultures that received plain round glass 12-mm diameter coverslips; (ii) alendronate: cultures that received round glass 12-mm diameter coverslips coated with the alendronate paste. An immediate or short-term response of the cells was analyzed. This assay aimed to test the cytotoxicity of an alendronate formulation (alendronate plus PEG) as it would be used *in vivo*. The controls with plain glass coverslips analyzed the cell viability of cultures submitted only to the mechanical aggression of the glass coverslips.

Short-term assay (cell viability)

Cells (1×10^4) were plated on 35 mm Petri dishes. Three days later, the cultures received the coverslips. After 0, 6 and 12 h the cells of three dishes per group were counted, and viability curves were plotted. As 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 (11).

Morphological analysis

The overall morphology of the cultures (untreated and alendronate) were 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 were analyzed. Phase photomicrographs were obtained from a phase inverted microscope TMS (Nikon Co., Tokyo, Japan).

Cell viability curves

Growth curves were constructed as described in other studies (12–14). Briefly, the cell number was determined by counting the viable cells in a hemocytometer using the Trypan blue dye exclusion assay. The cells were harvested from the culture dishes using a 25% pancreatin solution in Earle's Balanced Salt Solution (EBSS) (Sigma). For each time period, cells from three dishes of each group were counted. The number of viable cells harvested from each Petri dish was obtained by the following mathematical equation: $UC \times D \times 10^4$/no. of SQ, where UC is the unstained cell count (viable cells); D, the dilution of the cell suspension; no. of SQ, number of squares of the hemocytometer counted.

The viability percentage of the cell population of each Petri dish was obtained by the following mathematical equation: $UC/TC \times 100$, where TC is the total cell count (stained plus unstained cells).

Statistical analysis

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

In vivo assay (tissue response)

For the *in vivo* study six male Wistar rats (*Rattus norvegicus albinus*) weighting an average of 180–200 g were used. The rats were anesthetized prior to surgery using a mixture of xylazin and ketamine (0.15 ml per 100 g of rat bodyweight).

Substance placement

The alendronate paste was introduced into polyethylene tubes using lentulo bur. The tubes were of 12-mm height with 1-mm internal diameter, containing at maximum 4.2 mm^3 of alendronate paste. One end of the tube was closed with cyanoacrylate. These closed ends were then considered as controls, because cyanoacrylate is a biocompatible substance (14). The tubes were implanted into the rat back subcutaneous 15 mm from the skin incision. After the implantation of the tubes, the skin incision was closed using cyanoacrylate.

Histological preparation

The rats were killed by anesthetic overdose 7 and 14 days later. The skin containing the tubes and the surrounding tissues were cut and immersed into 10% buffered formalin for 48 h. After water washing the specimens were dehydrated and embedded in paraffin. Tissue specimens were sectioned at 5 μm and stained with hematoxylin–eosin for routine histological examination.

Evaluation criteria

The qualitative histological analysis was carried out considering the presence or absence and the extension of tissue necrosis and/or inflammation.

Results

In vitro assay

Throughout the experimental time (0–12 h), control cultures maintained stable cell viability (80.26 ± 0.17 to 89.16 ± 5.53%), whereas treated cultures (alendronate) displayed a striking drop in cell viability (%) reaching almost total cell death at 12 h (23.66 ± 8.57%) (Table 1; Fig. 1).

Table 1. Descriptive statistical analysis of the viability curves

Experimental time (h)	DF	SS	MS	*F*	*P*-value
Zero	1	41.8	41.8	0.51	0.51
6	1	1080.04	1080.04	16.34	0.016
12	1	5784.61	5784.61	45.55	0.003

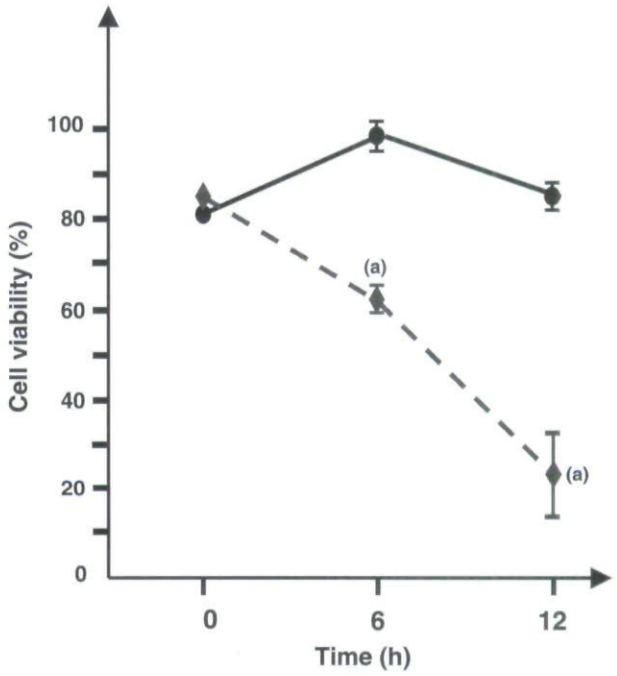

Fig. 1. Graphic representation of the viability curves of Cips cells in the short-term assay. Note the decrease of cell viability in 6 and 12 h at the alendronate group curve. Results are the mean + SEM of three experiments. (a) Significantly different from control.

Phase microscopy

Representative phase micrographs of Cips cells, control and treated with alendronate paste, illustrating the overall morphology of the cell line during the short-term assay are shown in Fig. 2. The control cultures exhibited cells with the usual morphology during whole experimental time (Fig. 2a). These cells were ovoid in shape with ample cytoplasm. The attached cells formed a homogeneous and continuous monolayer after confluence when they stopped growing because of contact inhibition. When in contact with the alendronate paste, at the first experimental time (0 h) the cells individually and the cell monolayers were similar to those of control cultures. After 6 h of contact with the substance some cells presented cytoskeletal changes assuming a picnotic shape (e.g. round cell with scarce cytoplasm and hyperchromatic nucleus). These cells were detached from the plastic substrate and formed clusters in suspension (Fig. 2b). At this experimental time it was still possible to find cells with the usual morphology and attached to the substrate. After 12 h of contact with the alendronate paste most cells were detached from the substrate forming suspended cell clusters (Fig. 2c,d). At this experimental time it was difficult to find attached cells in these alendronate cultures.

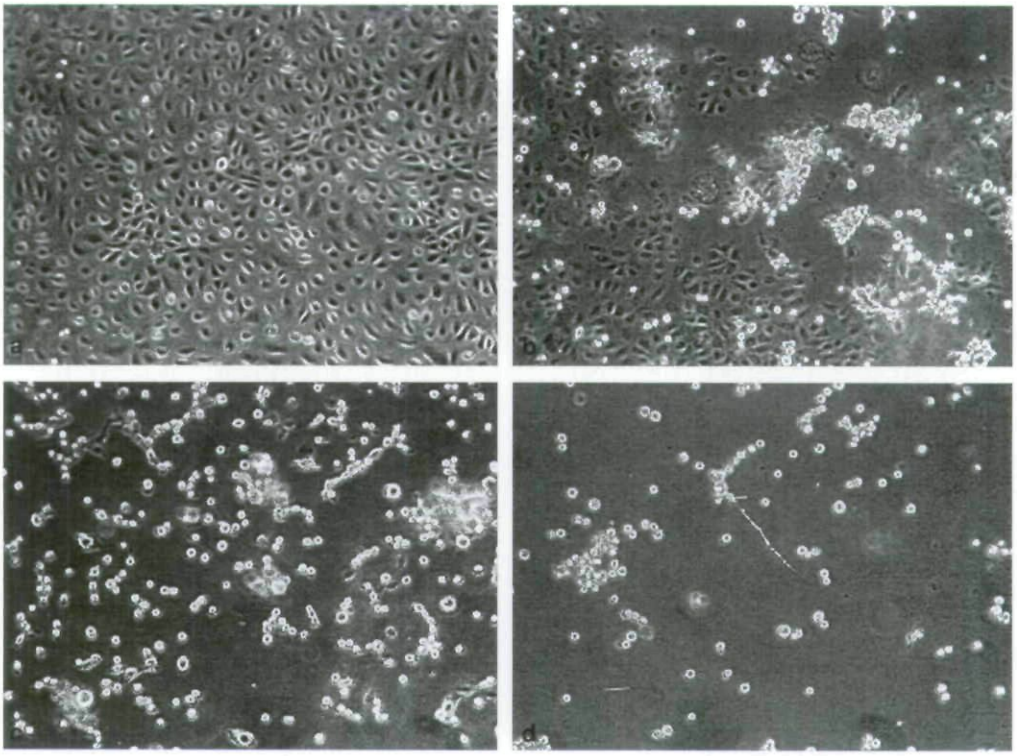

Fig. 2. Phase photomicrographs of Cips cells, (a) control: culture in the absence of alendronate at 6 h; (c,d) cultures in the presence of alendronate, 12 h (×100).

In vivo assay

Representative micrographs of the histological findings are shown in Fig. 3. The findings were similar at both experimental times (7 and 14 days). The histological analysis showed at the rat subcutaneous tissue surrounding the closed end of the tube – considered as control for the experiments – formation of granulation tissue with discrete inflammatory infiltrate (Fig. 3a,b). In the same animals the tissue reaction around at the open end of the tube containing the alendronate paste was much more exuberant. The reaction to the alendronate paste after the tube implantation presented at 7 days did not change even after 14 days of contact. Figure 3 (c–k) exemplify these reactions.

The tissue in direct contact with the alendronate paste appeared necrotic and dark stained (Fig. 3c,e). In continuation with this stained area the tissue exhibited extensive coagulation necrosis (Fig. 3d,f). At the distance, surrounding this necrotic area, a granulation tissue with intense inflammatory process was observed. These mononuclear inflammatory cells infiltrated the muscle (Fig. 3g,h) causing disarrangement of the muscle fibers (Fig. 3h). Commonly the necrosis reached the muscle layers destroying their cells. The inflammatory infiltrate also reached the fat lobules at the hypodermis causing similar effects to these cells as those observed at the muscle layer (Fig. 3i). In focal areas micro-abscesses were observed. In these areas collections of neutrophils were found (Fig. 3j). At the subcutaneous tissue it was still possible to observe extensive areas of necrotic tissue even far from the polyethylene tube (Fig. 3k).

Discussion

In cases of dental avulsion, before replantation of the tooth, the root canal preparation may be necessary. In order to inhibit a future root resorption, alendronate could be used as a root canal medication. In this case, the application of alendronate as a paste would be indicated. To obtain an appropriated density of the alendronate for filling the root canal the concentration of 2 g of alendronate for ml of PEG was chosen. The vehicle was elected because it is nontoxic (15) and has other favorable characteristics.

For the clinical application of a substance as alendronate it is advantageous to associate the substance with a vehicle in order to make easy the insertion of the substance into the root canal and promote the flowing to the apical region. The success of the endodontic treatment relies not only on the active substance itself, but also on the vehicle employed for preparing the paste, once the time of permanence of the medication at its action place is

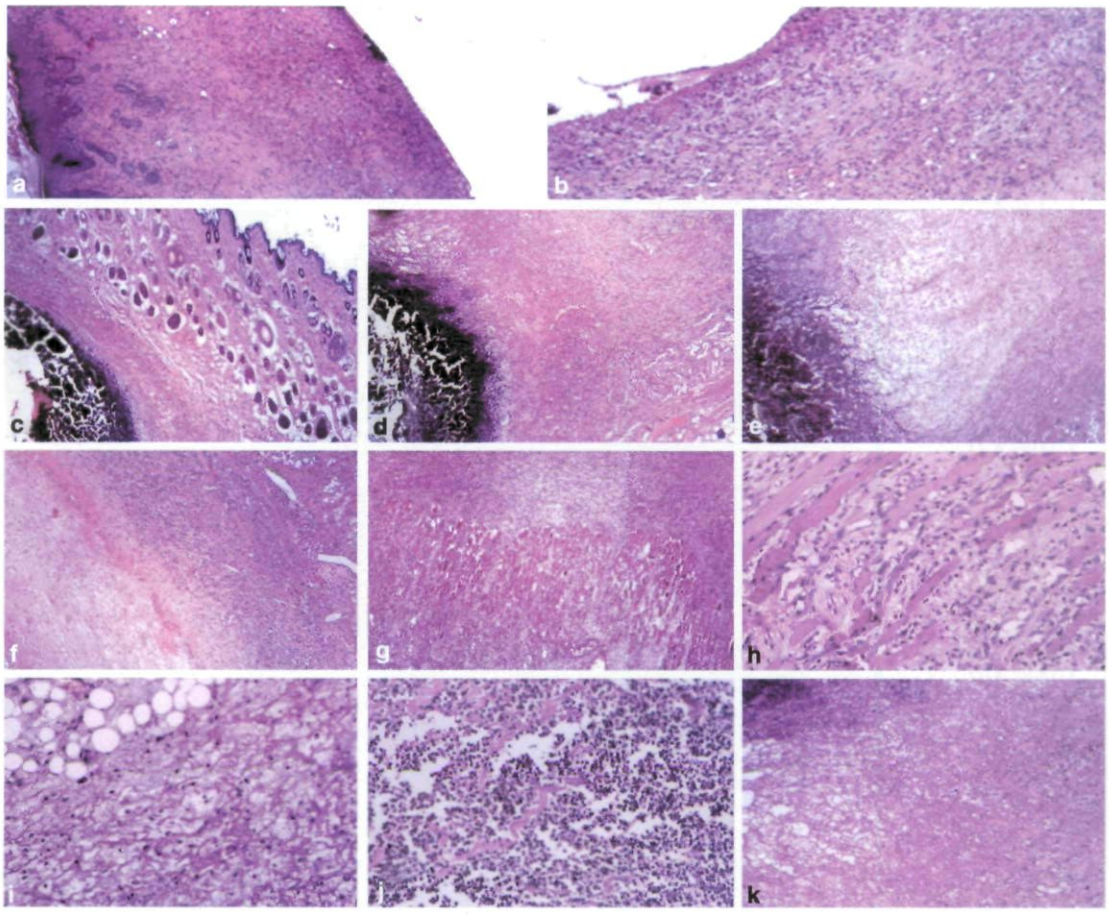

Fig. 3. Photomicrographs of the histological findings at the rat subcutaneous tissue. (a,b) control: granulation tissue with discrete inflammatory infiltrate; (d–k) tissue response to the alendronate paste; (c–e) necrosis is observed; (g, h) mononuclear inflammatory cells infiltrating the muscle, causing disarrangement of the muscle fibers (i). The inflammatory infiltrate reaching the fat lobules at the hypodermis. In focal areas micro-abscesses were observed (j). Far from the polyethylene tube observe presence of extensive areas of necrotic tissue (k). (H & E; a, d, e, f, g, i, j × 40; b, c, h, k × 100).

determined fundamentally by the vehicle. Polyethylene glycol either liquid or solid has been commonly used as vehicle for endodontic drugs.

The cytotoxic effects of alendronate were tested *in vitro* using an endothelial cell line and *in vivo* at the rat subcutaneous tissue. For the *in vitro* study the paste was applied using glass coverslips coated with the substance, whereas the application *in vivo* was carried out by implanting polyethylene tubes filled with the paste. These methods are reliable for analyzing cytotoxicity of several therapeutic agents in endodontics.

For the *in vitro* study an endothelial cell line (clone Cips) was used because this is the cell type targeting for the side effect of alendronate *in vivo* (16). The alendronate presents not only anti-osteoclastic action but also an anti-angiogenic characteristic. This aspect is of importance because the alveolus that will receive the replanted tooth is vascularized and the placement of a paste containing alendronate could cause a direct effect of this drug on the blood vessels. Although *in vitro* studies present limitations, our results pointed out for a highly cytotoxic effect of the alendronate at the conditions here applied (e.g. concentration and formulation). There was death at the Cips treated cultures of approximately 80% of the cells in 12 h of contact with the drug. Following the *in vitro* cytotoxic analysis protocol of the FDA at this study the drug was applied to the cell cultures at the same formulation indicated for clinical purpose. This study could also be carried out using culture medium conditioned by the substance, however, the direct contact with the paste was chosen because tooth avulsion normally occurs in young people that presents immature dental root and the contact of the paste with the periodontal tissues are more likely to take place.

Our results could not be directly compared with other studies of the literature because most of them have used different cell types, mostly osteoclasts (17–19). Additionally, those studies have used different concentrations and formulations of the alendronate.

However, we could compare our findings with those of Kum et al. (17) and Breuil et al. (18) that showed diminishing of osteoclast number as a result of contact with alendronate in concentrations of 10^{-5} M. Other authors stated that diminishing of osteoclasts could be the result of inhibition of recruitment of osteoclast or a direct cytotoxic effect (19). Additionally, high concentrations of other types of bisphosphonates, including pamidronate, chlodronate and risedronate provoked apoptosis in osteoclasts (19).

Study conducted by Wood et al. (16) in endothelial cells in culture showed a proliferation inhibition induced by alendronate applied at the concentrations of 10^{-3} to 10^{-10} M. These findings showed the anti-angiogenic action of the bisphosphonates that are important for chemotherapy of malignancies. However, this property is dangerous for the blood vessels leading to tissue avascular necrosis (8, 9). The existence of spontaneous necrosis in patients because of vascular commitment prompt us to test the effect of alendronate topically applied as it would be used in endodontics.

At the *in vivo* study a 1-mm diameter tube of polyethylene was used to deliver the alendronate paste to the subcutaneous tissue mimicking the relationship between the apical end of the root canal and the periapical tissues.

The histological analysis was qualitative. In fact, at first there was an intention to perform a quantitative analysis using scores for the histological findings, such as number of inflammatory cells, number of affected vessels, etc. as was performed by Kaynak et al. (20). However, the histological findings were very expressive and the differences between control and treated areas were evident supporting the observation of the cytotoxic effect of the alendronate.

The reaction to the cyanoacrylate at the control side of the tube was compatible with the trauma of the implantation. This result was expected because this substance is biocompatible (14).

The extensive necrosis of the subcutaneous tissue did not repair even after 14 days of contact with the drug. It means that alendronate, at the conditions here applied, has a long-term effect that could compromise the apical regeneration after tooth replantation.

Kaynak et al. (20) applied alendronate topically at the rat bone showing different findings; instead of necrosis they found excessive fibrosis and minimal inflammatory infiltrate. These differences could be the result of differences in doses, formulation and rat tissue used. While these authors used 0.6×10^{-6} M in the present study 0.6×10^{-5} M was used. Both concentrations of alendronate are known to inhibit the osteoclast activity (17, 18). Kaynak et al. (20) worked in rat bone, while we analyzed rat subcutaneous, both tissues with differences in vascularization and possibilities of drug metabolization. Moreover, they applied a liquid solution whereas we used paste, this occurred in the function of the applicability of the drug that in periodontics is performed in sponge gelatin and in endodontics in a paste presentation. On the contrary, the differences could reside at the experimental times; Kaynak et al. (20) analyzed the results 1 week after our last experimental time. Knowing that rats present high metabolic activity, the fibrosis observed by Kaynak et al. (20) could be the resultant of the cicatrisation of the extensive necrosis areas observed in our samples. Thus, we intend to keep studying the effect of alendronate, now extending our experimental time.

Levin et al. (4) immersed dog dental roots into alendronate solution and after replantation they demonstrated osteoclast inhibitory activity, enhanced healing and preservation of tooth mass subsequent to replantation. This form of alendronate application would be interesting to inhibit root resorption. However, it did not completely inhibit the root resorption, because they still occurred. May be this result could be due to the time of the contact with the drug that was not enough for incorporating the alendronate into the dental mineralized tissues (cement and dentin). The use of a paste would be more appropriated leading more time of the contact of alendronate and the dental root tissues.

It is not known if the incorporation of alendronate from the root canal would be effective in order to reach the more external tissues such as cement. However, even if alendronate does not pass through the dentin, it can reach the periodontal ligament through the apical end of the root canal.

The conclusion of this study is that alendronate in the form of paste in polyethylene glycol (2 g ml^{-1}, 0.6×10^{-5} M) showed to be highly cytotoxic *in vitro* as well as *in vivo*. However, it is important to take into account that alendronate is a potent inhibitor of osteoclast activity and could be of importance for several clinical applications in dentistry. Perhaps, the exuberance of the reactions observed were result of the concentration of the drug in the paste. This paste was manipulated more in function of density than concentration. Then, new researches must be carried out in order to find a concentration which is able to inhibit osteoclast activity after tooth replantation without causing any undesirable side effect such as bone necrosis.

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