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# Systemically Alendronate was Incorporated Into Dental Tissues but did not Cause Morphological or Mechanical Changes in Rats Teeth

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**KEY WORDS** sodium alendronate; bone mineral density; mineralized dental tissues

**ABSTRACT** This study evaluated the effect of the systemic use of sodium alendronate in rats in vivo. Forty-five Wistar rats aged 36 to 42 days and weighing 200 to 230 g were randomly assigned to a control group ( $n = 20$ ), which received distilled water, and an experimental group ( $n = 25$ ), which received 2 weekly doses of 1 mg/kg of chemically pure sodium alendronate. The animals were killed after 60 days of treatment. The tibias were removed for analysis of bone mineral density by dual-energy X-ray absorptiometry (DXA). Then, the maxillary incisors were extracted for analysis of the mineralized dental tissues using fluorescence spectroscopy (FS), scanning electron microscopy (SEM), bright field microscopy (BFM), and cross-sectional microhardness (CSMH) testing. DXA and CSMH data were subjected to statistical analysis by Kruskal-Wallis test (5% significance level). The experimental group presented higher bone mineral density than the control group by DXA. FS analysis revealed presence of alendronate in the mineralized dental tissues of the specimens of the experimental group. Significant morphological differences were not found by SEM and BFM. Enamel and dentin (100 and 300  $\mu\text{m}$  from the dentinoenamel junction) CSMH data did not show significant difference between the control and experimental groups. Based on the obtained results, we conclude that while alendronate increased the bone mineral density and was incorporated into the mineralized dental tissues it did not cause significant alterations in the morphology and microhardness of rat incisor enamel and dentin. *Microsc. Res. Tech.* 75:1265–1271, 2012. © 2012 Wiley Periodicals, Inc.

## INTRODUCTION

Bisphosphonates are a class of analogs of pyrophosphate with high affinity to hydroxyapatite (Bradaschia-Correa et al., 2007), which act on bone metabolism and are widely used in the prevention and treatment of osteopenic and osteoporotic conditions, such as osteoporosis, Paget's disease, and hypercalcemia related to malignancy (Russell et al., 2007, 2008; Vasikaran, 2001).

Sodium alendronate, a recently developed aminobisphosphonate, is a compound that strongly binds to the hydroxyapatite of the bone, preferentially to crystals at the sites of active resorption, and inhibits the formation, aggregation, and dissolution of the crystals (Russell et al., 2008). It also causes toxic effects on osteoclasts, resulting in disorders in their cytoskeleton and intracellular signaling, inhibition of adhesion mechanisms, structural changes in the ruffle border, and decrease of

their acid production (Bradaschia-Correa et al., 2007). As a result, osteoclasts may become latent or inactive and undergo apoptosis (Vasikaran, 2001), inhibiting osteoclastic bone resorption (Fleisch, 2007). The affinity of sodium alendronate for calcium phosphate and its strong binding to hydroxyapatite promotes a rapid incorporation of this compound to the tissues. Its half-life in serum is short, a few hours or less, whereas the half-life in the bone may be several years depending on the rate of bone remodeling (Srivastava and Alon, 2003).

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This drug is indicated in the therapy of several pediatric bone disorders (Srivastava and Alon, 2003), such as childhood osteoporosis (Bachrach and Ward, 2009), osteogenesis imperfect (Ward et al., 2011), vitamin D intoxication (Orbak et al., 2006), primary hyperparathyroidism (Mallet, 2008), hypercalcemia and hypercalciuria (Freundlich and Alon, 2008). It is possible that the alendronate administration to children or adolescents, in whom odontogenesis and/or tooth eruption are still taking place, might induce structural alterations in the teeth or even the failure of tooth eruption (Bradaschia-Correa et al., 2007).

Although the mechanism of action of sodium alendronate has been extensively investigated in bone (Azaras and Rezvani, 2010; Diab et al., 2009; Enjuanes et al., 2010; Ward et al., 2011), little is known about its effects in the formation of mineralized dental tissues, limited to enamel (Espírito Santo et al., 2010; Massa et al., 2006). To the best of our knowledge there are no studies investigating the effects of sodium alendronate on dentin.

We hypothesize that sodium alendronate could be incorporated into enamel/dentin, causing changes in these tissues that could be associated with higher risk to caries development. In this *in vivo* study we systemically administered sodium alendronate to rats and examined the alteration of bone mineral density of the proximal tibial metaphysis by dual-energy X-ray absorptiometry (DXA) to confirm that the drug was bioavailable and absorbed in the bones. In addition, we evaluated: (1) incorporation of the drug to the mineralized dental tissues, using fluorescence spectroscopy (FS); (2) structural and mechanical alterations in these tissues, using scanning electron microscopy (SEM), bright field microscopy (BFM), and cross-sectional microhardness (CSMH) testing.

## MATERIAL AND METHODS

Forty-five 36- to 42-day-old male Wistar rats (*Rattus norvegicus albinus*) (200–230 g) were used, after approval of the study by the Institutional Ethics Committee for Animal Care and Research Use (Protocol 10.1.468.53.5). The animals were housed in cages with natural lightening and mean temperature of  $24 \pm 0.5^\circ\text{C}$ , and were fed standard rat chow and water *ad libitum*.

The animals were randomly assigned to a control group ( $n = 20$ ), which received distilled water, and an experimental group ( $n = 25$ ), which received chemically pure sodium alendronate (alendronate monosodium trihydrate; Galena Química e Farmacêutica Ltda., SP, Brazil) at a dose of 1 mg/kg body weight diluted in distilled water. As the metabolic rate is twice as faster in rats than in humans (Keidel, 1971), the drug was administrated twice a week (Tuesdays and Fridays) always in the same period of the day to reach a dose compatible to the one given to human patients (1 mg/kg body weight once a week). A volume of 0.3 mL was administered at each day by gavage. A 3-cm long silicone gastrointestinal tube (#6 Levin tube) coupled to a disposable 1 mL syringe was used to inject the solution directly into the gastrointestinal tract. The control animals received only distilled water by the same route of administration. The animals were weighed every week in an electronic precision scale (Model 3400, Toledo do

TABLE 1. Analyses employed for evaluation of the tibias and maxillary incisors and number of specimens used in each analysis

Specimen	Type of analysis	No. specimens	
		Experimental group	Control group
Tibia	DXA	16	10
Maxillary incisor	FS	9	9
	SEM	10	10
	BFM	3	3
	CSMH	20	12

Brasil Ind. de Balanças Ltda., Toledo, PR, Brazil) in order to adjust the dose of the sodium alendronate solution according to the body weight of the animals.

Rats were killed after 60 days of treatment with a lethal injection of a mixture of ketamine hydrochloride (Ketamina Agener<sup>®</sup>, União Química Farmacêutica Nacional, Embu-Guaçu, SP, Brazil; 300 mg/mL) and xylazine hydrochloride (Dopaser<sup>®</sup>; Caleir S.A., Barcelona, Spain; 30 mg/mL). The tibias were removed and the maxillary incisors were extracted, which had been previously submitted to the randomization to be allocated to the further different analyses (Table 1), using the SAS (Statistical Analysis System) software for Windows version 9.1.3 (SAS Institute Inc., Cary, NC).

### DXA Analysis

Sixteen tibias of the experimental group and 10 of the control group were surgically removed and the proximal tibial metaphysis was analyzed with a dual-energy X-ray densitometer (Dual Lunar PIXImus; PIXImus Corp. Headquarters, Madison, WI) to record the bone mineral density in  $\text{g}/\text{cm}^2$ . Data were analyzed using the Lunar PIXImus software, version 2.2 (Lunar PIXImus Corp. Headquarters).

### FS Analysis

A calibration curve was first constructed using 10, 20, 30, and 40 mL of a sodium alendronate solution at 0.086 mg/mL, which was subjected to the derivatization reaction (De Marco et al., 1989) in order to emit fluorescence, since sodium alendronate does not have chromophoric properties. Then, three maxillary incisors of each group were triturated in a grill and agate pistil to increase the contact surface with the solution. The study was performed in triplicate, totalizing nine teeth per group. The resulting tooth fragments were placed in beakers containing 5 mL of sodium borate under agitation during 24 h to allow for extraction of the sodium alendronate that could have been incorporated to the dental tissues during the mineralization phase. Next, the remaining solution of each sample was collected and transferred to a flat-bottom glass balloon for the derivatization reaction. After addition of 5 mL of sodium citrate and 4 mL of 9-fluorenylmethyl chloroformate (FMOC), the solution was agitated for 30 s and, after 30 min had elapsed for the reaction to occur, 25 mL of dichloromethane were added to extract the excess of FMOC reagent. The balloon with the solution was then agitated in an automatic agitator for 30 s, left rest for 5 min and, then centrifuged at 1,000 rpm for 5 min to eliminate the excess of reagent. The upper portion of the aqueous phase of each sample was transferred to

another flask for the reading. The sodium alendronate/FMOC derivative formed after reaction has luminescence upon excitation at 266 nm, with emission spectra at 315 nm (De Marco et al., 1989). The excitation and emission spectra of the sodium alendronate/FMOC derivative were analyzed with a spectrofluorometer (Shimadzu RF-5301PC Series; Shimadzu, Kyoto, Japan) equipped with a 150 W ozone-free xenon arc lamp. The results were evaluated by analysis of the obtained spectra, determining the percentage of sodium alendronate incorporated to the teeth (amount of sodium alendronate per weight of each tooth).

### SEM Analysis

In preparation for the SEM analysis, 10 maxillary incisors of both groups were cleaned and immersed in modified Karnovsky solution (8% glutaraldehyde, 12% paraformaldehyde, and 0.2 M sodium cacodylate; pH 7.2–7.4) for fixation during 5 days. After this period, they were washed in 0.1 M sodium cacodylate buffer, air-dried, mounted on metallic stubs, and sputter-coated with gold (Emitech K650 Sputter Coater; London, England) until obtaining an approximately 200- $\mu\text{m}$  thick coating, and were examined with a scanning electron microscope (DSM 940A, Zeiss, Oberkochen, Germany). Five maxillary incisors of each group were used to analyze the structure of the external enamel surface at 50 $\times$ , 500 $\times$ , and 1,500 $\times$  magnifications, having the incisal border as the reference point for recording the SEM images. The other five maxillary incisors of each group had the mesial surface ground until exposing dentin to analyze enamel and dentin structure in depth at 500 $\times$  and 2,000 $\times$  magnifications, having the DEJ as the reference point for analysis. The SEM micrographs were examined for the topographical and structural aspects of enamel and dentin.

### BFM Analysis

Three maxillary incisors of each group had the mesial and distal surfaces of the teeth ground with 400-, 600-, 1,200-, and 4,000-grit silicon carbide paper (Saint-Gobain Abrasivos Ltda.) until obtaining specimens with thickness of 100  $\mu\text{m}$  in the central portion, as measured with a digital caliper (Vonder Instrumentos de Mediçao, São Paulo, SP, Brazil). The specimens were maintained in 0.02% sodium azide solution during preparation, and then washed with distilled water and mounted in microscopic slides for qualitative analysis by BFM using an Axioskop 40 microscope (Carl Zeiss, Germany). The analyzed parameters were enamel and dentin structure and iron deposition on enamel surface.

### CSMH Analysis

Thirty-two maxillary incisors (20 from the experimental group and 12 from the control group) were used for this analysis. For preparation of specimens, the teeth were embedded in chemically activated polyester resin with the mesial surface exposed (Mioflex<sup>®</sup>; Mioflex General Tintas and Vernizes Ltda, Jandira, SP, Brazil). After resin polymerization, the specimens were ground with 600- and 1,200-grit silicon carbide paper (Saint-Gobain Abrasivos Ltda., Jundiaí, SP, Brazil) in a water-cooled polishing machine (Struers A/S, Copen-

hagen, Denmark) to expose a flat dentin surface. The specimens were polished with 0.3  $\mu\text{m}$  alumina slurry (Alpha and Gamma Micropolish, Buehler, Lake Bluff, IL) on a felt polishing wheel (Buhler<sup>®</sup>, Germany), and then rinsed with deionized water, ultrasonically cleaned with deionized water for 10 min and stored at 37°C in relative humidity until the moment of test, within 7 days.

Specimens were tested for CSMH using a Shimadzu HMV-2 hardness meter (Shimadzu Corp., Kyoto, Japan) with a Knoop indenter under a load of 25 g in enamel and 10 g in dentin (Faraoni-Romano et al., 2008) for 10 s in each substrate. Knoop microhardness (KHN) was measured using the Newage C.A.M.S software (Computer Assisted Measurement System; Newage Testing Instruments, Inc., Southampton, PA). In enamel, five indentations spaced 200  $\mu\text{m}$  from each other were made in each specimen equidistantly between the dentinoenamel junction (DEJ) and the external surface of the tooth, based on a previous analysis of the enamel CSMH in rat teeth (Vieira et al., 2005). The average of the five CSMH values was calculated. In dentin, five indentations spaced 200  $\mu\text{m}$  from each other were made at 100  $\mu\text{m}$  and 300  $\mu\text{m}$  from the DEJ. The average of the five CSMH values was calculated for each depth.

### Statistical Analysis

DXA and CSMH data were analyzed statistically by the Kruskal-Wallis test at 5% significance level using the SAS software.

## RESULTS

The animals in both groups had a similar gradual weight gain during the experiment (data not shown). This indicates that the administration of sodium alendronate did not affect growth.

### DXA Analysis

Figure 1A presents the bone mineral density values of the proximal metaphysis of the tibia obtained by DXA ( $\text{g}/\text{cm}^2$ ) in both groups at day 60. The group treated with sodium alendronate had significantly higher bone mineral density than the control group ( $P < 0.05$ ), which confirms that the alendronate administration protocol was adequate, as treatment resulted in significant increase in bone mineral density.

### FS Analysis

The emission spectra of the control and experimental groups are shown in Figure 2. The experimental group spectrum presented an emission band with peak at 315 nm which, compared with the calibration curve, suggesting that sodium alendronate was incorporated to the dental tissues. This was not observed in the emission spectrum of the control group (distilled water). From the analysis of the calibration curve, it was possible to determine the amount of sodium alendronate incorporated to the mineralized tissues of each tooth (0.0018%).

The presence of alendronate in our tooth samples has also been independently confirmed by qualitative

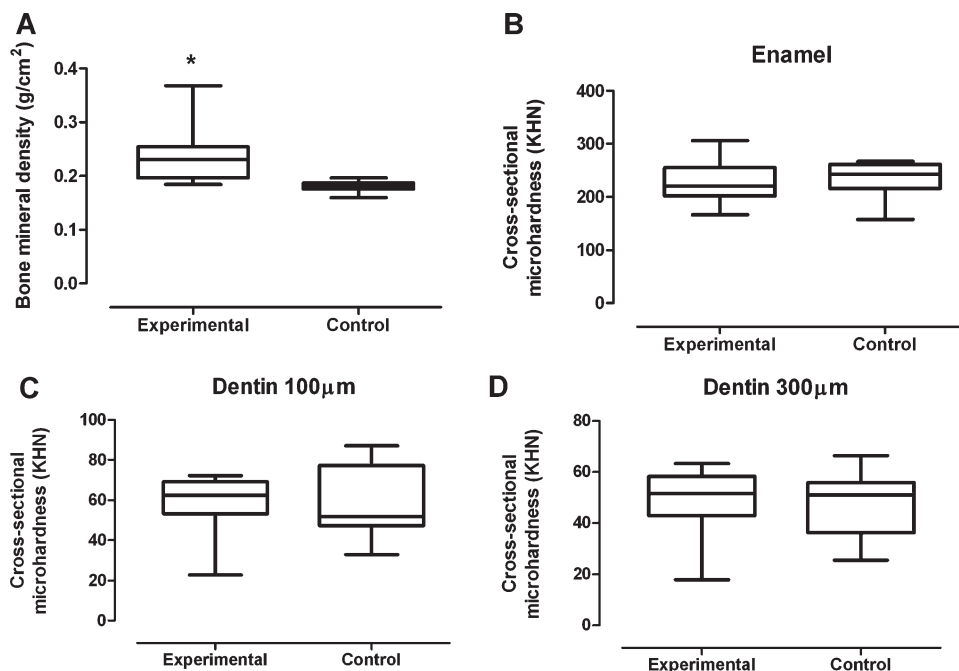


Fig. 1. Statistical box plots expressing minimum values, first quartiles, medians, third quartiles, and maximum values of DXA and CSMH data. Bone mineral density values of proximal tibial metaphysis obtained by DXA ( $\text{g}/\text{cm}^2$ ) (A) and CSMH (KHN) of enamel (B), den-

tin at 100  $\mu\text{m}$  from the dentinoenamel junction (C), and dentin at 300  $\mu\text{m}$  from the dentinoenamel junction (D) in the experimental (administration of sodium alendronate) and control (administration of distilled water) groups. \*Statistically significant difference.

Fourier transform infrared spectroscopy (data not shown).

### SEM Analysis

SEM micrographs of the external enamel and internal enamel and dentin of the rat incisors in the experimental and control groups are presented in Figures 3A–3C and Figures 3E–3G, respectively. In both groups, the external surface enamel was homogeneous,

with regular contour and no structural alterations. Internal examination of enamel and dentin did not reveal differences between the groups, and the mineralized dental tissues exhibited normal characteristics.

### BFM Analysis

Dentin and enamel exhibited normal appearance. There were no alterations in iron pigmentation on enamel surface after administration of sodium alendronate, compared with the control group. Comparison of the images obtained in the experimental (Fig. 3D) and control groups (Fig. 3H) did not reveal structural alterations or evidence of increased retention of organic components in the mature enamel or dentin. Other morphological changes that are common when dentin has mineralization problems, such as increased areas of interglobular dentin, were not detected. Changes in the mineralization pattern, such as lines of increased or decreased mineral content, were not observed either. The morphological analysis showed three histological layers on enamel (outer, central, and inner). As described before, the central layer had the highest mineral content, followed by the outer and inner layers (Saiani et al., 2009).

Polarized light microscopy has also been carried out (data and images not shown), and this technique as well did not point out any morphological/structural alterations same as SEM and BFM.

### CSMH Analysis

Figures 1B–1D present the CSMH values (KHN) of enamel (between the DEJ and the external tooth sur-

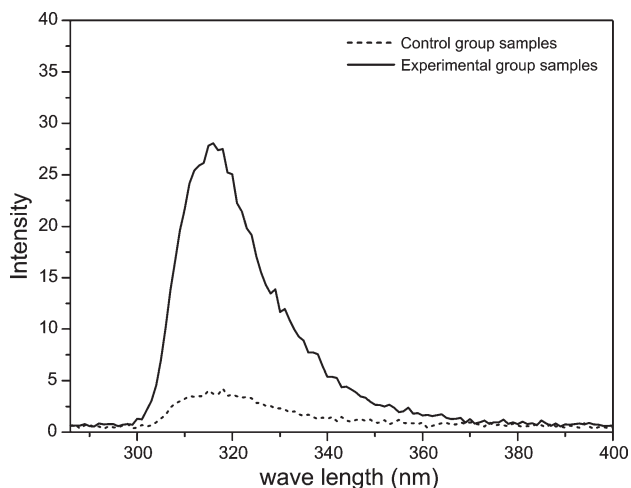


Fig. 2. Emission spectra ( $\lambda_{\text{ex}} = 266 \text{ nm}$ ) of the solutions obtained from samples of the control and experimental groups, showing the incorporation of alendronate into the mineralized dental structure.

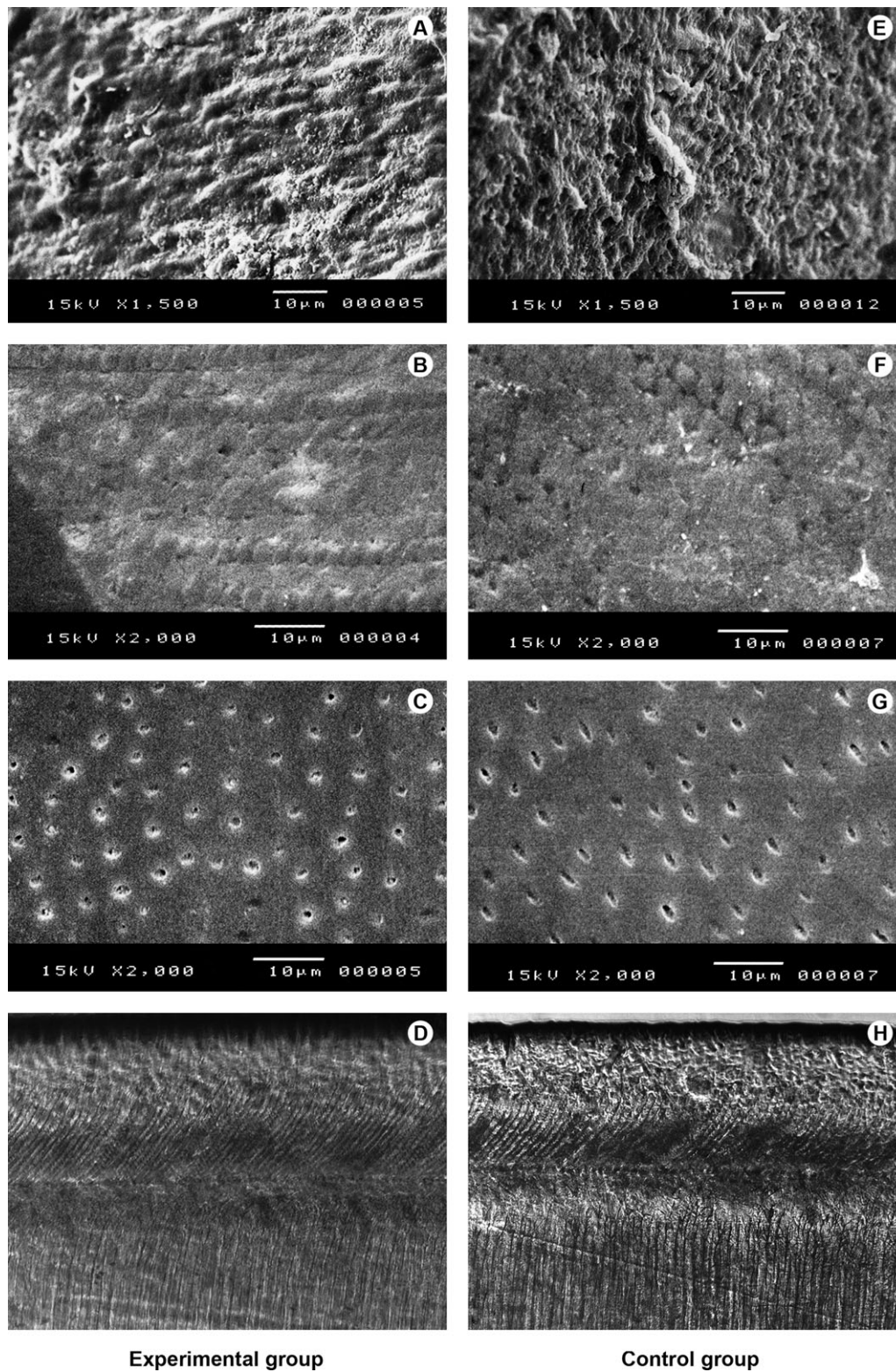


Fig. 3. Experimental group (administration of sodium alendronate) (A–D) and control group (administration of distilled water) (E–H). SEM micrographs showing homogeneous buccal external enamel surface, with regular contour and no structural alterations (A and E).

SEM micrographs of internal regions of enamel (B and F) and dentin (C and G) showing normal morphology. Cross-section of enamel and dentin by bright field microscopy (D and H) in which no structural alterations is displayed.

face) and dentin (100 and 300  $\mu\text{m}$  from the DEJ) in the experimental and control groups. No statistically significant difference ( $P > 0.05$ ) was found between the groups either in enamel or in dentin (both depths), demonstrating that the administration of sodium alendronate did not alter the enamel and dentin (at 100 and 300  $\mu\text{m}$  from the DEJ) CSMH compared with the control group.

## DISCUSSION

Odontogenesis involves a series of complex interactions among different cell types and between cells and the extracellular matrix. These interactions result in the differentiation of odontoblasts and ameloblasts, which form dentin and enamel, respectively (Thesleff et al., 1995).

A great variety of conditions and drugs may disturb the normal formation and mineralization of teeth (Small and Murray, 1978). For instance, the effects of etidronate (HEBP), a nonaminobisphosphonate, on collagen fibrillogenesis, matrix synthesis, and mineralization of dentin have been reported (Beertsen et al., 1985; Larsson, 1974; Ogawa et al., 1989; Sakai et al., 1999; Takano et al., 1998). HEBP has also been shown to affect enamel mineralization (Espírito Santo et al., 2010; Fouda et al., 1989, 1991, 1992; Weile et al., 1990, 1993), ameloblast morphology (Fejerskov et al., 1990; Josephsen et al., 1990), and ameloblast functions, including effects on protein synthesis and secretion (Fuangtharathip et al., 2000; Yamada et al., 2000), with development of hypoplasia and formation of subameloblastic cysts (Fejerskov et al., 1990; Fouda et al., 1989, 1991, 1992; Simmelink, 1987).

Sodium alendronate, on the other hand, belongs to the group of aminobisphosphonates and it has been widely prescribed for patients with bone disorders (Inoue et al., 2008; Kawate et al., 2010; Orbak et al., 2006). In the present study, the DXA analysis revealed an increase in the bone mineral density of the proximal tibial metaphysis after exposure to sodium alendronate, which confirms previous reports (Freundlich and Alon, 2008; Inoue et al., 2008; Kawate et al., 2010). Despite being widely used, few studies evaluated the effect of sodium alendronate in dental hard tissue formation *in vivo*, and those available are limited to enamel (Espírito Santo et al., 2010; Massa et al., 2006).

In the present study, quantitative chemical analysis by FS revealed the presence of sodium alendronate in the mineralized dental tissues of rat teeth (0.0018% per tooth) after systemic administration of this drug, which indicates that it is bioavailable in the organism during formation of the teeth. To the best of our knowledge, this is the first study reporting such observation. Unlike other mineralized tissues, in which the mineral is deposited toward the direction the organic matrix, enamel loses most of the proteins and water present in its organic matrix during amelogenesis, becoming the most mineralized tissue in mammals. For this reason enamel does not normally undergo remodeling once its formation is completed and incorporated substances or structural alterations become permanent (Gerlach et al., 2002).

Microhardness values are usually employed to evaluate the structural quality of enamel (Gerlach et al., 2002) and dentin. In the present study, it was not possible to observe significant differences in the CSMH of enamel (between the DEJ and the external tooth surface) or dentin (100 and 300  $\mu\text{m}$  from the DEJ) comparing the control and alendronate-treated groups. Neither were observed any structural alterations in the mineralized tissues by SEM after exposure to systemic sodium alendronate.

Finally, the analysis of the enamel and dentin by BFM showed no morphological alterations in the extracellular matrix. A recent study (Espírito Santo et al., 2010) evaluated the birefringence of enamel organic extracellular matrix during the secretory stage and the mechanical properties of the enamel of maxillary incisors of rats treated with subcutaneously injected sodium alendronate (30  $\mu\text{g}/\text{kg}/\text{day}$ ). The morphological alterations in the extracellular matrix reported by those authors were not observed in the present study probably because they evaluated immature enamel while we evaluated mature enamel in addition to using different alendronate dose and route of drug administration. In the present study, a dose of about 286  $\mu\text{g}/\text{kg}/\text{day}$  was administered to the rats, which is almost 10 times higher as compared to the 30  $\mu\text{g}/\text{kg}/\text{day}$  of the cited study; however, different routes of administration were used in these studies. As mentioned before, the alendronate dose used in the present study was calculated to reach a dose compatible to the one given to human patients. Additionally, administration by gavage aims at reproducing in a more reliable form the route of administration used in patients (oral route).

The amount of alendronate found in this study was 0.0018% per tooth, after systemic administration of this drug, and probably was not sufficient to cause significant alterations according to the tested parameters. However, it is a valuable finding that confirms the strong affinity of alendronate for hydroxyapatite and its rapid incorporation to the matrix of mineralized tissues, including teeth, during the mineralization phase. The literature has shown that, similarly, other elements are incorporated to the dental tissues and might cause several effects. An excellent example is fluoride, which is known to reduce the solubility of dental substrate to acid attacks from cariogenic challenge, playing a key role in the prevention and control of dental caries (Leite et al., 2011). Likewise, lead is incorporated to calcified dental tissues, which allows assessing subclinical past systemic exposure to this ion (Sawan et al., 2010). Thereby, further studies are needed to investigate what effects can appear after alendronate incorporation to the dental tissues, for example, whether it may alter their resistance to acid demineralization, making the tooth more or less susceptible to caries disease or erosive processes.

Based on the obtained results, we conclude that although sodium alendronate increased the bone mineral density and was incorporated to the mineralized dental tissues, there were no significant structural, morphological, and mechanical alterations in mature enamel and dentin of rat incisors, suggesting that the use of alendronate clinically in patients could be safe.

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