Chronic treatment with zoledronic acid alters the expression levels of inflammatory, bone, and apoptotic markers and Toll-like receptors 2 and 4 in rat dental pulp

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Objective. The aim of this study was to evaluate the immunostaining of inflammatory, apoptotic, and bone markers, as well as Toll-like-receptors (TLRs) 2 and 4 in the dental pulp in rats treated with zoledronic acid (ZA).

Study Design. We administered 4 intravascular infusions of saline (control group) or 0.20 mg.kg^{-1} ZA in Wistar rats (n = 6/group). After 70 days, the 3 rights molars (n = 18/group) were microscopically evaluated (presence of ectasic/dilated blood vessels and inflammatory cells). Immunohistochemistry was performed for tartrate resistant acid phosphatase 5 (TRAP; cell counting), cyclo-oxygenase-2 (COX-2), interleukin-6 (IL-6), TLR2, TLR4, receptor activator of nuclear kappa B ligand (RANKL), osteoprotegerin (OPG), and caspase-3 (scored 0−3 in odontoblast and nonodontoblast dental pulp cells). Mann-Whitney and Fisher's exact tests and Spearman's correlation were used (GraphPad Prism 5.0).

Results. There was no alteration in ectasic/dilated blood vessels ($P = .101$) or inflammatory cells ($P = .500$), but the number of TRAP-positive cells was reduced in the ZA-group ($P = .027$). In ZA-group odontoblasts, immunostaining for COX-2 ($P = .044$), TLR4 ($P = .003$), OPG ($P = .035$) and caspase-3 ($P = .039$) increased, and that for RANKL ($P = 0.045$) decreased. In nonodontoblast dental pulp cells, RANKL immunostaining decreased ($P = .009$). In the ZA group, the RANKL/OPG ratio decreased in odontoblast ($P = .022$) and nonodontoblast dental pulp cells ($P = .007$). IL-6 did not differ between the groups.

Conclusions. ZA increases the expression levels of inflammatory, apoptotic markers, and TLR4 and alters bone makers in the dental pulp of rats. (Oral Surg Oral Med Oral Pathol Oral Radiol 2019;128:139−145)

Zoledronic acid (ZA) is a potent antiresorptive drug used in the treatment of bone disorders and is strongly associated with the development of bisphosphonate-related osteonecrosis of the jaws, a specific type of medication-related osteonecrosis of the jaws. There are several bisphosphonates involved in the pathogenesis of this disease, but ZA is the most strongly associated one.[1]

Although bisphosphonate-related osteonecrosis of the jaws is the primary side effect of treatment with bisphosphonates, a recent adverse effect on the dental organ has been described: chronic treatment with bisphosphonates increases the expression levels of inflammatory markers in dental pulp cells. ZA treatment resulted in increases in the levels of tumor necrosis factor alpha (TNF-α), interleukin-1 beta, and inducible oxide nitric synthases (iNOS) and an influx of macrophages after long-term treatment in rats,[2] and these findings of bisphosphonate toxicity in teeth are not isolated.

In rats, sodium alendronate interacts with teeth, promoting teratogenic effects[3] and inducing denticle and odontoma formation.[4] In cell cultures, the treatment of dental pulp cells with ZA shows toxicity that is dose and time dependent[5] and results in the overactivation of proapoptotic proteins, such as caspase 3.[6] In humans, the use of alendronate reduces root resorption,[7] and chronic treatment with bisphosphonates leads to internal dystrophic calcifications in the dental pulp of patients.[8]

Inflammatory alterations in the dental pulp can lead to the overexpression of receptors of innate immunity, such as Toll-like receptors (TLRs) 2 and 4, which are constitutively expressed in odontoblasts to recognize bacterial caries.[9] These immune alterations predispose cells to increase production of cytokines[10] and help initiate pulpitis in odontoblasts.[11] Additionally, in bone, bisphosphonate treatment increases the expression of osteoprotegerin (OPG) and reduces the receptor

Statement of Clinical Relevance

The increases in the expression levels of inflammatory and apoptotic markers and inversion of the RANKL/OPG axis in the dental pulp after zoledronic acid infusion can predispose the dental pulp to tissue damage and pulpal calcifications.

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activator of nuclear kappa B ligand (RANKL) and OPG expression altering the RANKL/OPG axis.[12] These alterations are important in teeth because dental pulp cells produce and are dependent on RANKL.[13]

It has been suggested that bisphosphonates could alter the expression levels of inflammatory and bone markers, elevate apoptosis rates, and compromise the pulp immune response. However, studies investigating changes in the inflammatory profile of the dental pulp are restricted to animals submitted to caries models or dental pulp cells cultures treated with compounds derived from cariogenic bacteria.[10] Therefore, the purpose of this study was to evaluate the expression levels of inflammatory, apoptotic, and bone markers, as well as TLR2 and TLR4, in rat dental pulp treated with ZA.

MATERIALS AND METHODS

Ethical approach and sample size calculation

Approval for experimental use of laboratory animals was obtained from the local (Federal University of Ceará, Brazil) Ethics Committee on Animal Use (protocol 26/13) and was in compliance with the Federal Law No. 11794 of October 8, 2008, and the Decree No. 6689, July 15, 2009 that regulated the law in 11,794; available at http: Www.planalto.gov.brccivil03 Ato2007- 20102008 LeiL11794.htm. The animals (n = 6/cage) were kept in polypropylene cages (49 × 34 × 16 cm), in a 12-hour light/dark cycle, temperature of 23 ± 2°C, and unrestricted access to filtered water and sterile extruded feed.

This study was designed to minimize the number of animals required for the experiments. Therefore, a sample size calculation was designed on the basis of a previous study,[2] which showed an increase in the prevalence of inflammatory cells in the dental pulp of rats treated with ZA (100%) compared with a saline-treated group (16.7%), using a power of 80% and a confidence interval of 95%. Thus, we defined a sample of 6 animals by group (Kesley method, χ^2 test).

Animals, doses, and experimental protocols

Male Wistar (*Rattus norvegicus*) rats (n = 6/group), weight 200 ± 13 g and 49 ± 2 days old, were randomly selected, divided by using the random command in Microsoft Excel (Microsoft Corp., Redmond, WA) and kept in the experiment room of the Department of Physiology and Pharmacology of the Federal University of Ceará. Rats of this age were selected because by age 30 days, all molars had erupted.[14]

After anesthesia with xylazine (20 mg/kg) and ketamine (80 mg/kg) intraperitoneally, providing approximately 20 minutes of deep anesthesia, the animals received 3 consecutive weekly intravascular (penile access) infusions of 0.1 mL/kg saline or 0.20 mg/kg of ZA (Eurofarma, São Paulo, SP, Brazil), as previously descripted.[15] The infusions were performed on days 0, 7, and 14, and on day 49, an additional dose was given. Three weeks later (day 70), the animals were euthanized, and the right hemimandibles were fixed in 10% neutral buffered formalin. The animals remained in good health from the start of the study through the experiment.

After fixation (24 hours), the right hemimandibles were decalcified (ethylenediaminetetraacetic acid 10%; pH 7.3) for 30 days to prepare the tissue for microscopic slides. Microscopic slides (4 μm) were deparaffinized, dehydrated, and cored by using the conventional hematoxylin and eosin method for histologic analysis. Additional tissue sections were performed for immunohistochemical reactions.

Immunohistochemical reaction

After deparaffinization and rehydration of tissue sections for immunohistochemistry (IHC), antigenic recuperation was performed by heating the sections in citrate solution, pH 6.0, for cyclooxygenase-2 (COX-2; Abcam, Cambridge, MA), interleukin-6 (IL-6; Abcam), TLR2 (Abcam), TLR4 (Abcam), RANKL (Abcam), OPG (Abcam) and tartrate resistant acid phosphatase 5 (TRAP) (Abcam), or in tris- ethylenediaminetetraacetic acid, pH 9.0, for caspase 3 (Abcam). After reaching room temperature, the slides were blocked in peroxidase with 3% hydrogen peroxide (H_2O_2) and diluted in phosphate buffered saline for 30 minutes.

After blocking with albumin for 1 hour, the slides were incubated overnight with the following primary antibodies: COX-2 (1:200), IL-6 (1:300), TLR2 (1:300), TLR4 (1:300), RANKL (1:200), OPG (1:250), TRAP (1:100), and caspase 3 (1:50). After washing with phosphate buffered saline, the slides were incubated with Universal Immune-peroxidase Polymer (Histofine, Nicherei, Seattle, WA) for 1 hour, and 5,5-diaminobenzidine tetra hydrochloride (DAB; Abcam) was used to identify positive cells. Murine spleen was used as positive control for COX-2, IL-6, caspase 3. Small intestine was used as positive control for TLR4 and TLR2 and the periodontium of each rat was used as positive control for RANKL, OPG and TRAP.

Histologic and immunohistochemical analysis

The 3 right mandibular molars from each rat were blindly analyzed by optic microscopy at × 400 magnification (5 microscopic fields per tooth in 18 total teeth for each group were evaluated). To characterize the cell profiles of the pulp after hematoxylin and eosin staining, the presence of ectasic or dilated blood vessels and inflammatory cells was evaluated. The total number of TRAP-positive cells (IHC) was also counted.

To characterize the inflammatory profile of the pulp, the molars were evaluated, the percentage of odontoblast and nonodontoblast cells (mesenchymal cells, such as fibroblasts and inflammatory cells) was determined, and the cytoplasmic detection of each antibody was characterized as follows: 0 = no positive cells; 1 = mild, 1% to 33% of positive cells; 2 = moderate, 34% to 66% of positive cells; and 3 = intense, 67% to 100% positive cells. The final score was that agreed upon by 2 observers (κ= 0.935).[2] The factorial scores for RANKL and OPG were calculated to compare the RANKL/OPG ratios between the 2 groups.

Statistical analysis

The Mann-Whitney test and Spearman's correlation were used for scores (median [minimum−maximum] or mean [mean ± standard error of the mean] analysis); Fisher's exact test (absolute and percentage frequency of the animals) was used for categorical analysis in GraphPad Prism 5.0 software ($P < .05$).

Power size calculation

On the basis of the mean number of TRAP-positive cells that were found in the dental pulp of the ZA-treated group (0.50 ± 0.34) compared with the saline group (3.33 ± 1.80) and considering the sample of 6 animals per group (n = 6), a power of 96.6% to reject the null hypothesis of this study was calculated (*t* test).

RESULTS

Effect of ZA in the dental pulp

Histologically, there were no changes in the dentin structure of the ZA-treated teeth. Tubular dentin and pulps of unchanged volume were observed on all teeth. The number of dental pulps exhibiting dilated and ectasic blood vessels ($P = .101$) or inflammatory cells ($P = .500$) was similar between the ZA-treated and saline-treated groups. However, the ZA-treated group (0.50 ± 0.34) had significantly fewer TRAP-positive cells compared with the saline-treated group (3.33 ± 1.80) ($P = .027$) (Table I; Figure 1).

Effect of ZA in odontoblast cells

In odontoblast cells, immunostaining for inflammatory markers showed a contrasting response. Although the COX-2 expression level was increased in the ZA-treated group (3; 2−3) compared with the saline-treated group (2; 1−3) ($P = .044$), immunostaining for IL-6 in these cells did not differ significantly between the 2 groups (median = 1) ($P = .464$). Additionally, the proapoptotic protein caspase 3 had higher levels of immunostaining in the odontoblasts from the ZA-treated group (2; 1−3) than in the odontoblasts from the saline-treated group (1; 0−3) ($P = .039$) (see Table I; Figure 2).

Table I. Inflammatory markers, bone markers, and microbial recognition receptor profiles in the dental pulp from rats chronically treated with ZA

	Saline	*ZA*	*P value*
Ectasic/Dilated blood vessels	18 (100%)	14 (77.8%)	.101*
Inflammatory cells	9 (50%)	6 (33.3%)	.500*
TRAP-positive cells	3.33 ± 1.80	0.50 ± 0.34	*.027*†,‡
Odontoblast pulp cells			
COX-2	2 (1−3)	3 (2-3)	*.044*†,‡
IL-6	1 (0−2)	1 (1−2)	.464‡
TLR2	1 (0−3)	1.5 (0−3)	.478‡
TLR4	1 (0−2)	2 (1−3)	*.003*†,‡
RANKL	2 (1−3)	1 (0−3)	*.045*†,‡
OPG	2 (0−3)	3 (1−3)	*.035*†,‡
Caspase 3	1 (0−3)	2 (1−3)	*.039*†,‡
Nonodontoblast pulp cells			
COX-2	2.5 (13)	2 (1−3)	.713‡
IL-6	1 (0−1)	1 (0−1)	.464‡
TLR2	0 (0−1)	0 (0−1)	.945‡
TLR4	0 (0−1)	1 (0−2)	.113‡
RANKL	1 (1−2)	0 (0−1)	*.009*†,‡
OPG	1 (0−2)	1 (1−3)	.232‡
Caspase 3	1 (0−1)	1 (0−1)	.550‡

*Fisher's exact test (n, %).
†$P < .05$ (n = 18 teeth). Scores = (0) no positive cells; (1 = mild) 1% to 33% of positive cells; (2 = moderate) 34% to 66% of positive cells; and (3 = intense) 67% to 100% positive cells.
‡Mann-Whitney test (median [minimum − maximum] or mean ± standard mean of error).
COX, cyclooxygenase; *IL,* interleukin; *OPG,* osteoprotegerin; *RANKL,* receptor activator of nuclear kappa B ligand; *TLR,* Toll-like receptor; *ZA,* zoledronic acid.

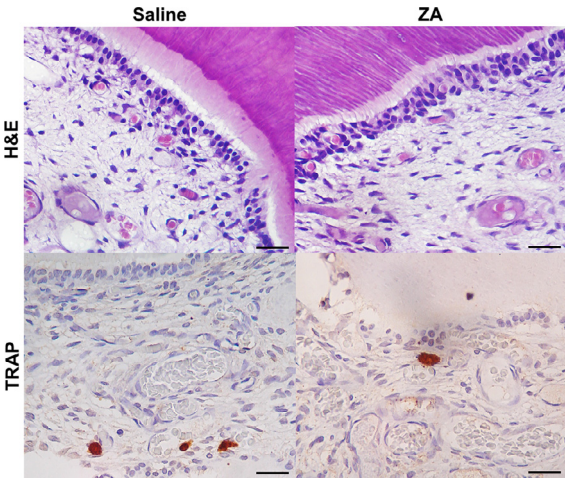

Fig. 1. The cellular profile of dental pulp from rats chronically treated with zoledronic acid (ZA) shows no modifications in the histologic profile, but a reduction in the number of TRAP-positive cells is observed (original magnification × 400; hematoxylin and eosin [H&E] and immunohistochemistry [IHC]).

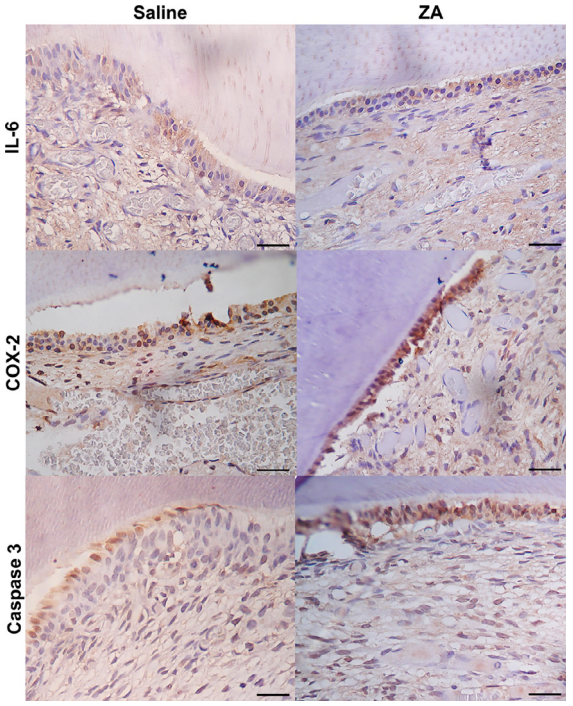

Fig. 2. The inflammatory profile of dental pulp from rats chronically treated with zoledronic acid (ZA) shows an increase in immunostaining for cyclooxygenase-2 (COX-2) in odontoblast dental pulp cells, without any variations in interleukin-6 (IL-6) immunostaining (original magnification × 400, immunohistochemistry [IHC]).

RANKL immunostaining was reduced in the odontoblasts from the ZA-treated group (1; 0−3) compared with those from the saline-treated group (2; 1−3) ($P = .045$). OPG immunostaining was increased in the ZA-treated group (3; 1−3) compared with the saline-treated group (2; 0−3) ($P = .035$) (see Table I; Figure 3). The RANKL/OPG ratio was significantly decreased in the ZA-treated group (0.46 ± 0.07) compared with the saline-treated group (1.78 ± 0.64) ($P = .022$) (Figure 4).

The immunostaining for TLR2 did not differ between the ZA- and saline-treated groups ($P = .478$), but the TLR4 expression level increased in the ZA-treated group (2; 1−3) compared with the saline-treated group (1; 0−2) ($P = .003$) (see Table I and Figure 3).

Effect of ZA in nonodontoblast cells

In nonodontoblast dental pulp cells, there were no observed differences in immunostaining for COX-2 ($P = .713$), IL-6 ($P = .464$), TLR2 ($P = .495$), TLR4 ($P = .113$), OPG ($P = .232$), or caspase 3 ($P = .550$). Only the immunostaining for RANKL was reduced in nonodontoblast dental pulp cells from the ZA-treated group (0; 0−1) compared with the saline-treated group (1; 1−2) ($P = .009$) (see Table I and Figures 2 and 3).

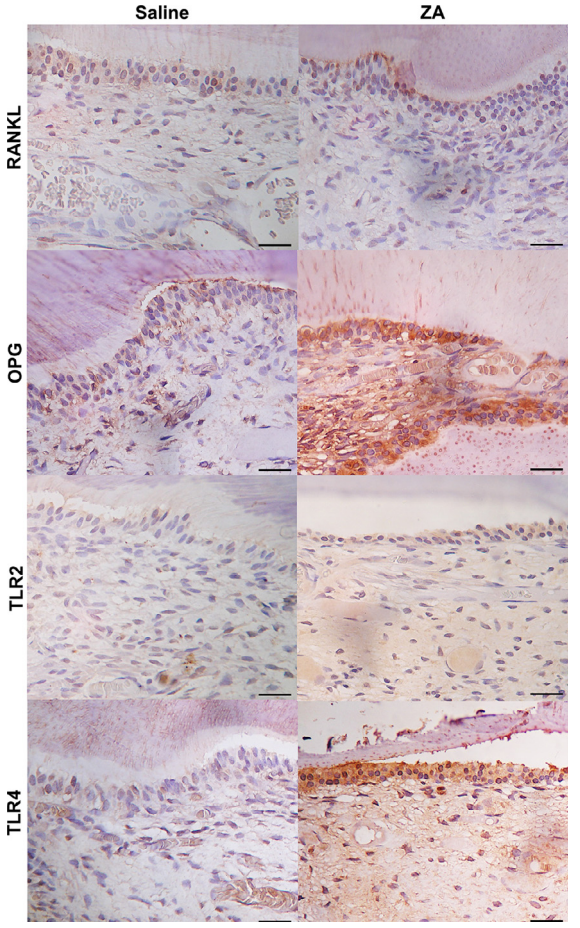

Fig. 3. Bone markers and microbial-recognition receptor profiles of the dental pulp from rats chronically treated with zoledronic acid (ZA) shows a reduction in the receptor activator of nuclear kappa B ligand (RANKL) expression level in odontoblasts and nonodontoblast dental pulp cells and increases in the levels of osteoprotegerin (OPG) and Toll-like receptor 4 (TLR4) immunostaining (original magnification × 400, immunohistochemistry [IHC]) in odontoblasts.

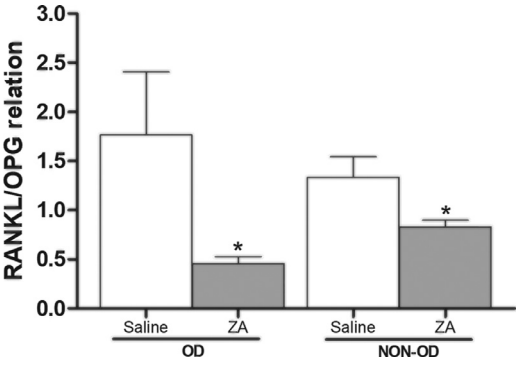

Fig. 4. The receptor activator of nuclear kappa B ligand/osteoprotegerin (RANKL/OPG) ratio in OD (odontoblasts) and NON-OD (nonodontoblasts) dental pulp cells from rats chronically treated with zoledronic acid (ZA). *$P < .05$ vs saline group (Mann-Whitney test; mean ± standard error of mean).

The RANKL/OPG ratio was reduced in the nonodontoblast dental pulp cells from the ZA-treated group (0.83 ± 0.07) compared with those from the saline-treated group (1.33 ± 0.21) ($P = .007$). (see Figure 4).

Correlation between the markers in odontoblast and nonodontoblast dental pulp cells

OPG immunostaining in odontoblasts was directly cofractionated with TLR4 ($P = .019$; $r = 0.426$), and there was a negative correlation between OPG and TLR4 ($P = .008$; $r = -0.525$) in nonodontoblast dental pulp cells. The other markers showed no statistically significant correlations (Table II).

DISCUSSION

ZA is the strongest bisphosphonate and acts through powerful mechanisms to increase the production of proinflammatory cytokines and to reduce angiogenesis and cell viability.[16,17] Because of its proapoptotic properties, ZA is even used as an antineoplastic drug.[18] However, many tissues and organs are affected by the effects mediated by ZA, causing adverse events, such as anemia, leukocytosis, gastric disorders, and delay in weight gain,[15] and alterations in teeth are not an exception.[2]

Although previous studies have shown that treatment with bisphosphonates interferes with molar tooth formation,[14] we did not obtain these findings in our study because we selected rats with fully erupted molars. This suggests that treatment with bisphosphonates only interferes in morphology during dental development. In most cases, bisphosphonates are used after total dental eruption. At this time, inflammatory changes seem to be the more important issue. Cvikl et al.[5] were the first to describe the toxic effects of bisphosphonates in dental pulp cell lines, and several reports of pulpal changes have been described. These findings cause great concern because the proinflammatory state induced by ZA in the

Table II. Correlation between inflammatory, bone, and apoptotic markers and the expression levels of receptors of innate immunity in odontoblast and nonodontoblast dental pulp cells from rats chronically treated with ZA

		COX-2	*IL-6*	*TLR2*	*TLR4*	*RANKL*	*OPG*	*Caspase 3*	*TRAP*
Odontoblast									
COX-2	r	–	0.269	−0.259	0.254	0.486	0.320	0.112	−0.386
	P value	–	.238	.245	.230	.082	.128	.612	.069
IL-6	r	–	–	−0.122	0.114	0.081	0.187	−0.052	−0.027
	P value	–	–	.620	.624	.773	.418	.827	.911
TLR2	r	–	–	–	0.222	0.273	0.374	0.336	0.294
	P value	–	–	–	.255	.219	.050	.087	.136
TLR4	r	–	–	–	–	−0.105	*0.426**	0.144	−0.235
	P value	–	–	–	–	.625	*.019*	.455	.229
RANKL	r	–	–	–	–	–	−0.022	0.260	0.028
	P value	–	–	–	–	–	.920	.230	.900
OPG	r	–	–	–	–	–	–	0.341	−0.188
	P value	–	–	–	–	–	–	.070	.339
Caspase 3	r	–	–	–	–	–	–	–	−0.253
	P value	–	–	–	–	–	–	–	.203
TRAP	r	–	–	–	–	–	–	–	–
	P value	–	–	–	–	–	–	–	–
Nonodontoblast									
COX-2	r	–	−0.046	−0.313	−0.116	0.268	0.117	−0.122	−0.447
	P value	–	.844	.156	.588	.281	.587	.580	.065
IL-6	r	–	–	−0.108	0.160	0.054	0.327	−0.252	0.201
	P value	–	–	.659	.489	.848	.148	.285	.396
TLR2	r	–	–	–	0.107	0.017	−0.106	0.060	0.253
	P value	–	–	–	.589	.942	.591	.767	.202
TLR4	r	–	–	–	–	*−0.525**	−0.031	0.188	−0.084
	P value	–	–	–	–	*.008*	.873	.328	.672
RANKL	r	–	–	–	–	–	−0.206	−0.224	0.024
	P value	–	–	–	–	–	.334	.304	.917
OPG	r	–	–	–	–	–	–	0.086	0.048
	P value	–	–	–	–	–	–	.657	.808
Caspase 3	r	–	–	–	–	–	–	–	0.034
	P value	–	–	–	–	–	–	–	.866
TRAP	r	–	–	–	–	–	–	–	–
	P value	–	–	–	–	–	–	–	–

*$P < .05$, Spearman correlation.

COX, cyclooxygenase; *IL,* interleukin; *OPG,* osteoprotegerin; *RANKL,* receptor activator of nuclear kappa B ligand; *TLR,* Toll-like receptor; *ZA,* zoledronic acid.

dental pulp could predispose to an overlap of response with dental caries.[2]

In our study, we observed that treatment with ZA increases COX-2 immunostaining but not IL-6 immunostaining in odontoblasts. COX-2 production is stimulated by odontoblasts in contact with microorganisms in the depths of dental caries,[10] leading to TNF-α, IL-6, and prostaglandin E2 production and pulp inflammation.[19] In bone cells, exposure to bisphosphonate elevates the COX-2 level.[20] However, in odontoblasts, an increase in the COX-2 expression level is dependent on the Smad2 protein,[21] which is inversely associated with IL-6 production.[22]

In the dental pulp, an increase in the COX-2 expression level leads to oxidative stress and a reduction in cell viability.[23] Although these findings were associated with exposure to lipopolysaccharides or microorganisms, we found that ZA treatment can increase the expression levels of COX-2 and caspase 3 in odontoblasts. In a previous study, it was shown that the exposure of dental pulp cells to ZA increases the caspase 3 expression level.[6] Thus, this effect has been demonstrated not only in vitro but also in vivo.

In odontoblasts, the overexpression of oxidative stress molecules iNOS as a result of ZA exposure was recently described.[2] Although the oxygen free radicals in odontoblasts occur as a defense mechanism against caries, they can negatively affect pulp cell differentiation,[24] and iNOS overexpression occurs in parallel with TLR overexpression.[25] TLR2 and TLR4 are constitutively expressed in odontoblasts, and TLR2 is first activated by gram-positive bacteria, and shortly thereafter by TLR4 activation by gram-negative bacteria.[9] In caries, the increase in TLR4 activation leads to the overproduction of TNF-α and chemokines, which cause pulp inflammation.[10] In odontoblast cultures, bisphosphonates increase TLR4-dependent cytokine production,[26] leading to the intense overproduction of proinflammatory cytokines.[27] We did not study the overlap of inflammatory responses of dental caries in the teeth of rats treated with ZA. This is a limitation of our study and a knowledge gap in the literature. However, it is probable that there is an overlap of the caries-induced inflammatory response and ZA treatment.

In our study, TLR4 was directly correlated with OPG and inversely correlated with RANKL. In bone, TLR4 interferes with osteoclastogenesis[28] and is indispensable to bone deposition and maturation. In vivo experiments demonstrated that TLR4-deficient mice did not express osteopontin (a protein strongly dependent on a low RANKL/OPG ratio) and did not form enough bone to close calvarial defects.[29]

In our study, there was an increase in the TLR4 expression level in odontoblasts and a reduction in the RANKL/OPG ratio and the number of TRAP-positive cells, confirming the findings of Zhao and Ivashkiv[28] and Wang et al[29], and demonstrating that ZA not only deregulates the RANKL/OPG axis in bone but also does so in the tooth. There are no in vivo studies showing that treatment with ZA leads to a reduction in the RANKL/OPG ratio in the dental pulp, but it is known that in bone, a reduction in the RANKL/OPG ratio leads to bone formation.[30] However, reduction in osteoclast counting in apical lesion in rats treated with alendronate[31] and increase in necrotic bone area in mice treated with ZA and submitted to apical lesion induction[32] have previously been described.

In the dental pulp, this reduction leads to internal dental calcification[33] and a reduction in the internal and external root resorption through the negative regulation of odontoclast cells.[34] This partially explains why patients receiving chronic treatment with bisphosphonates develop more dystrophic calcifications in the dental pulp.[8]

CONCLUSIONS

The findings of this study reinforce previous findings indicating that ZA deregulates the immunity of the dental pulp by increasing the expression levels of inflammatory mediators (COX-2), proapoptotic proteins (caspase 3), and receptors of innate immunity (TLR4), contributing to the hypothesis that ZA increases the likelihood of developing pulpitis in the presence of inflammatory caries. Additionally, this is the first in vivo study showing that chronic treatment with ZA reduces the RANKL/OPG ratio and the number of TRAP-positive cells in the dental pulp, and this can partially explain the pathogenesis of pulpal calcifications found in patients receiving bisphosphonate treatment and the reduction in root resorption observed after bisphosphonate treatment.

DISCLOSURE

This study was partially funded by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) with protocol number 446338/2014-1 of MCTI/CNPq/Universal.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.oooo.2019.01.069.

REFERENCES

1. Barasch A, Cunha-Cruz J, Curro FA, et al. Risk factors for osteonecrosis of the jaws: a case-control study from the CONDOR dental PBRN. *J Den Res*. 2011;90:439-444.
2. Barros Silva PG, Verde MEQL, Brizeno LAC, et al. Immune cell profile of dental pulp tissue treated with zoledronic acid. *Int Endod J*. 2017;50:1067-1076.

3. Hiraga T, Ninomiya T, Hosoya A, Nakamura H. Administration of the bisphosphonate zoledronic acid during tooth development inhibits tooth eruption and formation and induces dental abnormalities in rats. *Calcif Tissue Int*. 2010;86:502-510.
4. Massa LF, Bradaschia-Correa V, Arana-Chavez VE. Immunocytochemical study of amelogenin deposition during the early odontogenesis of molars in alendronate-treated newborn rats. *J Histochem Cytochem*. 2006;54:713-725.
5. Cvikl B, Agis H, Stögerer K, Moritz A, Watzek G, Gruber R. The response of dental pulp-derived cells to zoledronate depends on the experimental model. *Int Endod J*. 2011;44:33-40.
6. Pourgonabadi S, Mousavi SH, Tayarani-Najaran Z, Ghorbani A. Effect of zoledronate, a third-generation bisphosphonate, on proliferation and apoptosis of human dental pulp stem cells. *Can J Physiol Pharmacol*. 2018;96:137-144.
7. Komatsu K, Shimada A, Shibata T, et al. Long-term effects of local pretreatment with alendronate on healing of replanted rat teeth. *J Periodontal Res*. 2008;43:194-200.
8. Camargo Moraes P, Silva CAB, Soares AB, et al. Tooth alterations in areas of bisphosphonate-induced osteonecrosis. *Clin Oral Investig*. 2015;19:489-495.
9. Mutoh N, Tani-Ishii N, Tsukinoki K, Chieda K, Watanabe K. Expression of Toll-like receptor 2 and 4 in dental pulp. *J Endod*. 2007;33:1183-1186.
10. Veerayutthwilai O, Byers MR, Pham T-TT, Darveau RP, Dale BA. Differential regulation of immune responses by odontoblasts. *Oral Microbiol Immunol*. 2007;22:5-13.
11. Elsalhy M, Azizieh F, Raghupathy R. Cytokines as diagnostic markers of pulpal inflammation. *Int End J*. 2013;46:573-580.
12. Martini G, Gennari L, Merlotti D, et al. Serum OPG and RANKL levels before and after intravenous bisphosphonate treatment in Paget's disease of bone. *Bone*. 2007;40:457-463.
13. Morikawa T, Matsuzaka K, Nakajima K, Yasumura T, Sueishi K, Inoue T. Dental pulp cells promote the expression of receptor activator of nuclear factor-κB ligand, prostaglandin E2 and substance P in mechanically stressed periodontal ligament cells. *Arch Oral Biol*. 2016;70:158-164.
14. Bradaschia-Correa V, Massa LF, Arana-Chavez VE. Effects of alendronate on tooth eruption and molar root formation in young growing rats. *Cell Tissue Res*. 2007;330:475-485.
15. Silva PG, Ferreira Junior AE, Teófilo CR, et al. Effect of different doses of zoledronic acid in establishing of bisphosphonate-related osteonecrosis. *Arch Oral Biol*. 2015;60:1237-1245.
16. Jacobs C, Schramm S, Dirks I, et al. Mechanical loading increases pro-inflammatory effects of nitrogen-containing bisphosphonate in human periodontal fibroblasts. *Clin Oral Investig*. 2018;22:901-907.
17. Sharma D, Ivanovski S, Slevin M, et al. Bisphosphonate-related osteonecrosis of jaw (BRONJ): diagnostic criteria and possible pathogenic mechanisms of an unexpected anti-angiogenic side effect. *Vasc Cell*. 2013;5:1.
18. Gschwantler-Kaulich D, Weingartshofer S, Grunt TW, et al. Estradiol impairs the antiproliferative and proapoptotic effect of Zoledronic acid in hormone sensitive breast cancer cells in vitro. *PLoS One*. 2017;12:1-13.
19. Noguchi F, Kitamura C, Nagayoshi M, Chen KK, Terashita M, Nishihara T. Ozonated water improves lipopolysaccharide-induced responses of an odontoblast-like cell line. *J Endod*. 2009;35:668-672.
20. Valenti MT, Giannini S, Donatelli L, et al. The effect of risedronate on osteogenic lineage is mediated by cyclooxygenase-2 gene upregulation. *Arthritis Res Ther*. 2010;12:1-13.
21. Lin PS, Cheng RH, Chang MC, et al. TGF-β1 stimulates cyclooxygenase-2 expression and PGE2 production of human dental pulp cells: role of ALK5/Smad2 and MEK/ERK signal transduction pathways. *J Formos Med Assoc*. 2017;116:748-754.
22. Walia B, Wang L, Merlin D, Sitaraman SV. TGF-beta down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2. *FASEB J*. 2003;17:2130-2132.
23. Soares DG, Basso FG, Scheffel DS, Hebling J, De Souza Costa CA. Responses of human dental pulp cells after application of a low-concentration bleaching gel to enamel. *Arch Oral Biol*. 2015;60:1428-1436.
24. Krifka S, Seidenader C, Hiller KA, Schmalz G, Schweik H. Oxidative stress and cytotoxicity generated by dental composites in human pulp cells. *Clin Oral Investig*. 2012;16:215-224.
25. Farges JC, Bellanger A, Ducret M, et al. Human odontoblast-like cells produce nitric oxide with antibacterial activity upon TLR2 activation. *Front Physiol*. 2015;6:1-9.
26. Hojo K, Tamai R, Kobayashi-Sakamoto M, Kiyoura Y. Etidronate down-regulates Toll-like receptor (TLR) 2 ligand-induced proinflammatory cytokine production by inhibiting NF-κB activation. *Pharmacol Rep*. 2017;69:773-778.
27. Horst OV, Tompkins KA, Coats SR, Braham PH, Darveau RP, Dale BA. TGF-β1 inhibits TLR-mediated odontoblast responses to oral bacteria. *J Dental Res*. 2009;88:333-338.
28. Zhao B, Ivashkiv LB. Negative regulation of osteoclastogenesis and bone resorption by cytokines and transcriptional repressors. *Arthritis Res Ther*. 2011;13:1-10.
29. Wang D, Gilbert JR, Shaw MA, et al. Toll-like receptor 4 mediates the regenerative effects of bone grafts for calvarial bone repair. *Tissue Engineer Part A*. 2015;21:1299-1308.
30. Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Biochem Biophys*. 2008;473:139-146.
31. Xiong H, Wei L, Hu Y, Zhang C, Peng B. Effect of alendronate on alveolar bone resorption and angiogenesis in rats with experimental periapical lesions. *Int Endod J*. 2010;43:485-491.
32. Kang B, Cheong S, Chaichanasakul T, et al. Periapical disease and bisphosphonates induce osteonecrosis of the jaws in mice. *J Bone Miner Res*. 2013;28:1631-1640.
33. Pan KQ, Zhang PM, Deng J, Lou XX, Meng Y, Liu GR. Expression of OPG/RANK/RANKL in the rat dental pulp tissue of periodontitis combined with vascular calcification and its clinical significance. *Shanghai Kou Qiang Yi Xue*. 2016;25:391-395.
34. Lin YP, Love RM, Friedlander LT, Shang HF, Pai MH. Expression of Toll-like receptors 2 and 4 and the OPG-RANKL-RANK system in inflammatory external root resorption and external cervical resorption. *Int Endod J*. 2013;46:971-981.

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