Effects of Rosiglitazone on the Outcome of Experimental Periapical Lesions in Mice

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Abstract

Introduction: The purpose of this study was to evaluate a protocol for systemic administration of rosiglitazone in mice in order to stimulate apoptosis of osteocytes in the jaws and to evaluate the effect of osteocyte apoptosis induced by rosiglitazone in the progression of periapical lesions in mice at 7, 21, and 42 days.

Methods: C57BL/6 mice at 4–5 weeks of age were used. In phase 1, mice (n = 24) were treated with rosiglitazone (gavage, 10 mg/kg dose) or without (phosphate-buffered saline + 10% dimethyl sulfoxide) for 1, 2, or 3 weeks. We used the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling and 4′,6-diamidino-2-phenylindole methods for quantification of apoptotic cells. In phase 2, mice (n = 30) received rosiglitazone for 2 weeks or just vehicle for 1 week (n = 30), and periapical lesions were induced for 7, 21, or 42 days. We performed the measurement of periapical lesions, tartrate-resistant acid phosphatase staining, dual-energy x-ray absorptiometry for the evaluation of bone mineral density (BMD) in long bone, and gene evaluation using real time quantitative polymerase chain reaction of osteocyte markers (Sost, Hyou1, and Dmp1) and receptor activator of nuclear factor kappa-B ligand (RANKL) (Tnfsf11). Results: It was observed that systemic administration of rosiglitazone for 2 weeks showed apoptosis of osteocytes in a more expressive manner. In phase 2, in the groups that received rosiglitazone (gavage, 10 mg/kg dose) or without (phosphate-buffered saline) for 1, 2, or 3 weeks, we observed significant differences in RANKL and Dmp1 expression or in the BMD of femurs. Conclusions: Rosiglitazone stimulated apoptosis of osteocytes, interfering in the progression of periapical lesions in mice. (J Endod 2017;43:2061–2069)

Key Words

Apoptosis, gene expression, mice, microscopic analysis, osteocytes, periapical lesion, rosiglitazone

Significance

Rosiglitazone presents side effects in bone cells. To date, the effect of rosiglitazone-stimulated osteocyte apoptosis in the formation and progression of periapical lesions has not been studied. We investigated the effect of rosiglitazone on periapical lesions in mice.

Effects of Rosiglitazone

The periapical lesion develops from the bacterial invasion in the pulp tissue, which triggers a chronic infectious and immunoinflammatory process, resulting in resorption in the apical and periapical tissues. This is characterized by an imbalance between the number and virulence of the microorganisms present in the root canal system with respect to host resistance (1). The immunoinflammatory process in lesions of endodontic origin involves the participation of different cell types such as the activation of endothelial cells, neutrophils, macrophages, lymphocytes, and osteoclasts simultaneously contributing to the destruction of bone tissue (2).

Osteocytes represent the most abundant cell type in bone tissue (approximately 95% of the cells present) and have been considered for a long time as static or inactive cells with limited physiological and pathological interest, usually characterized by their morphology and location and rarely by their function (3). In addition, the presence of osteocytes in the mineralized bone matrix makes the study of these cells technically difficult, which represents a challenge not yet completely solved (4).

Osteocytes are considered the orchestrators of many bone functions because they are indispensable for adult skeletal homeostasis (3) and contribute to the regulation of the availability of calcium in the mature skeleton by extracting this mineral from the perilacunar space (5). In addition, they are considered the initiator and the center of the process of bone remodeling (6), being the main factor responsible for the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) (osteoclastogenesis activating cytokine) and sclerostin (inhibitor of osteoblasts) (7).

The protein sclerostin, encoded by the Sost gene, is related to the differentiation of osteoblasts into mature osteocytes (8) and stimulates osteoclastogenesis by increasing RANKL expression (9). In addition to sclerostin, among the proteins expressed by osteocytes, we can highlight the protein of the dentin matrix 1 (DMP1) and the protein regulated by oxygen 150 (ORP150). DMP1 was originally identified in the teeth as an acid extracellular matrix protein (10) and was subsequently identified as being...
highly expressed in odontoblasts and osteoblasts/osteocytes (11). In turn, ORP150 has recently been studied for playing an important role in cell adaptation to hypoxia and in the prevention of cell apoptosis in various tissue types. This protein is present in mature osteocytes embedded in the mineralized bone matrix (12).

Side effects in bone tissue cells have been reported after the use of a class of drugs indicated for the treatment of type 2 diabetes mellitus called thiazolidinediones (TZDs) (13–17). Rosiglitazone, which belongs to this class of drugs, is a synthetic agonist with high affinity for the peroxisome proliferator-activated receptor gamma. This receptor is involved in the differentiation of progenitor cells into adipocytes or osteoblasts, which can subsequently be differentiated into osteocytes. Thus, the continuous use of rosiglitazone may stimulate osteocyte apoptosis (13, 16, 17). However, to date, the effect of rosiglitazone-stimulated osteocyte apoptosis in the formation and progression of periapical lesions has not been studied.

Thus, the objective of the present study was to evaluate in vitro a protocol of systemic administration of rosiglitazone in mice in order to stimulate the apoptosis of osteocytes in the jaws and the formation and progression of periapical lesions in different experimental periods (7, 21, and 42 days) induced in wild-type (C57BL/6) mice with or without rosiglitazone.

Materials and Methods

All the procedures performed on the animals were conducted according to the ethical guidelines of the Animal Use Ethics Committee of the University of São Paulo, Ribeirão Preto, São Paulo, Brazil (# 2014.1.450.58.8), and based on the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for the reporting of animal studies (18). Male C57BL/6 mice, 4–5 weeks old and weighing on average 15 g, were purchased from the Central Facility of the University of São Paulo (Ribeirão Preto campus). The animals were kept in polypropylene cages (5 animals per cage) with a temperature of 22°C ± 2°C and air relative humidity of 55% ± 10% in a dark-light cycle of 12:12 hours in the Facility of the School of Dentistry of Ribeirão Preto, University of São Paulo, with food and water ad libitum.

Phase 1: Definition of a Protocol of Systemic Administration of Rosiglitazone to Stimulate Apoptosis Osteocytes in Mice Jaws

For this phase, 24 mice were divided into 4 groups and submitted to the effects of rosiglitazone (Sigma-Aldrich, St Louis, MO). The animals were randomized in a simple way using a random number table. The administration protocol was based on previous studies (13–15). The oral route (gavage, final volume of 165 μL/mice/dose) at 10 mg/kg body weight (phosphate-buffered saline [PBS] + 10% dimethyl sulfoxide [DMSO] as vehicle) daily (1 dose/day) was used for 1, 2, or 3 weeks in order to identify the best period of administration of rosiglitazone capable of stimulating apoptosis of osteocytes more significantly in the jaws. Table 1 shows the distribution of the experimental groups according to the solution and periods given to the mice.

### TABLE 1. Description of the Experimental Groups and Drug Administration

<table>
<thead>
<tr>
<th>Groups</th>
<th>Solution</th>
<th>Drug administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Vehicle (PBS + 10% DMSO) (control group)</td>
<td>1 week</td>
</tr>
<tr>
<td>G2</td>
<td>Rosiglitazone (10 mg/kg) + PBS + 10% DMSO</td>
<td>1 week</td>
</tr>
<tr>
<td>G3</td>
<td>Rosiglitazone (10 mg/kg) + PBS + 10% DMSO</td>
<td>2 weeks</td>
</tr>
<tr>
<td>G4</td>
<td>Rosiglitazone (10 mg/kg) + PBS + 10% DMSO</td>
<td>3 weeks</td>
</tr>
</tbody>
</table>

DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline.

Histotechnical Processing

After the administration periods of rosiglitazone for each group, we waited 42 days to simulate a late period of assessment of the periapical lesion, and the animals were killed by means of anesthesia with ketamine hydrochloride (ketamine 10% [150 mg/kg body weight]; Agener União Química Farmacêutica Nacional S/A, Embu-Guacu, SP, Brazil) and xylazine 2% (7.5 mg/kg body weight) (Dopaser; Laboratories Callier SA, Barcelona, Spain) and inhalation of CO₂ in a specific chamber. After euthanasia, the mandibles were removed with sterile surgical scissors and divided in the incisor region. The hemiarcades were fixed by immersion in 10% buffered formalin for 24 hours at room temperature and washed for approximately 4 hours in running water. Then, for decalcification, a solution based on EDTA at 4.13% (pH = 7–7.4) was used. The samples were kept in this solution at room temperature and changed weekly until their complete decalcification, which occurred after a period of approximately 30 days. The degree of decalcification of the mineralized structures was tested by inserting a needle into the tissues to verify their consistency. Afterward, the samples were washed in running water for 2 hours, dehydrated in alcohol of increasing concentrations (70% and 95% for 30 minutes each, 2 changes of 100% for 20 minutes each, and two 100% alcohol exchanges for 40 minutes each), diaphanization in xylene (2 baths of 20 minutes and 1 of 40 minutes), and inclusion in paraffin. The blocks containing the hemiarches were cut longitudinally into a microtome (Leica RM2145; Leica Microsystems GmbH, Wetzlar, Germany) in the mesiodistal direction. Semiserial cuts of 5 μm were obtained including the first and second molars and the alveolar bone tissue. Ten to fifteen slides were obtained, with 3 cuts in each slide (19).

The Terminal Deoxynucleotidyl Transferase-mediated Deoxyuridine Triphosphate Nick End Labeling Method

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method was performed using a kit for the detection of apoptotic cell-derived DNA fragments (Apop Tag-Plus Kit; Oncor Inc, Gaithersburg, MD) according to the manufacturer’s protocol. The cuts were pretreated in 20 μg/mL protease K (protein-digesting enzyme, Oncor Inc) for 15 minutes at 37°C and, after several washes in distilled water, were immersed in 3% hydrogen peroxide in PBS, 50 mmol/L sodium (pH = 7.4), and 200 mmol/L sodium chloride for 15 minutes and then immersed in kit-specific equilibration buffer. After incubation in terminal deoxynucleotidyl transferase enzyme at 37°C for 1 hour in a humidified chamber, the reaction was stopped by immersion in stop/wash buffer at 37°C for 15 minutes. The sections were then washed in PBS for 10 minutes and incubated in antidigoxigenin peroxidase at 37°C for 1 hour in a humidified chamber. After washing in PBS, the sections were treated with 0.06% solution of 3,3,5-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) in the presence of 0.1% hydrogen peroxide for 5 to 10 minutes at room temperature. The sections were counterstained with hematoxylin. As a positive control, slides with cuts of mammary glands from the kit were used. Negative controls were obtained by omission of the terminal deoxynucleotidyl transferase enzyme (20).
4′-6-diamidino-2-phenylindole Staining

Staining with 4′-6-diamidino-2-phenylindole (DAPI) was used to analyze the condensation of DNA of the apoptotic cells. The dewaxed slides were washed 3 times with PBS for 5 minutes each, waterproofed with 0.5% PBS-Triton, and washed again 3 times with PBS for 5 minutes each. They were then incubated with 0.25 μg/mL DAPI dye in phosphate buffer for approximately 5 minutes at room temperature in a dark environment.

Quantification of Apoptotic Cells

All analyses were performed under a conventional optical microscope (Carl Zeiss MicrolImaging GmbH, Göttingen, Germany). Data were analyzed by a single experienced examiner blinded to the groups. No filter was used for the TUNEL analysis, whereas a blue specific microscope filter (DAPI, Carl Zeiss MicrolImaging GmbH) was used for the visualization of DAPI-stained slides. Five to 6 random microscopic fields were analyzed both in the alveolar bone between the molars and below their apexes in the jaws of each animal for cell counting (630× magnification).

The ratio of apoptotic osteocytes/total osteocytes in each field was calculated using Image J software (Version 1.49u; National Institutes of Health, Bethesda, MD). The mean of 2 slide ratios for each animal was calculated, and the values of all animals per group, at each drug administration period, were considered for statistical analysis.

TUNEL-positive osteocytes were considered when stained with brown color, showing disintegration or fragmentation of the nucleus. DAPI-positive osteocytes were considered when their nuclei had defined fluorescent spots, indicating the condensation of DNA in the nucleus. Results were expressed as the mean percentage of apoptotic osteocytes/total osteocytes (±standard deviation) for each group.

Phase 2: Evaluation of Progression of Induced Periapical Lesions in Mice with or without Rosiglitazone

Induction of Periapical Lesions. In the second phase of the study, after defining the best experimental period for the administration of rosiglitazone for the induction of osteocyte apoptosis in mice jaws (rosiglitazone 10 mg/kg + PBS + 10% DMSO once a day), periapical lesions were induced in the first lower molars of 60 mice. The animals were anesthetized intramuscularly with ketamine hydrochloride A (ketamine 10% [150 mg/kg body weight], Agener União Química Farmacêutica Nacional S/A) and 7.5 mg/kg xylazine 2% (Dopaser) before performing the operative procedures. To allow adequate visualization and easy access to the lower molars, a specific surgical table was used in which the animals were positioned to allow the maintenance of the buccal opening and correct visualization of the teeth.

A classic model for the induction of periapical lesion in rodents was used. The pulp tissues of the lower (left and right) mice first molars were exposed to the microbiota of the buccal cavity with the aid of a low-rotation spherical dental drill (#¼; KG Sorensen, Cotia, SP, Brazil) (Fig. 1A) coupled to a motor handpiece (Dabi Atlante Equipamentos Odontológicos, Ribeirão Preto, SP, Brazil) until root canal access was seen and verified with a #8-K endodontic file (Maillefer SA, Ecublens, Switzerland) (Fig. 1B). The teeth were exposed to the oral cavity to promote contamination by the oral microbiota. After 7, 21, and 42 days, the animals were killed through intramuscular anesthesia with ketamine and xylazine and inhalation of CO2 in a specific chamber. The animals (N = 60) were randomized using a random number table and divided into 6 groups according to administration with rosiglitazone (10 mg/kg + PBS + 10% DMSO) or without (PBS + 10% DMSO) and the period of periapical lesion development (7, 21, or 42 days).

Figure 1. (A) Coronal opening of the lower molar with a low-rotation spherical drill. (B) Verification of the entrance of the root canals with a type K endodontic file #8.
After euthanasia of the animals, the mandibles were removed with sterile surgical scissors and divided in the region of incisors, obtaining 2 hemimandibles. The left one was submitted to histotechnical processing, whereas the right one was stored in RNA later solution (Life Technologies, Carlsbad, CA) for evaluation through real time quantitative polymerase chain reaction (qRT-PCR) technique.

**Histotechnical Processing and Microscopic Evaluation.**

The left hemimandibles were fixed and submitted to routine histotechnical processing as previously described. Initially, the representative sections of each experimental group were stained with hematoxylin-eosin (HE) and submitted to conventional light microscopy for description of the apical and periapical regions. Additionally, analysis of the periapical lesion area was performed under fluorescence microscopy. All analyses were performed by a single expert evaluator with no previous knowledge of the group to be analyzed using the Axio Imager. M1 microscope (Carl Zeiss MicroImaging GmbH) attached to a camera (AxioCam MRC5, Carl Zeiss MicroImaging GmbH). The distal roots of the lower first molars were evaluated in sections showing simultaneously the coronary, middle, and apical thirds of the root canal, the apical foramen, and the alveolar bone.

**Descriptive Microscopic Analysis of Periapical Region Characteristics.**

Descriptive analysis of the apical and periapical regions was performed in the representative sections stained in HE of each period and in each experimental group, mainly evaluating the periodontal ligament, apical cementum (characteristics of the surface), and alveolar bone (the presence of resorption and characteristics of the lacunae).

**Morphometry in Fluorescence Microscopy.**

Morphometric evaluation of the periapical lesions was performed in the HE-stained slides using the Axio Imager. M1 microscope (10 × magnification) operating in the fluorescence mode (Alexa Fluor 488 filter, Carl Zeiss MicroImaging GmbH) with G365 excitation, FT395 reflectors, and LP420 emission. The area of the periapical lesions was delineated and measured in μm² using specific software (AxioVision Rel. v 4.8, Carl Zeiss MicroImaging GmbH). We evaluated 2 to 3 central sections of each specimen, obtaining the mean for each animal. Delimitation of the lesion excluded intact structures (periodontal ligament, cementum, and alveolar bone), easily identified by strong green fluorescence, as well as areas of resorption, fibrillar dissociation, and inflammatory infiltrate, identified by darkened appearance and the absence of fluorescence (26).

**TRAP Histoenzymology.**

The activity of tartrate-resistant acid phosphatase (TRAP) was used for the labeling of multinucleated giant cells. The sections were dewaxed (2 xylol baths of 5 minutes each) and hydrated (100% alcohol twice for 5 minutes each; 95%, 70%, and 50% alcohol for 2 minutes each; and distilled water for the same period). The slides were then placed in 50% alcohol/aceton solution for 1 minute and air dried. Then, a solution of 10 mL acetic acid buffer, 0.1 mL dimethylformamide, 5 mg Fast Red (Sigma-Aldrich), and 1 mL naphthol AS-BI phosphoric acid (Sigma-Aldrich) was pipetted onto slides protected from light in an oven at 57°C for enough time to observe the labeling (approximately 1 minute). After incubation, the counterstaining with Fast Green (Merck KGaA, Darmstadt, Hessen, Germany) was performed for 1 minute. The slides were then assembled and evaluated under Axio Imager. M1 under conventional light for counting the number of TRAP-positive cells present in resorption Howship lacunae in contact with the alveolar bone surrounding the periapical lesion and then expressed as the total number of cells (19).

**Technology of Dual-energy X-ray Abstractometry.**

After euthanasia, the femurs of the animals in all groups were surgically removed. A femur from each animal was analyzed using the Lunar PIXImus dual-energy X-ray absorptiometer (Lunar PIXImus Corp Head-quarters, Madison, WI) for recording bone mineral density (BMD) in g/cm² of the distal femoral metaphysis. Data were analyzed using Lunar PIXImus software (2.2v, Lunar PIXImus Corp Headquarters) (27). The groups were compared with each other to investigate whether the administration of rosiglitazone affected long bone BMD.

**qRT-PCR Array.**

The right jaws were submitted to a specific kit for the isolation of nucleic acids (total RNA extraction) (PureLink RNA Mini Kit; Ambion, Life Technologies, Carlsbad, CA). The procedures were performed according to the manufacturer’s protocol. The extracted RNA content was measured in a spectrophotometer with a wavelength of 260 nm. Subsequently, RNA was converted into complementary DNA with the use of a kit (High Capacity CDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Briefly, messenger RNAs of Dmp1 (DMP1 gene), Sost (sclerostin gene), HYOU1 (ORP150 gene), and TGFβ1 (RANKL gene) were evaluated. Primers for the amplification of the osteocyte genes in real-time polymerase chain reaction (Applied Biosystems). GAPDH and Actb were used as reference genes. qRT-PCR reactions were performed in duplicate using SYBR Green reagent (Applied Biosystems). Relative quantification was performed using the 2^-ΔΔCt method.

**Statistical Analysis.**

All statistical analyses (of both phases) were performed using Graph Pad Prism 4 software (Graph Pad Software Inc, San Diego, CA). A significance level of 5% was established for all analyses. Once data presented a normal distribution (the Shapiro-Wilk test), the 1-way analysis of variance (ANOVA) test was used followed by the more appropriate multiple-choice posttest suggested by the software (Sidak or Tukey). For phase 1, comparisons were made between all the groups evaluated. For phase 2, comparisons were made between groups that did not receive rosiglitazone (control 7 days, control 21 days, and control 42 days) at different periods of periapical lesion progression and groups that did receive rosiglitazone (rosiglitazone 7 days, rosiglitazone 21 days, and rosiglitazone 42 days) along with comparison between the experimental periods (7, 21, and 42 days).

**Results**

**Phase 1: Definition of a Protocol of Systemic Administration of Rosiglitazone to Stimulate Osteocyte Apoptosis in Mice Jaws**

The control group showed a significantly lower proportion of apoptotic osteocytes/total osteocytes when compared with the experimental groups, independent of the evaluated technique (TUNEL or DAPI). TUNEL-stained sections showed approximately 17% (0.174 ± 0.04) of TUNEL-positive osteocytes in the control group, whereas the right one was submitted to a specific kit for the isolation of nucleic acids (total RNA extraction) (PureLink RNA Mini Kit; Ambion, Life Technologies, Carlsbad, CA). The procedures were performed according to the manufacturer’s protocol. The extracted RNA content was measured in a spectrophotometer with a wavelength of 260 nm. Subsequently, RNA was converted into complementary DNA with the use of a kit (High Capacity CDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Briefly, messenger RNAs of Dmp1 (DMP1 gene), Sost (sclerostin gene), HYOU1 (ORP150 gene), and TGFβ1 (RANKL gene) were evaluated. Primers for the amplification of the osteocyte genes in real-time polymerase chain reaction (Applied Biosystems). GAPDH and Actb were used as reference genes. qRT-PCR reactions were performed in duplicate using SYBR Green reagent (Applied Biosystems). Relative quantification was performed using the 2^-ΔΔCt method.

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**Phase 2: Evaluation of Progression of Induced Periapical Lesions in Mice with or without Rosiglitazone**

**Descriptive Microscopic Analysis of Periapical Region Characteristics.**

In the control groups, it was possible to observe that, after 7 days of pulp exposure, the cemental root surface was...
Morphometry in Fluorescence Microscopy.

The HE slides areas, and increased periodontal ligament (Fig. 3 periodontal ligament was amply increased, with the presence of mixed and mononuclear inflammatory cells. The alveolar bone had resorption areas on its surface. On the other hand, at 42 days, the surface of the cementum had extensive resorption areas, and the periodontal ligament was enlarged, with edema, dissociation of collagen fibers, and predominantly mononuclear inflammatory cells. The alveolar bone had resorption areas on its surface. On the other hand, at 42 days, the surface of the cementum had extensive resorption areas, and the periodontal ligament was amply increased, with the presence of mixed and dense inflammatory infiltrate, alveolar bone with reabsorption areas, and increased periodontal ligament (Fig. 3A, C, and E). In the 2-week rosiglitazone groups, at 7 days, regularity on the surface of the cementum, the presence of few inflammatory cells, and regular alveolar bone were also observed. At 21 days, several areas of cementum and alveolar bone resorption could be observed, with the presence of osteoclasts in Howship lacunae. At 42 days, the periodontal ligament was severely enlarged, with extensive areas of edema, fibrillar dissociation, and predominantly mononuclear inflammatory cells. The surface of the cement had several resorption areas with some empty lacunae. The alveolar bone was severely reabsorbed with osteoclasts in the Howship lacunae quite distant from the apex because of the presence of the inflammatory process in the apical region (Fig. 3G, I, and K).

Morphometry in Fluorescence Microscopy. The HE slides were observed under a fluorescence microscope filter. In the control groups, a gradual increase during the periods of 7, 21, and 42 days after periapical lesion induction was observed. The mean values of the lesion area were 65042.21 μm², 110623.76 μm², and 294696.22 μm², respectively. Statistical analysis performed using 1-way ANOVA and post-test of multiple comparisons of Sidak showed a statistically significant difference between the periods of 7 and 42 days and 21 and 42 days (P < .05) (Fig. 3B, D, and F). In the rosiglitazone groups, the mean values of the lesion area were 66501.52 μm², 169480.66 μm², and 328520.37 μm², respectively, for the periods of 7, 21, and 42 days. In these groups, it was also observed that the progression of the periapical lesions presented a time-dependent pattern. A statistically significant difference was found by the posttest of multiple comparisons of Sidak also between the periods of 7 and 42 days and 21 and 42 days (P < .05) (Fig. 3H, J, and L). In comparison with the control groups, there was a trend toward greater lesions in the rosiglitazone groups but without a statistically significant difference (P > .05) (Fig. 3S).

Figure 2. (A) A representative image of the 2-week rosiglitazone group using the TUNEL method. The red arrows indicate nonapoptotic osteocytes with healthy nuclei and blue coloration. The yellow arrows indicate apoptotic osteocytes with a brownish coloration. (B) A representative image of the 2-week rosiglitazone group using the DAPI technique. The red arrows indicate nonapoptotic osteocytes with healthy (homogeneously fluorescent or nonfluorescent) nuclei, whereas yellow arrows indicate areas of nuclei DNA condensed seen by defined fluorescent spots on apoptotic osteocytes. The bar in the upper right corner of the figures = 20 μm. Graphic representation of apoptotic osteocytes/total osteocytes in the control and experimental groups, respectively, evaluated by the (C) TUNEL and (D) DAPI techniques. #A statistically significant difference between the control group and all other groups. *A statistically significant difference between the 1-week rosiglitazone and 2-week rosiglitazone groups (P < .05).
Figure 3. (A–F) Photomicrographs of microscopic sections of the measurement of periapical lesions in animals in the control and experimental groups at (A and B) 7 days, (C and D) 21 days, and (E and F) 42 days. (A, C, and E) HE-stained sections and (B, D, and F) the same sections observed in the fluorescent mode. (G–L) Photomicrographs of microscopic sections of the measurement of periapical lesions in animals with rosiglitazone at (G and H) 7 days, (I and J) 21 days, and (K and L) 42 days. (G, I, and K) HE-stained sections and (H, J, and L) the same sections observed in the fluorescent mode. (M–R) Photomicrographs of the microscopic sections of the (M, O, and Q) control and (N, P, and R) rosiglitazone groups at 7, 21, and 42 days stained by TRAP for osteoclast counting (HE 10×, Carl Zeiss MicroImaging GmbH). The bar in the upper right corner of each photomicrograph = 50 μm. (S) Graphic representation of the values obtained after the measurement of the periapical lesions experimentally induced in the control animals and those that received rosiglitazone after the experimental periods of 7, 21, and 42 days. (T) Graphic representation of the comparison of osteoclast counts between groups. *A statistical difference (P < .05).
Dual-energy X-ray Abstractometry. Figure 4A shows the BMD values of the distal femoral metaphysis of the mice obtained by the dual-energy X-ray abstractometry technique in the control and rosiglitazone groups. It was not possible to observe a statistically significant difference between groups (P > .05) showing that the systemic use of rosiglitazone for 2 weeks did not stimulate the apoptosis of osteocytes in long bone.

qRT-PCR Array. Comparing the groups with or without rosiglitazone, RT-PCR analysis showed that Sost expression was higher in the 21-day period in the rosiglitazone group, showing a trend to decrease according to the progression of the lesion (P < .05) (Fig. 4B). Regarding the expression of the Hyou1 gene, a greater expression was observed when comparing the control with the rosiglitazone groups at 21 days (P < .05) (Fig. 4C). For the Dmp1 gene, it was not possible to find a statistically significant difference when comparing the control with the rosiglitazone groups; however, a decrease in expression was observed between the groups of 7 and 21 days and 7 and 42 days in the control groups (P < .05) (Fig. 4D). For the Tnfsf11 gene, it was not possible to find a statistically significant difference when comparing the control with the rosiglitazone groups (Fig. 4E).

Discussion

In the present study, the first aim was to evaluate a protocol of systemic administration of rosiglitazone to stimulate the apoptosis of osteocytes in mice jaws. The administration of rosiglitazone in mice to induce apoptosis of osteocytes is not very well established in the literature and has been described only in a few studies (13, 14, 28). Therefore, after phase 1 of this study, it was decided to give rosiglitazone for 2 weeks to the mice because osteocyte apoptosis occurred more significantly when compared with administration for 1 week without a significant difference when compared with the 3-week period.

The progression of the periapical lesion was evaluated for 7, 21, and 42 days in mice with or without rosiglitazone for 2 weeks. TZD administration was used as a form of osteocyte apoptosis induction because this effect has already been reported previously in the literature (13, 16, 17). The initial hypothesis was that the animals that received TZD would present larger periapical lesions because the apoptosis of osteocytes leads to a greater recruitment of osteoclasts to the region, triggering greater bone destruction (29). It was possible to observe a gradual increase in the area of the lesions with their progression in the control and rosiglitazone groups. A trend toward greater lesions.
in the groups receiving rosiglitazone was observed but without a statistically significant difference ($P > .05$). Thus, it is noteworthy that the rate of osteocyte apoptosis observed in jaws induced by oral rosiglitazone for 2 weeks was not sufficient to statistically alter the size of the periapical lesion. From this finding, 2 hypotheses seem to arise. The first is that apoptosis of osteocytes does not actually interfere in the development of the periapical lesion and, second, that the rate of osteocyte apoptosis observed in the present study was not sufficient to influence the development of the periapical lesion. In addition, it is worth mentioning that osteocyte death could stimulate, besides alteration in the cytokine profile expressed by this cellular type, compensatory mechanisms in the context of periapical lesion development.

The number of osteoclasts adjacent to the periapical lesion was also evaluated. It was observed that, similarly to the pattern observed in the measurement of periapical lesions, a progressive increase in the number of osteoclasts was observed throughout the periods in the control and rosiglitazone groups, with more osteoclasts present in the group receiving rosiglitazone at 21 days ($P > .05$). This finding corroborates studies that have shown increased bone loss in mice after the administration of TZD (14, 28) and increased osteoclastogenesis. It has been suggested that apoptotic osteocytes signal to neighboring osteocytes in order to increase RANKL expression, leading to increased osteoclastogenesis and bone resorption (29). Although it did not present a statistically significant difference, in the period of 42 days, an increase in the number of osteoclasts was also observed in the rosiglitazone group. This may have occurred because, in the face of an infection, the organism initially responds more expressively in an attempt to combat it followed by a period of “accommodation” or “adaptation” of this process.

Regarding the evaluation of the femurs’ BMD, no significant difference was observed between the groups despite a tendency to decrease in the rosiglitazone groups ($P > .05$). This finding may be because of the fact that, for osteocyte apoptosis to have a massive effect on bone mineral density in long bones, a longer period of administration of the drug would be necessary because Broulik et al (28) reported a significant decrease of the BMD of mice after the administration of rosiglitazone for 9 weeks compared with control mice.

The study of osteocytes still presents some difficulties, as previously mentioned, and the interest in the study of these cells has been widely awakened in recent years (30). Some studies report heterogeneity in the expression patterns of osteoblast and osteocyte proteins and genes, raising the possibility of specific subpopulations of these cell types, with different final destinations (31, 32). Thus, several markers of osteocytes have been described, such as E11/gp38, CD44, MEPE, PHEx, FGF-23, and CapG, but without well-established definition for proteins expressed strictly by osteocytes to be used as isolated markers.

Osteocytes are an abundant source of sclerostin, the main inhibitor of osteoblast activity (33), and stimulate the formation of clasts by increasing RANKL expression. Using RT-PCR evaluation, we observed that the expression of Sost was higher at 21 days in the rosiglitazone group and was accompanied by more osteoclasts, which is in agreement with findings in the literature (9).

A recent study evaluated the expression of ORP150, a protein expressed by osteocytes, mediated by conditions of hypoxia in vitro. The authors reported a relative increase in ORP150 messenger RNA, which supports its cytoprotective role under oxygen deprivation conditions (12). As in the literature, our results showed that at 21 days there was an increase in Hyou1 expression in the rosiglitazone group. Regarding the expression of Dmp1, we found no significant difference between the control or rosiglitazone groups. This finding may have occurred because DMP1 is considered a marker of young osteoblasts not yet fully embedded in the mineralized matrix, and, therefore, they may not have been influenced by rosiglitazone administered for 2 weeks (8).

The findings of the present study are in agreement with the literature because it has been shown that TZDs present an increase in osteoclastogenesis (13, 34) and sclerostin expression (16) and bone loss in mice by means of osteoblast and bone formation suppression (14).

One mechanism by which rosiglitazone may stimulate osteocyte apoptosis is the induction of the differentiation of progenitor cells into adipocytes in detriment to osteoblasts, as suggested by previous studies (13). However, in the present study, the body weight of mice registered for an initial pilot phase did not present a significant difference between the control and rosiglitazone groups (data not shown).

Our study cannot be directly compared with others in the literature because there are no reports of the evaluation of osteocytes during the development of periapical lesions. However, evaluating the participation of osteocytes in the immune-inflammatory process, Pesce-Viglietti et al (35) evaluated whether Brucella abortus infection would modify osteocyte function. The authors reported that the infection could alter the function of osteocytes, contributing to damage in the bone tissue. Thus, more studies are needed in this context to try to elucidate the participation of osteocytes during the installment and progression of chronic infectious processes of the oral cavity, such as in endodontic infection and periapical lesions.

Conclusion

From the results of phase 1 of this study, we conclude that 2 weeks, as compared with 1 week and 3 weeks, is the optimum time period for the systemic administration of rosiglitazone to stimulate apoptosis of osteocytes in mice. In phase 2 of the study, the systemic administration of rosiglitazone for 2 weeks altered the number of osteoclasts and the expression of the Sost and Hyou1 genes without affecting the expression of Dmp1 and Tnfsf11. It also contributed to a tendency toward greater periapical lesions and lower BMD in long bones. These results lead us to conclude that within the constraints of the parameters and methodologies used in this study rosiglitazone interfered with the progression of induced periapical lesions in mice.

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