Archives of Oral Biology 102 (2019) 147–154

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Teriparatide improves alveolar bone modelling after tooth extraction in orchiectomized rats

Igor de Oliveira Puttini[a], Pedro Henrique da Silva Gomes-Ferreira[a,*], Danila de Oliveira[b], Jaqueline Suemi Hassumi[b], Paulo Zupelari Gonçalves[a], Roberta Okamoto[a]

[a] Department of Surgery and Integrated Clinic, Aracatuba Dental School, Univ. Estadual Paulista–UNESP, Aracatuba, Sao Paulo, Brazil
[b] Department of Basic Sciences, Aracatuba Dental School, Univ. Estadual Paulista–UNESP, Aracatuba, Sao Paulo, Brazil

ARTICLE INFO

Keywords:
Bone and bone
Teriparatide
Parathyroid hormone (1-34) amide
Men
Osteoporosis

ABSTRACT

Objective: The aim of this study was to evaluate bone metabolism in the alveolar repair process in orchiectomized male rats treated with teriparatide.

Materials and methods: A total of 78 rats were divided into three groups: 26 oriectomized rats treated with teriparatide (ORQTRAT), 26 untreated orchiectomized rats (ORQ), and 26 rats which had undergone sham surgical procedures (SHAM), all these animals underwent extraction of the central incisor tooth. Thus, a histological analysis was performed (42 days). Real Time PCR (14 and 42 days) and immunohistochemical (42 days) analyses were performed based on the expression of RANK-L and osteoprotegerin (OPG). The calcein and alizarin were analyzed via laser confocal microscopy to verify alveolar bone turnover, and microtomographic analysis was performed to determine volume and bone quality (calcified tissues). In this analysis, the euthanasia period was 60 days post-extraction. The quantitative data were submitted to the statistical test and the significance level of 5% was adopted.

Results: Teriparatide increased bone turnover with a higher relative gene expression of RANKL and OPG at 14 days, and at 42 days, there was a significant decrease of RANKL and an increase of OPG, this standard can also be evaluated in the ratio of RANKL to OPG. Greater values of area and bone mineral deposition were found for the ORQTRAT group ($p < 0.05$), along with higher bone volume values ($p < 0.05$). The immunolabels revealed greater intensity in the relationship of RANKL and OPG, which led to intense remodeling aiding in the process of bone formation.

Conclusion: The teriparatide treatment in orchiectomized rats increases bone volume and decreases the porosity, in addition to promoting greater intensity in bone turnover during alveolar repair.

1. Introduction

Although most research on osteoporosis in the past focused on women, osteoporosis is becoming a relevant problem in men, too. One in eight men over the age of 50 will have a fracture related to osteoporosis (Cooper & Melton, 1992), and this has a likelihood of increasing with the aging of the male population (Burge et al., 2007). Osteoporosis in the male gender can be classified as primary or secondary, and in the primary category, it may still be divided into idiopathic and age-related osteoporosis (Khosla, Amin, & Orwoll, 2008).

Bisphosphonate therapy has been shown to be effective in increasing bone mineral density (BMD) in men with primary osteoporosis, as well as secondary osteoporosis resulting from hypogonadism (Orwoll et al., 2000; Ringe, Faber, Farahmand, & Dorst, 2006). However, in several cases of osteoporosis, stopping bone loss may not be enough. In these cases, treatments that stimulate bone neoformation may become a viable treatment option (National Cancer Institute–EUA–2001). In men, the impairment of bone formation is an important etiological factor; therefore, an anabolic treatment is a logical approach (Soen, 2016). Teriparatide is an anabolic agent approved for the treatment of osteoporosis in men; its main function is to increase osteoblastic and indirect activity, as well as increase the intestinal absorption of calcium and the renal tubular resorption of calcium, thus increasing the blood serum level (Cheng & Gupta, 2012; Kendler et al., 2018).

The bone anabolic function of teriparatide extends not only to the long bones and vertebrae, but its function in the post-extraction alveolar repair process has been proven, increasing bone formation (de Oliveira et al., 2018). Even in the case of osteoporosis/osteopenia,

* Corresponding author at: Rua José Bonifácio, 1193, 16015-050, Vila Mendonça, Aracatuba, Sao Paulo, Brazil.
E-mail address: pedroferreirabmf@gmail.com (P.H.d.S. Gomes-Ferreira).

https://doi.org/10.1016/j.archoralbio.2019.04.007
Received 4 December 2018; Received in revised form 5 April 2019; Accepted 11 April 2019

teriparatide can restore the bone condition of a healthy individual during its administration (de Oliveira et al., 2018). Proteins such as WNT, alkaline phosphatase, osteocalcin and TRAP, expressed on extracellular matrix in bone show this performance, which is corroborated by an increase in bone tissue evaluated by computerized microtomography (de Oliveira et al., 2018).

The increase in bone formation after extraction during therapy with teriparatide, is no longer a question. But how would this remodeling process be? Could this process be signaled by resorption and neoformation? Would the calcium precipitation be the only responsible for this bone mineral deposition process? To answer these points, the present work has the aim to evaluate bone metabolism in the alveolar repair process in orchiectomized male rats treated with teriparatide

2. Materials and methods

The Ethics Committee on Animal Use of the Araçatuba Dental School (CEUA-00706-2015) approved this study. The study was conducted in the Department of Surgery and Integrated Clinical of the Araçatuba Dental School, according to the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010)

For this study, a total of 78 adult male rats (Rattus novergicus albinus, Wistar) weighing approximately 300 g at 6 months of age were used. They were divided into three groups of random form (n = 26 per group): SHAM, representing the animals that underwent sham surgery only to simulate surgical stress; ORQ, a group of animals orchiectomized to induce osteoporosis, and without treatment; and ORQTRAT, the animals that were orchiectomized but treated with teriparatide. Sixteen animals per experimental group were divided into two subgroups for analyses. Eight were in the subgroup of the decalcified tissues analyzed via histology in hematoxylin and eosin and immunohistochemistry, with a 42-day euthanasia period. In addition, eight rats were in the subgroup of calcified tissues, where an analysis was performed by confocal laser microscopy and microCT, with a 60-day euthanasia period. For RT-PCR analysis, the animals were euthanized at 14 (n = 5) and 42 days (n = 5). These animals were kept in cages and were fed with a Nuvilab feed (Nuvilab CR-1- Nuvital Nutrientes Ltda®, PR, Brazil) containing 1.4% Ca, 0.8% P, and water ad libitum, which did not undergo additional stress.

2.1. Orchiectomy

For the induction of osteoporosis, the animals within the ORQ and ORQTRAT groups were anesthetized with Coopazine (Xylazine-Coopers, Brazil, Ltda, Osasco, SP, Brazil) and Vetaset (Intake ketamine Hydrochloride, Fort Dodge, Animal Health Ltda, Campinas, SP, Brazil). Then, incisions were made in both scrotal sacs to expose the testicles. With hemostatic tweezers, the spermatic funiculus was presented with concomitant individualization and the lacquering of the vas deferens and the vascular pedicle, and then, it was sectioned. The testicles were removed, and the surgical wound was sutured with 4-0 silk thread (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brazil). Seifi, Ezzati, Saedi, and Hedayati (2015) validated this procedure in 2015 after an Elisa test proved that endogenous testosterone had decreased. The animals within the SHAM group underwent a sham surgery that involved only the exposure, rather than the removal, of the testicles so that they experienced the same stress as the other groups did.

2.2. Pharmacological treatment

Immediately after the surgical procedure, the rats were subjected to daily subcutaneous injections of teriparatide at a concentration of 0.5 μg/kg/day diluted in a sterile saline solution at 0.1 mg/ml until the end of the experimental periods (de Oliveira et al., 2018).

2.3. Tooth extraction

One month after initiation of drug therapy with teriparatide, the rats were anesthetized with Coopazine (Xilazine-Coopers, Brazil, Ltda) and Vetaset (Ketamin Chloridrate, injectable, Fort Dodge, Saúde Animal, Ltda) using the manufacturer's recommended dosage. Then, antisepsis with polyvinylpyrrolidone iodide (Indústria Química e Farmacêutica Rioquímica Ltda, Brazil) was performed, followed by right-upper-incisor extraction using an instrument specially adapted for this purpose (Okamoto & de Russo, 1973). The gingival mucosa was sutured with polyglactin 910910 (Vicryl 4.0–Johnson & Johnson, New Brunswick, NJ, USA).

For the calcified groups, 14 days after the tooth extraction, 20 mg/kg of the fluorochrome calcein was administered intramuscularly (Luvizuto, Dias, Okamoto, Dornelles, & Okamoto, 2011; Ramalho-Ferreira, Faverani, Prado, Garcia, & Okamoto, 2015). After an additional 28 days (42 days after tooth extraction), alizarin red fluorochrome was administered at a dose of 20 mg/kg for each animal (Luvizuto et al., 2011; Ramalho-Ferreira, Faverani, Prado et al., 2015). The euthanasia of these animals was performed 60 days after tooth extraction (18 days after alizarin administration).

For the analyses of decalcified tissue, euthanasia occurred at 42 days after exodontia.

2.4. RT-PCR

PCR analysis was performed at 14 and 42 days post-extraction to evaluate the ribonucleic acid (RNA) present in the period of the greatest bone repair response (14 days) and the final period of repair (42 days). On euthanasia day, the animals were anesthetized, and the right maxilla of each animal was removed, washed in phosphate buffer saline (PBS), frozen in liquid nitrogen, and stored at −80° in a freezer for total RNA extraction with Trizol reagent (Life Technologies Invitrogen, Carslbad, CA, USA). For extraction, the SV Total RNA Isolation System kit (Promega, Madison, Wisconsin, USA) was used, followed by quantification, concentration, and purity analysis.

The normalization of the total RNA concentration was performed (Sigma-Aldrich, St. Louis, Missouri, EUA), followed by the manufacturing of complementary deoxyribonucleic acid (cDNA) strands, and RT-PCR expression related to bone repair was made by using the SybrGreen system (Applied Biosystems, Foster City, California, USA). The reactions were performed in quadruplicate, and the Taqman Universal PCR Master Mix was added to the plates containing the genes of interest. The cDNA volumes were calculated according to the quantification of samples.

The amplification reaction was performed and the results analyzed based on the cycle threshold (Ct) value, which allowed for the quantitative analysis of the genes of interest expression, as well as the related genes on the plate (Hassumi et al., 2018; Oliveira et al., 2017).

The RT-PCR analysis was performed with the aim of detecting relative gene expression related to RANKL, osteoprotegerin (OPG), and their ratio (RANKL/OPG).

2.5. Processing for histological technique

For the analysis of decalcified tissues, eight animals from all experimental groups were euthanized via anesthetic overdose at 42 days post-extraction, and the right maxilla was removed for the histological analysis (paraffin cuts).

The samples were fixed in 10% buffered formalin for 48 h and were washed in running water for 24 h. After they underwent decalcification in ethylenediaminetetraacetic (18%), they were dehydrated using a sequence of alcohols. After these steps, diaphanization was performed with xylol for later inclusion in paraffin to obtain a cut 5 μm in thickness, and it was mounted on slides. Some slides were separated for staining in hematoxylin and eosin, and others for immunohistochemical

reactions.

For the histometric and immunohistochemical analyses, only one examiner performed the analyses, and the same was unknown to the respective section group. In this way there are no biased results.

2.6. Immunohistochemical analysis

The immunohistochemical processing was performed in the Laboratory for the Study of Mineralized Tissues of the Faculty of Dentistry of Araçatuba–UNESP.

Endogenous peroxidase activity was inhibited with hydrogen peroxide. Next, the slides underwent the antigenic recovery step with phosphate citrate buffer (pH 6.0). The primary antibodies used were against OPG and RANK-L (Santa Cruz Biotechnology, Paulinia, SP, Brazil), with the objective of analyzing cellular responses regarding the process of bone remodeling.

Immunohistochemical experiments were performed using immunoperoxidase as a detection method. Biotinylated secondary rabbit anti-goat antibody (Pierce Biotechnology, São Paulo, Brazil) was used, the amplifier was Streptavidin and Biotin (Dako, Tokyo, Japan), and diaminobenzidine (Dako, Tokyo, Japan) was the chromogen. For each of the antibodies used, the expression of these proteins was evaluated semi-quantitativelyDifferent scores (light, moderate, and intense) were assigned according to the number of cells immunolabeled in the alveolar repair process. The analysis was performed under an optical microscope (LeicaR DMLB, Heerbrugg, Switzerland) by manually counting the labeled cells in previously determined areas, which were known to be involved in the dynamics of bone tissue. The analyzer was submitted to the Kappa test, with above 0.8 index obtained, showing that the observed scores were consistent. The diaminobenzidine markings were considered to be positive.

Goat isotype igG control was a substitute for the primary antibody and carried out negative controls reactions.

2.7. Microtomographic analysis (micro-Ct)

For the analysis of micro-CT, euthanasia was performed at 60 days. At the end of the fixation step with 10% formaldehyde for 48 h, the maxilla of eight animals for the calcified tissue analyses were washed for 24 h in running water and stored in 70% alcohol for analysis by X-ray beam scanning in a computerized microtomography system (FOP/UNICAMP). The specimens were scanned with the SkyScan microtome (SkyScan 1176 Bruker MicroCT, Aatselaar, Belgium, 2003) by using 9-μm slices (50Kv and 500 μ), with a copper and aluminum filter and a rotation step of 0.3 mm. The images obtained via the projection of X-rays in the samples were stored and reconstituted to determine the area of interest through the software NRecon (SkyScan, 2011; Version 1.6.6.0). In the Data Viewer software (SkyScan, Version 1.4.4 64-bit), the images were reconstructed to fit the standard positioning for all samples and could be observed in three planes (transverse, longitudinal, and sagittal). Then, with the software of CTAnalyser–CTAn (2003-11SkyScan, 2012 Bruker MicroCT Version 1.12.4.0) the region of interest (ROI) was defined for three-dimensional evaluation. The CTAn software analyzes and measures an image according to the gray scales (threshold). The threshold used in the analysis was 25–90 shades of gray, which made it possible to obtain the volume of bone formed in the alveoli under repair (de Oliveira et al., 2018). The parameters used were bone volume and percentage of total porosity.

2.8. Laboratory procedures for calcified tissue

After the end of the micro-CT analyses, the maxillas were dehydrated in an increasing sequence of alcohols of 70–100%. The pieces were then soaked in a solution of methyl methacrylate (MMAL) (Classic, Classic Dental Articles, São Paulo, SP, Brazil). This sequence was followed by three MMAL baths. The benzolium peroxide catalyst (1%, Riedel-de Haën AG, Seelze-Hannover, Germany) was added to the latter bath. The samples were introduced into an essay tube soaked in the solution and were kept in an oven at 37 °C for five days until the final polymerization of the resin.

After the polymerization the resin blocks were cut in the sagittal plane with the aid of a Maxicut mounted on a bench motor (Kota–São Paulo–SP, Brazil). The pieces were then bilaterally scrubbed with 120, 300, 400, 600, 800, and 1200 crescent granulations mounted on an automatic polishing machine (ECOMET 250PRO/AUTOMET 250, Buehler, Lake Bluff, Illinois) until the cuts reached a thickness of 80 μm. For the measurement, a digital caliper was used (Mitutoyo, Pompeia, SP, Brazil).

The slices were mounted on glass slides and mineral oil (liquid petroleum, Mantecor, Taquara, RJ, Brazil) and were sealed with a glass cover and clear enamel to prevent oil leakage and the possible dehydration of the samples (Ramalho-Ferreira, Faverani, Grossi-Oliveira, Okamoto, & Okamoto, 2015; Ramalho-Ferreira, Faverani, Prado et al., 2015).

2.9. Confocal laser microscopy

Longitudinal sections of the area of interest (bone adjacent to the apical third of the maxillary central incisor) were obtained using a Leica CTR 400 CS SPE microscope (Leica Microsystems, Heidelberg, Germany) through a 10x objective (original magnification 100x). The bone near the right upper incisor was taken as a reference. Thus, the area of the bone around this tooth was evaluated in each specimen.

The images obtained via confocal laser microscopy were reconstructed through the software grid installed to manipulate the microscope (Leica CTR 4000 CS SPE, Leica Microsystems, Heidelberg, Germany). The images obtained showed two colors representing calcium precipitation after the administration of calcein (green) at 14 days following tooth extraction (Fig. 1A) and alizarin (red) at 42 days after extraction (Fig. 1B). The software overlay the images and presented both overlapping fluorochromes (Fig. 1C). The prominence of green color represented a greater amount of an older bone, whereas a red exaltation represented a greater amount of a younger bone (Ramalho-Ferreira, Faverani, Grossi-Oliveira et al., 2015; Ramalho-Ferreira, Faverani, Prado et al., 2015).

These images were saved in TIFF format and transported to ImageJ software (Processing Software and Image Analysis, Ontario, Canada). With the "color threshold" tool, each image was standardized according to hue, saturation, and brightness to reveal fluorochromes. The Principle, the "free hands" tool, was selected; the calcein was highlighted; and the "measure" tool was used to calculate the area in μm2. The same procedure was performed with alizarin, with data related to the dynamics of the bone tissue in the region being obtained (Ramalho-Ferreira, Faverani, Grossi-Oliveira et al., 2015; Ramalho-Ferreira, Faverani, Prado et al., 2015).

To achieve the mineral apposition rate from the fluorochrome labeling, images were imported to Image J software (Processing Software and Image Analysis, Ontario, Canada), and the image's standardization was performed through the overlapping of both files containing green and red labeling. The "straight" tool was selected, and a line was drawn from the beginning of the alizarin precipitation (red label) to the end of the calcein precipitation (green label) in five regions randomly. The mean values were divided by 28, representing the period between both fluorochrome injections, thus leading to the value of the mineral apposition rate (Dempster et al., 2013; Faverani et al., 2018).

2.10. Statistical analysis

GraphPad Prism 7.03 (GraphPad Software, La Jolla, USA) was used to perform the statistical analysis. The homoscedasticity was assessed via the Shapiro-Wilk test to distinguish the parametric and non-parametric data. After this test it was possible to observe that all data were

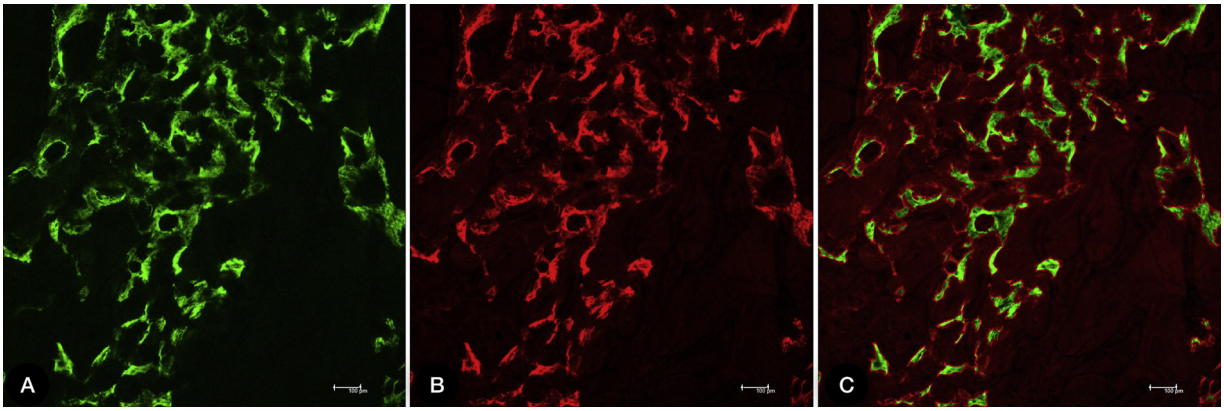

Fig. 1. Images of alveolar bone obtained by laser confocal microscopy. All fluorochromes labeled fluoride represent the precipitation of calcium present in bone tissue. **(A)** bone area marked by calcein (green); **(B)** alizarin (red) and **(C)** overlap of both fluorochrome markers (calcein and alizarin) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

parametric. For micro-CT, bone volume, percentage of total porosity, and Mineral apposition rate and PCR, the analysis of variance (ANOVA) one-way test and Tukey post-test were used. For the area of fluorochromes, the ANOVA two-way test and Tukey post-test were used. A significance level of $p < 0.05$ was considered to be significant for all tests.

3. Results

3.1. Histological analysis

A photomicrograph of the histological slides from a period of 42 days was performed in the most representative area of the cytoarchitecture of each group through a 25x objective, which allowed for analyzing if the SHAM group had good bone neoformation with little connective tissue, which represents little trabecular space (Fig. 2A). In the images obtained from the ORQ group, it was possible to observe poor bone neoformation and richness in connective tissue, characterizing large intertrabecular spaces (Fig. 2B). The slides of the animals treated with teriparatide (ORQTRAT) yielded good histological characteristics, and it was possible to notice great bone neoformation and small areas of connective tissue (Fig. 2C) presented good histological characteristics

3.2. RT-PCR

In the relative gene expression of RANKL at 14 days, it was possible to observe higher values for ORQTRAT (4.30), followed by ORQ (2.24), and lower values for SHAM (1.00), with no significant statistical difference between groups ($p > 0.05$, Tukey). At 42 days, there was a significant increase of RANKL for ORQ (3.37), with a significant statistical difference when compared with ORQTRAT (1.39) ($p = 0.0243$, Tukey) (Fig. 3A).

For OPG at 14 and 42 days, the ORQ group presented the lowest results, which resulted in a lower 1.00 of the standard group (SHAM 14 days). The ORQTRAT group presented the highest results with 1.24 (14 days) and 2.36 (42 days). In the periods of 14 and 42 days, a significant statistical difference was found in the comparison between ORQ and ORQTRAT ($p < 0.05$, Tukey) (Fig. 3B).

The ratio of RANKL to OPG can be evaluated as 1. Values greater than or equal to 1 indicate bone resorption. It was possible to evaluate that the ORQ (7.46) and ORQTRAT (3.02) groups were in a reabsorption period at 14 days, however ORQ presented more intense relative expression being statistically significant when compared to SHAM ($p = 0.0091$, Tukey) and ORQTRAT ($p = 0.0145$, Tukey). At 42 days, this resorption state was still present in the ORQ group (10.40) but even more intense, and the bone resorption process was maintained similar for SHAM (1.02) ($p = 0.0168$, Tukey) and stopped for ORQTRAT (0.58) ($p = 0.0196$, Tukey) (Fig. 3C).

3.3. Immunohistochemical analysis

The immunolabeling analysis was performed at 42 days in all experimental groups to evaluate the process of bone remodeling through the labeling of the OPG and RANK-L proteins (Fig. 4).

SHAM Group:

Labeling by OPG: it was possible to evaluate light labeling.

Labeling by RANK-L: it was possible to evaluate moderate labeling.

ORQ Group:

Labeling by OPG: it was possible to evaluate moderate labeling.

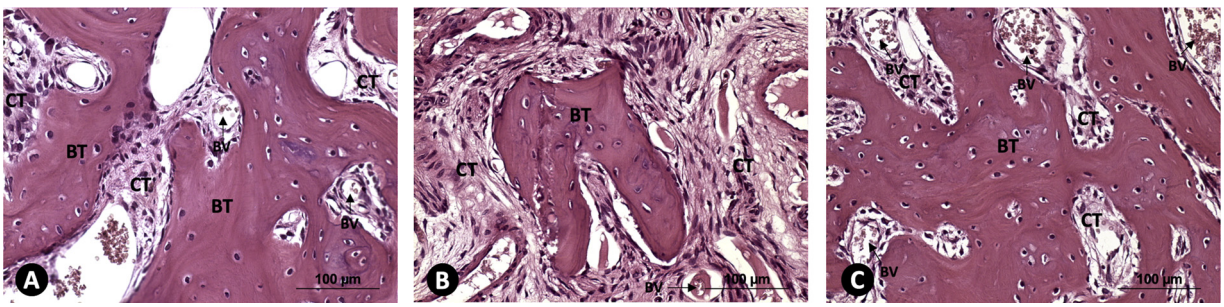

Fig. 2. Histological images of alveolar bone under repair in the period of 42 days after the tooth extraction of groups **(A)** SHAM - Presence of a greater amount of mineralized bone matrix with lamellar tissue, normal bone cytoarchitecture; **(B)** ORQ - Higher presence of connective tissue, less amount of mineralized tissue, cytoarchitecture shows impairment in bone formation after the exodontia; and **(C)** ORQTRAT - greater bone formation resembling the SHAR group cytoarchitecture, less quantified connective tissue (Original, 25x). (CT: Connective tissue, BT: Bone tissue, BV: Blood vessel).

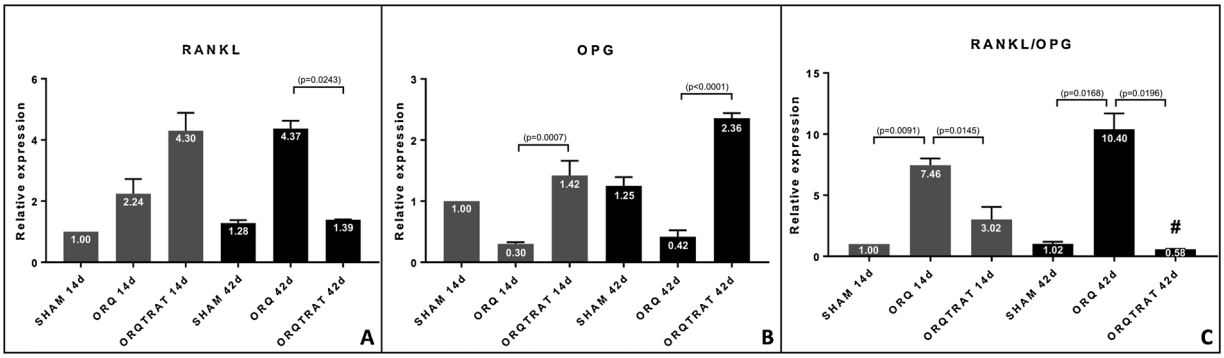

Fig. 3. Relative gene expression of RANKL (A) and OPG (B) over 14 and 42 days in the SHAM, ORQ and ORQTRAT groups ([p < 0.05). **C:** RANKL/OPG ratio indicating presence of resorption under 1. Only the ORQTRAT group presented the results with (#) absence of active process of bone resorption. Results represented by mean, error bar by S.EM.

Labeling by RANK-L: it was possible to evaluate moderate labeling.
ORQTRAT Group:
Labeling by OPG: it was possible to evaluate intense labeling.
Labeling by RANK-L: it was possible to evaluate intense labeling.

3.4. Microtomographic analysis (micro-Ct)

Quantitatively, the bone volume in the SHAM group was a mean of 124.120 mm^3; in the ORQ group, it was 65.006 mm^3; and in the ORQTRAT group, it was 157.915 mm^3, with the volume being similar to that of the SHAM group, but with a significant statistical difference from the ORQ group ($p < 0.05$, Tukey). (Fig. 5).

In relation to the bone porosity of the alveolus in repair, as evaluated by the percentage of total porosity, the values were 46.292% (SHAM group), 40.310% (ORQ group), and 32.812% (ORQTRAT group), showing a lower percentage of porosity in the ORQTRAT group ($p = 0.496$, ANOVA one-way test) (Fig. 6).

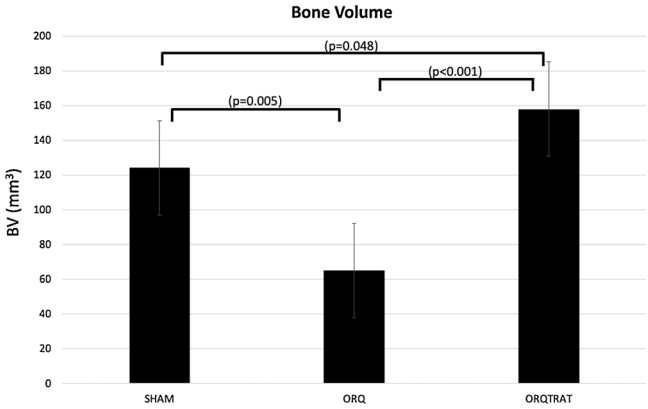

Fig. 5. Quantification of bone volume of SHAM, ORQ and ORQTRAT groups ([p < 0.05, Tukey). Results represented by mean, error bar by S.EM.

3.5. Laser confocal microscopy

In the photomicrographs of the fluorochromes, there were no significant statistical results in the intra-group comparison ($p > 0.05$, Tukey). In the intergroup comparison, it was possible to observe a real loss of calcium deposition in bone that suffered from the decrease of testosterone by orchiectomy, with lower values for the ORQ group, and the SHAM group presented better results than ORQ did ($p = 0.0149$, Tukey). Teriparatide supplementation in the orchiectomized animals

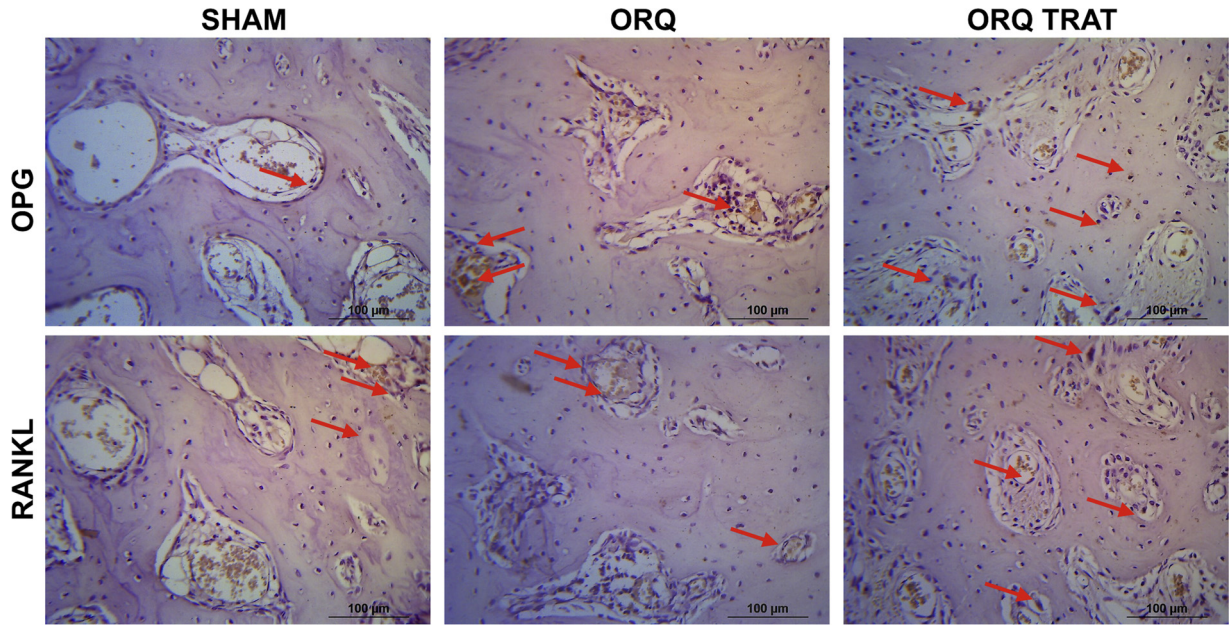

Fig. 4. immunolabeling of the OPG and RANKL proteins for the SHAM (light and moderate), ORQ (moderate and moderate) and ORQTRAT (intense and intense) groups of the alveolus under repair of 42 days after the exodontia (Original, 25x).

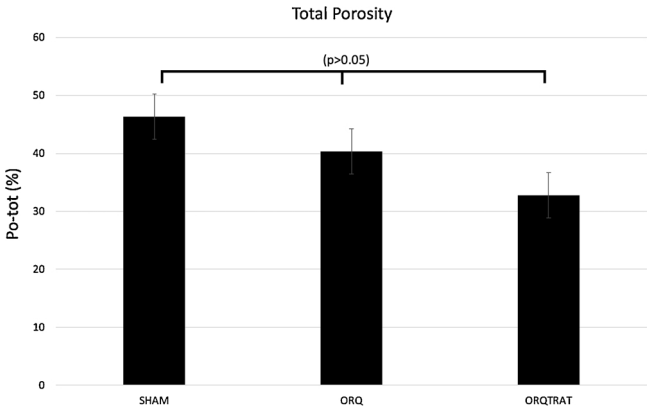

Fig. 6. Quantification of total porosity of SHAM, ORQ and ORQTRAT groups. ($p > 0.05$, Tukey). Results represented by mean, error bar by S.EM.

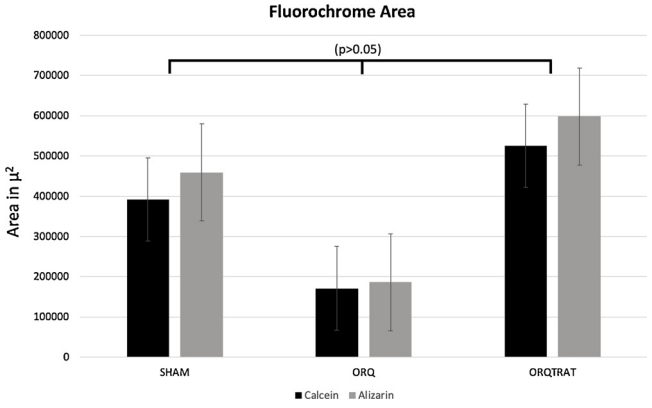

Fig. 7. Fluorochromes area (μm^2) of SHAM, ORQ and ORQTRAT groups, by the expressions calcein and alizarin ([$= p < 0.05$). Results represented by mean.

(ORQTRAT) showed a relevant result in comparison with both ORQ ($p = 0.0061$, Tukey) and SHAM ($p = 0.047$, Tukey) (Fig. 7).

The mean values of the mineral apposition rate found in the ORQTRAT group (5.24 µm/day) were higher when compared with the SHAM group rate (4.44 µm/day) and also the ORQ group rate (1.53 µm/day). The results of the mineral apposition rate of the ORQTRAT, ORQ, and SHAM groups showed significant statistical differences in the comparison of the three groups ($p < 0.05$, Tukey) (Fig. 8).

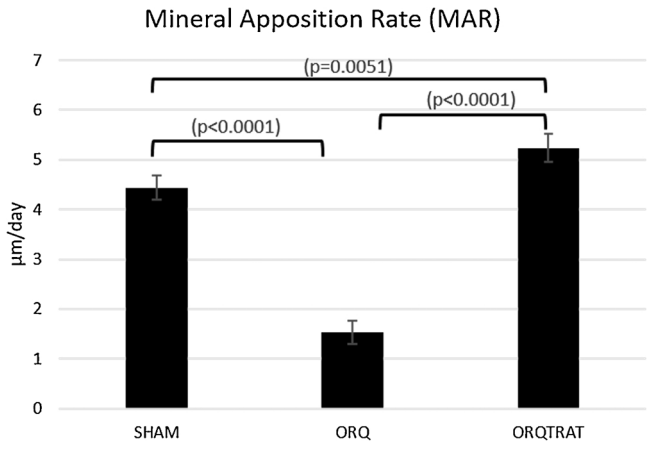

Fig. 8. Mineral apposition rate per day (µm) of the SHAM, ORQ and ORQTRAT groups, evaluated linearly ([$= p < 0.05$). Results represented by mean, error bar by S.EM.

4. Discussion

Teriparatide has been shown to be effective in treating osteoporosis, reducing the risk of fractures, and increasing bone mineral density in long bones (Kim, Kang, Kim, Lim, & Hahn, 2017). However, little is known about the effects on the jaw bone.

The relative gene expression present in the alveolar repair of orchiectomized rats treated or not treated with teriparatide presents a very relevant context, which can be complemented by immunohistochemistry.

In the present study, the increase in relative gene expression relative to bone resorption (RANKL) was present in the initial period (14 days post-extraction) in the ORQ and ORQTRAT groups, while for OPG, ORQ was below 1 (0.30), showing that there is signaling for marked resorption 14 days, but ORQTRAT outperformed the SHAM group with results above 1 (1.42). At 42 days, it was possible to observe that the persistence of treatment with PTH 1–34 led to a reduction in the relative expression of RANKL in which ORQTRAT (1.39) and SHAM (1.28) presented similar results close to 1. As for OPG, ORQ remained as low as 0.42 and ORQTRAT reached even higher levels (2.36). These results show that there is a decrease in the bone resorption process and they help in the bone formation process as well, which corroborates with that found in the literature in which there is increase in WNT expression and decrease in TRAP (de Oliveira et al., 2018).

In this context, the RANKL to OPG ratio shows that the osteoporosis of the animals of the present study led to a marked bone resorption process at 14 days (ORQ: 7.46), which shows a growth over time of 10.40 at 42 days after tooth extraction. The SHAM group presented similar results for 14 and 42 days (1.00 and 1.02 respectively), showing the preservation of homeostasis in this process, whereas the ORQTRAT group keeps presenting a resorption process in the period of 14 days (3.02), which was reduced exponentially over time, presenting 0.58 in the period of 42 days. The value below 1, shows that the process of bone resorption in these animals is reduced even when compared to SHAM.

The immunolabeling pattern for RANK-L and OPG at 42 days was approximate between SHAM and ORQ (moderate), but the ORQ immunolabeling occurred in a region of immature connective tissue, different from SHAM, which showed tissue marking mature bone. The treatment with teriparatide daily appeared to intensify the immunoblotting, increasing the presence of these proteins in the ORQTRAT group, in agreement with what has been described in the literature regarding the intensification of the response in the process of formation and bone resorption (Murray, Rao, Divieti, & Bringhurst, 2005). de Oliveira et al. (2018) shows that PTH 1–34 also increases the expression of proteins such as WNT, alkaline phosphatase and osteocalcin when compared to healthy and osteoporotic animals, showing immunoblotting that leads to increased bone production as well as increased mineralization. In addition, it presents light immunolabeling for TRAP, which means less osteoclastic action, corroborating in this way with the present study.

These results corroborate directly those found in the confocal laser microscopy analysis, which showed a higher calcium deposition for the ORQTRAT group compared with the other groups ($p < 0.05$, Tukey). Intensification in the process of bone formation as well as mineralization can also be seen through mineral apposition rate, which values daily mineral deposition surpassing that of a SHAM rat. Bone anabolism caused by intermittently administered teriparatide may, in fact, be supported in the present results.

In addition, the standard histological of the alveolar bone in the repair of teriparatide-treated animals (ORQTRAT) could be evaluated by the histological slice, which showed a better trabecular pattern with the presence of little connective tissue. This is different from the untreated orchiectomized animal (ORQ), which had a greater presence of intertrabecular connective tissue.

The quality of bone tissue is fundamental to the success of

rehabilitation (Jaffin & Berman, 1991; Jemt, Book, Linden, & Urde, 1992). It has been suggested that bone density, bone volume, and trabecular bone volume are fundamental parameters in the success rate of osseointegrative implants (Parfitt et al., 1987). Microtomography is the gold standard for the evaluation of these parameters (Burghardt, Link, & Majumdar, 2011).

In this context, it can be noticed through microCT at 60 days of alveolar repair that the treatment with teriparatide intermittently was able to improve the bone volume when compared with the orquietomized rats without treatment (ORQ) and even when compared with salable animals (SHAM) ($p < 0.05$, Tukey). In addition, the decreasing bone porosity suggested that treatment with teriparatide can improve the microstructural characteristics of the alveolar bone under repair, characterizing greater volume and a better quality of alveolar bone.

This decrease in porosity is also confirmed when other parameters such as trabecular thickness are evaluated (de Oliveira et al., 2018), which show that teriparatide leads to a thicker bone production when compared to osteoporotic, and similar in comparison with animals healthy This means that PTH 1–34 administered intermittently in low doses can lead to normal trabecular thickness but with an even larger volume, obtaining a bone with characteristics of a healthy but higher volume animal.

Due to the high incidence of osteoporosis in men, a high number of treatments have been proposed (Riggs, Khosla, & Melton, 1998). However, little is discussed about the effect of these drugs on alveolar bone turnover. It is known that bisphosphonates, especially alendronate, are the most commonly used drugs for the treatment of osteoporosis, with good results in the prevention of vertebral fractures (Wells et al., 2008). However, alendronate binds to hydroxyapatite leading to apoptosis of osteoclasts, leading to a decrease in bone turnover, thus causing osteonecrosis of the mandibles (Ruggiero & Drew, 2007), which means it is not the ideal treatment for osteoporosis in men when evaluated in the dental context (Gagnon, Li, & Ebeling, 2008).

Thus, treatments that stimulate new bone formation require further studies. Treatments for osteoporosis have not yet been studied in men as in women, and only some therapies have been approved for application in men. These include bisphosphonates and teriparatide (Gagnon et al., 2008). Teriparatide is the only anabolic treatment approved for use in men with osteoporosis (Cheng & Gupta, 2012).

Considering that the patient needs an adequate occlusion for a better quality of life, the general concept of the treatment of osteoporosis, both in the prevention of vertebral fractures and long bones, should be extended not only to solve this problem, but to solve the problems of the patient as a whole. Therefore, when possible, teriparatide should be indicated in cases of osteoporosis in which the patient needs dental surgical treatments, thus minimizing cases of mandibular osteonecrosis due to the anabolic effect present (de Oliveira et al., 2018) and the great turnover evaluated in the present study.

5. Conclusion

In view of the above, it is possible to conclude that teriparatide treatment in orchiectomized rats increases bone volume and decreases the porosity, in addition to promoting greater intensity in bone turnover in alveolar repair, with increased signaling for production and decrease for resorption, so that in addition to treating osteoporosis, it does not negatively interfere with dental procedures

Conflict of interest

None.

Author contributions

RO, conceptualization of study, funding acquisition and project administration and conceptualization of study, data validation, data discussion, manuscript preparation and revision; IOP and DO, execution of experimental procedures, data collection and analysis, data discussion and manuscript preparation; PHSGF, supervision of data collection, supervision of data analysis, data validation, data discussion, manuscript preparation; PZG and JSH, data validation, data discussion, and performing PCR-RT analysis. All authors reviewed, edited and approved the final manuscript.

Acknowledgements

The authors would like to thank CAPES for granting a master's degree to the first author (Puttini, IO) and for the regular research assistance granted to the advisor of this research (Okamoto, R) (FAPESP # 2015 / 14688-0). To the Center of Electron Microscopy of the Institute of Biosciences of Botucatu, UNESP, thank you for the viability of the analysis via laser confocal microscopy.

References

Burge, R., Dawson-Hughes, B., Solomon, D. H., Wong, J. B., King, A., & Tosteson, A. (2007). Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025. *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research, 22*(3), 465–475. https://doi.org/10.1359/jbmr.061113.

Burghardt, A. J., Link, T. M., & Majumdar, S. (2011). High-resolution computed tomography for clinical imaging of bone microarchitecture. *Clinical Orthopaedics and Related Research, 469*(8), 2179–2193. https://doi.org/10.1007/s11999-010-1766-x.

Cheng, M. L., & Gupta, V. (2012). Teriparatide-indications beyond osteoporosis. *Indian Journal of Endocrinology and Metabolism, 16*(3), 343–348. https://doi.org/10.4103/2230-8210.95661.

Cooper, C., & Melton, L. J. (1992). Trends in endocrinology & metabolism. *Epidemiology of osteoporosis, 3*(6), 224–229. https://doi.org/10.1016/1043-2760(92)90032-V.

de Oliveira, D., de Oliveira Puttini, I., Silva Gomes-Ferreira, P. H., Palin, L. P., Matsumoto, M. A., & Okamoto, R. (2018). Effect of intermittent teriparatide (PTH 1-34) on the alveolar healing process in orchiectomized rats. *Clinical Oral Investigations*. https://doi.org/10.1007/s00784-018-2672-y.

Dempster, D. W., Compston, J. E., Drezner, M. K., Glorieux, F. H., Kanis, J. A., Malluche, H., ... Parfitt, A. M. (2013). Standardized nomenclature, symbols, and units for bone histomorphometry: A 2012 update of the report of the ASBMR histomorphometry nomenclature committee. *Journal of Bone and Mineral Research: the Official Journal of the American Society for Bone and Mineral Research, 28*(1), 2–17. https://doi.org/10.1002/jbmr.1805.

Faverani, L. P., Polo, T. O. B., Ramalho-Ferreira, G., Momesso, G. A. C., Hassumi, J. S., Rossi, A. C., ... Okamoto, R. (2018). Raloxifene but not alendronate can compensate the impaired osseointegration in osteoporotic rats. *Clinical Oral Investigations, 22*(1), 255–265. https://doi.org/10.1007/s00784-017-2106-2.

Gagnon, C., Li, V., & Ebeling, P. R. (2008). Osteoporosis in men: Its pathophysiology and the role of teriparatide in its treatment. *Clinical Interventions in Aging, 3*(4), 635–645.

Hassumi, J. S., Mulinari-Santos, G., Fabris, A., Jacob, R. G. M., Goncalves, A., Rossi, A. C., ... Okamoto, R. (2018). Alveolar bone healing in rats: Micro-CT, immunohistochemical and molecular analysis. *Journal of Applied Oral Science: Revista FOB, 26*, e20170326. https://doi.org/10.1590/1678-7757-2017-0326.

Jaffin, R. A., & Berman, C. L. (1991). The excessive loss of branemark fixtures in type IV bone: A 5-year analysis. *Journal of Periodontology, 62*(1), 2–4. https://doi.org/10.1902/jop.1991.62.1.2.

Jemt, T., Book, K., Linden, B., & Urde, G. (1992). Failures and complications in 92 consecutively inserted overdentures supported by branemark implants in severely resorbed edentulous maxillae: A study from prosthetic treatment to first annual checkup. *The International Journal of Oral & Maxillofacial Implants, 7*(2), 162–167.

Kendler, D. L., Marin, F., Zerbini, C. A. F., Russo, L. A., Greenspan, S. L., Zikan, V., & López-Romero, P. (2018). Effects of teriparatide and risedronate on new fractures in post-menopausal women with severe osteoporosis (VERO): A multicentre, double-blind, double-dummy, randomised controlled trial. *Lancet. 391*(10117), 230–240. https://doi.org/10.1016/S0140-6736(17)32137-2.

Khosla, S., Amin, S., & Orwoll, E. (2008). Osteoporosis in men. *Endocrine Reviews, 29*(4), 441–464. https://doi.org/10.1210/er.2008-0002.

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biology, 8*(6), e1000412. https://doi.org/10.1371/journal.pbio.1000412.

Kim, S. M., Kang, K. C., Kim, J. W., Lim, S. J., & Hahn, M. H. (2017). Current role and application of teriparatide in fracture healing of osteoporotic patients: A systematic review. *Journal of Bone Metabolism, 24*(1), 65–73. https://doi.org/10.11005/jbm.2017.24.1.65.

Luvizuto, E. R., Dias, S. S., Okamoto, T., Dornelles, R. C., & Okamoto, R. (2011). Raloxifene therapy inhibits osteoclastogenesis during the alveolar healing process in rats. *Archives of Oral Biology, 56*(10), 984–990. https://doi.org/10.1016/j.archoralbio.2011.03.015.

Murray, T. M., Rao, L. G., Divieti, P., & Bringhurst, F. R. (2005). Parathyroid hormone secretion and action: Evidence for discrete receptors for the carboxyl-terminal region

and related biological actions of carboxyl-terminal ligands. *Endocrine Reviews, 26*(1), 78–113. https://doi.org/10.1210/er.2003-0024.

Okamoto, T., & de Russo, M. C. (1973). Wound healing following tooth extraction. Histochemical study in rats. *Revista Da Faculdade de Odontologia de Aracatuba, 2*(2), 153–169.

Oliveira, D., Hassumi, J. S., Gomes-Ferreira, P. H., Polo, T. O., Ferreira, G. R., Faverani, L. P., & Okamoto, R. (2017). Short term sodium alendronate administration improves the peri-implant bone quality in osteoporotic animals. *Journal of Applied Oral Science: Revista FOB, 25*(1), 42–52. https://doi.org/10.1590/1678-77572016-0165.

Orwoll, E., Ettinger, M., Weiss, S., Miller, P., Kendler, D., Graham, J., ... Lombardi, A. (2000). Alendronate for the treatment of osteoporosis in men. *New England Journal of Medicine, 343*(9), 604–610. https://doi.org/10.1056/nejm200008313430902.

Parfitt, A. M., Drezner, M. K., Glorieux, F. H., Kanis, J. A., Malluche, H., Meunier, P. J., ... Recker, R. R. (1987). Bone histomorphometry: Standardization of nomenclature, symbols, and units. Report of the ASBMR histomorphometry nomenclature committee. *Journal of Bone and Mineral Research: the Official Journal of the American Society for Bone and Mineral Research, 2*(6), 595–610. https://doi.org/10.1002/jbmr.5650020617.

Ramalho-Ferreira, G., Faverani, L. P., Grossi-Oliveira, G. A., Okamoto, T., & Okamoto, R. (2015). Alveolar bone dynamics in osteoporotic rats treated with raloxifene or alendronate: Confocal microscopy analysis. *Journal of Biomedical Optics, 20*(3), 038003. https://doi.org/10.1117/1.jbo.20.3.038003.

Ramalho-Ferreira, G., Faverani, L. P., Prado, F. B., Garcia, I. R., Jr., & Okamoto, R. (2015). Raloxifene enhances peri-implant bone healing in osteoporotic rats. *International Journal of Oral and Maxillofacial Surgery, 44*(6), 798–805. https://doi.org/10.1016/j.ijom.2015.02.018.

Riggs, B. L., Khosla, S., & Melton, L. J., 3rd (1998). A unitary model for involutional osteoporosis: Estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. *Journal of Bone and Mineral Research: the Official Journal of the American Society for Bone and Mineral Research, 13*(5), 763–773. https://doi.org/10.1359/jbmr.1998.13.5.763.

Ringe, J. D., Faber, H., Farahmand, P., & Dorst, A. (2006). Efficacy of risedronate in men with primary and secondary osteoporosis: Results of a 1-year study. *Rheumatology International, 26*(5), 427–431. https://doi.org/10.1007/s00296-005-0004-4.

Ruggiero, S. L., & Drew, S. J. (2007). Osteonecrosis of the jaws and bisphosphonate therapy. *Journal of Dental Research, 86*(11), 1013–1021. https://doi.org/10.1177/154405910708601101.

Seifi, M., Ezzati, B., Saedi, S., & Hedayati, M. (2015). The effect of ovariectomy and orchiectomy on orthodontic tooth movement and root resorption in wistar rats. *Journal of Dentistry (Shiraz, Iran), 16*(4), 302–309.

Soen, S. (2016). Drug therapy for primary osteoporosis in men. *Clinical Calcium, 26*(July (7)), 1047–1052 CliCa160710471052. Japanese. PubMed PMID: 27346317.

Wells, G. A., Cranney, A., Peterson, J., Boucher, M., Shea, B., Robinson, V., ... Tugwell, P. (2008). Alendronate for the primary and secondary prevention of osteoporotic fractures in postmenopausal women. *The Cochrane Database of Systematic Reviews, 23*(1), CD001155. https://doi.org/10.1002/14651858.CD001155.pub2.