GUSTAVO ANTONIO CORREA MOMESSO

STRONTIUM RANELATE IMPROVES ALVEOLAR BONE HEALING IN OSTEOPENIC RATS

(RANELATO DE ESTRÔNCIO MELHORA REPARO ÓSSEO ALVEOLAR EM RATAS OSTEOPÊNICAS)

Araçatuba – SP

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Dissertação apresentada à Faculdade de Odontologia do Campus de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho"- UNESP, para obtenção do Título de MESTRE EM ODONTOLOGIA (Área de concentração em Cirurgia e Traumatologia Bucomaxilofacial)

Orientadora: Profª. Adj. Drª. Roberta Okamoto

Coorientador: Prof. Ass. Dr. Leonardo Perez Faverani

Araçatuba – SP

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Dedicatória

*D*edico este trabalho às pessoas mais importantes de minha vida:

*A*o meu amado pai, **Idanir Antonio Momesso Junior,** fonte de amor infindável. Infelizmente, o destino nos distanciou para que eu pudesse cumprir esta longa jornada, a qual representa um sonho nosso. Hoje completam sete anos que estou longe de casa e, apesar da saudade imensurável e as dificuldades aqui enfrentadas, a conquista de hoje remete à maneira preciosa que dedicastes sua vida inteira pelos seus filhos. Isso é inquestionável! Por muitas vezes o senhor abdicou de seus sonhos para que pudéssemos concretizar nossos anseios. Só Deus é capaz de saber os momentos mais complicados de nossas vidas quando nos encontramos sozinhos, mas o senhor nunca os deixou transparecer para sua família e os enfrentou com dignidade e honra. Um dia queria poder ser a metade do homem de família e pai que o senhor foi para mim. Obrigado por ser fonte eterna de sabedoria e principal referência em minha vida. Sem o seu apoio, persistência e vontade, nada disso seria possível.

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A minha irmã, **Marília Gabriela Correa Momesso,** por todo o apoio e amparo que sempre que me deu, desde meus tempos de colegial me incentivando e ajudando com os estudos, tornando-se uma grande referência em minha vida. Ter a mesma profissão que você é uma grande responsabilidade que carrego por toda sua competência e inteligência. Ao mesmo tempo, tudo se torna mais fácil pelo exemplo que tenho ao meu lado. Obrigado por todo carinho, amor e zelo que sempre teve por mim, como irmão mais novo. Você fez parte de toda minha formação como ser humano. Este título tem parte fundamental sua. Te amo para sempre!

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𝒜 **Deus,** por ser uma pessoa privilegiada. Alguns momentos de dificuldades em

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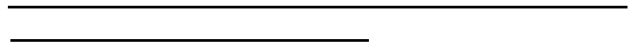
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Epígrafe

A grandeza não consiste em receber honras, mas em merecê-las

(Aristóteles)

Momesso, G.A.C. Stontium ranelate improves alveolar bone healing in osteopenic rats. ARACATUBA: UNESP – Univ. Estadual Paulista. 2017

ABSTRACT: This study aimed evaluate alveolar bone healing in osteopenic rats treated with strontium ranelate. Thirty-three three months's old female rats were selected and diveded into three groups: OVX (animals underwent to ovariectomy with no drug treatment); SHAM (animals underwent to fake surgery with no drug treatment) and OVX-SR (animals underwent to ovariectomy treated with strontium ranelate). Firstly, animals undewent to bilateral ovariectomy to induce osteopenic condition. Drug treatment started at 30 days after, during the all experimental period. Thirty days after, it was performed extraction of the right upper incisor tooth, to further evaluation of alveolar healing. Animals from decalcified group were euthanized at 14 days after tooth extraction, and its samples were destinated to histological and immunohistochemestry analysis. Animals from calcified group were euthanized at 60 days and its samples were destinated to confocal microscopy and micro-tomography analysis. Histological results showed that OVX-SR group had the better aspect of new bone formation, with few number of trabecular bone and poor presence of connective tissue compared to OVX group. Immunohistochemestry results showed an intense labeling of OPG for OVX-SR group and intense labeling of RANKL for OVX group. Regarding confocal microscopy analysis, it was possible observed that OVX-SR group showed a significance greater amount of alizarin precipitation compared to another both groups (Tukey test – $P < 0.05$). About micro-tomographic parameters, OVX-SR group showed high values for BV/TV (Tukey test – $P > 0.05$) and Tb.Th (Tukey test – $P < 0.05$) and lower valus for Tb.Sp, Po.Tot and Tb.N (Tukey test – $P > 0.05$). It was concluded that strontium ranelate

improves microscopy and morphologic aspects on alveolar bone healing of osteopenic rats.

Momesso, G.A.C. Ranelato de estrôncio melhora reparo ósseo alveolar em ratas osteopênicas. ARACATUBA: UNESP – Univ. Estadual Paulista. 2017

Este estudo objetivou avaliar o reparo ósseo alveolar em ratas osteopênicas tratadas com ranelato de estrôncio. Trinta e três ratas fêmeas com 3 meses de idade foram selecionadas e divididas em 3 grupos experimentais: OVX (animais submetidos à ovariectomia sem tratamento medicamentoso); SHAM (animais submetidos à cirurgia fictícia sem tratamento medicamentoso) e OVX-RE (animais submetidos à ovariectomia e tratados com ranelato de estrôncio). Inicialmente, os animais foram submetidos à cirurgia de ovariectomia bilateral para indução de condição osteopênica. O tratamento medicamentoso iniciou 30 dias após o procedimento cirúrgico com duração até o momento de eutanásia. Trinta dias após o início do tratamento, foi realizado a extração do incisivo superior direito dos animais para posterior avaliação do reparo alveolar. Os animais do grupo descalcificado foram submetidos à eutanásia aos 14 dias após a extração dentária, sendo as amostradas destinadas às análises histológica e imunoistoquímica. Os animais do grupo calcificado foram submetidos à eutanásia aos 60 dias após a extração dentária, sendo as amostras destinadas às análises por microscopia confocal e microtomográfica. Os resultados histológicos evidenciaram que o grupo OVX-RE demonstrou melhor aspecto de neoformação óssea, com trabéculas mais espessas e baixa presença de tecido conjuntivo, comaprado ao grupo OVX. Os resultados imunoistoquímicos demonstraram intensa marcação de OPG para o grupo OVX-RE e intensa marcação de RANKL para o grupo OVX. Já a análise por microscopia confocal

evidenciou que o grupo OVX-RE obteve quantidade significativamente maior de marcação para vermelho de alizarina comaprado aos outros dois grupos (Tukey test – $P < 0,05$). Em relação aos parâmetros microtomográficos, foi possivel observar maiores valores de BV/TV (Tukey test – $P > 0.05$) e Tb.Th (Tukey test – $P < 0.05$) e menores valores de Tb.Sp, Po.Tot e Tb.N (Tukey test – $P > 0.05$) para o grupo OVX-RE. Sendo assim, é possível concluir que o ranelato de estrôncio melhora os aspectos microscópicos e morfológicos do reparo ósseo alveolar em ratas osteopênicas.

Palavras-chave: Ranelato de estrôncio, osteoporose, ovariectomia, reparo ósseo, extração dentária.

Listas

Lista de Figuras

administration with a little amount of old bone. **(B)** OVX group showed a large amount of old bone, labeling by the calcium precipitation in the calcein (green) administration. OVX-SR group showed a large amount of new bone, labeling by alizarin red and low amount of old bone (green).

Lista de Abreviaturas

OVX =   Ovariectomy

SR =   Strontium ranelate

ROI =   Region of interest

mg/kg =   Miligram per kilogram

OPG =   Osteoprotegerin

i.m =   Intra-Muscular

RANKL =   Receptor activator of nuclear factor kappa-B ligand

mL =   Mililiters

mm =   Milímeters

µm =   Micrometers

p =   Unit of statistical relevance

E$_2$ =   Estrogen

BV/TV =   Bone volume percent

Tb.Th =   Trabecular thickness

Tb.Sp =   Trabecular space

Po.Tot =   Total porosity

Tb.N =   Trabecular number

SUMÁRIO

Strontium ranelate improves alveolar bone healing in osteopenic rats

(Ranelato de estrôncio melhora reparo ósseo alveolar em ratas osteopênicas)

Introduction
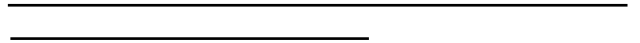
Introduction

After teeth loss, there is a need to maintenance of bone tissue quality, aiming the further maxillofacial rehabilitation with dental implants or bone grafts. However, concomitant emergence of systemic changes, such as diabetes, arterial hypertension and osteopenia could lead to bone dynamics decrease with the consequence of microarchitecture deterioration (Leslie et al. 2012, Hamann et al. 2012, Lerner 2006, Wu et al. 2016). The lack of estrogen on postmenopausal women stimulates osteoclastogenesis cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL6 and macrophage stimulating factor (M-CSF), besides the receptor activator of NF-kB ligand (RANKL), enhancing osteoclasts activity and bone resorption (Pacifici 1996, Manolagas, O'Brien, and Almeida 2013, Hofbauer et al. 2000) . This bone *turnover* unbalance may leads to a severe decreasing of bone mass, characterizing osteoporosis condition, which represents a high risk of bone fractures (Riggs, Khosla, and Melton 2002).

Data from world health organization shows that up to three hundred million people worldwide is affected by osteoporosis (Kanis 1994). Furthermore, experimental studies with ovariectomized rats observed that osteoporosis condition could affected the maxillofacial region, increasing its fragility and decreasing bone mass (Luvizuto, Queiroz, et al. 2010, Luvizuto, Dias, et al. 2010a). Therefore, there is many available alternatives for the treatment of osteoporosis. The mainly therapies adopted represents catabolic drugs, as estrogen replacement, selective estrogen-receptors modulator (SERMS), denosumab and bisphosphonates (Marie et al. 2005, Marie 2006). These drugs are beneficial to prevent bone fractures and bone mass loss, since inhibit osteoclast activity. However, the long-term use of bisphosphonates and denosumab have been shown an expressive reducing of bone *turnover*, which may develop medication-related osteonecrosis of the jaw (MRONJ) (Ruggiero 2013, Ruggiero et al. 2014).

On the other hands, new antiosteoporotic therapies have been emerging, between them, strontium ranelate (SR), which is an anabolic drug that acts reducing bone resorption as promotes bone formation. SR is composed by an organic molecule (ranelic acid) binding two stable strontium atoms (Marie 2006). The mechanism of action of this drug still controversial, however two hypothesis have been described. Some studies

suggest that SR activating calcium-sensing receptors (CaSR) presents on osteoblastic cells, stimulating bone formation (Chattopadhyay et al. 2007, Brennan et al. 2009). Moreover, it is believed that SR downregulates expression of RANKL and enhances the expression of osteoprotegerina (OPG), decreasing osteoclast activity (Atkins et al. 2009, Marie, Felsenberg, and Brandi 2011).

Relevant clinical trials evidenced that SR reduced the incidence of vertebral and hip fractures (Reginster et al. 2005, Kanis et al. 2011). Besides that, experimental studies showed that this drug was beneficial for ovariectomized rats (Zacchetti et al. 2014). However, there is no study evaluated the action of this drug on alveolar bone healing. Thus, this study aimed evaluate alveolar bone healing of osteopenic rats treated with strontium ranelate.

Materials and methods

Materials and methods

Experimental design

Animals

This study received the approval by Ethics Committee for the Use of Animals (000685/2015) (Attachment I) of São Paulo State University (Unesp), School of Dentistry, Araçatuba. Then, it was selected thirty-three three months' old female rats weighing approximately 350g. The animals were kept in cages with stable temperature ($22\pm2°$.C) and were fed with solid feed (NUVILAB, Curitiba PR, Brazil) water ad libitum for 10 days during acclimatization and after the start of the experiment.

The animals were divided into three groups: SHAM (positive control group, which animals underwent to fictional bilateral ovariectomy surgery with no drug treatment); OVX (negative control, which animals underwent to bilateral ovariectomy with no drug treatment) and OVX-SR (experimental group, which animals underwent to bilateral ovariectomy and treated with strontium ranelate). These animals were further divided into subgroups according to the euthanasia period and laboratorial analysis (Fig. 1).

EUTHANASIA	GROUP 1 SHAM	GROUP 2 OVX	GROUP 3 OVX/SR	ANALYSIS	
14 DAYS	5 SAMPLES	5 SAMPLES	5 SAMPLES	histology / immunohistochemistry	DECALCIFIED
60 DAYS	6 SAMPLES	6 SAMPLES	6 SAMPLES	Micro CT / Confocal microscopy	CALCIFIED

Figure 1 – Experimental groups according drug therapy, euthanasia period and laboratorial analysis.

Estrous cycle

Firstly, it was performed the estrous cycle analysis of the animals, according to Long & Evans technique (1922) (Evans and Long 1922), which was collected the vaginal

contents to immediately evaluation by electronic microscopy. The animals that presented a regular cycle were selected to study.

Bilateral ovariectomy

After selected the appropriate animals for study, it was performed bilateral ovariectomy to induce estrogen deficiency in animals from OVX and OVX-SR groups. Animals were anesthetized with xylazine (10mg/kg; p.c., i.m. Coopazine; Coopers Brasil Ltda, Campinas, São Paulo, Brazil) and ketamine hydrochloride injection (80mg/kg; Vetaset; Fort Dodge Saúde Animal Ltda, Campinas, São Paulo, Brazil), and incisions were made in both flanks, exposing ovaries followed by its removal. Tissue planes were sutured with silk thread 4-0 (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brazil) and administrated pentabiotic injection (0.1 ml/kg; Fort Dodge Saúde Animal Ltda) during the immediate postoperative period (Fig 2 A-D). The SHAM group underwent to a fake surgery, which were exposed the both ovaries, but not removed to simulate the same stress of other groups. This experimental model was already described in previous studies (Luvizuto, Dias, et al. 2010b, Luvizuto et al. 2011), proving the decreasing plasmatic concentration of estradiol after castration, and leading to an osteopenic condition.
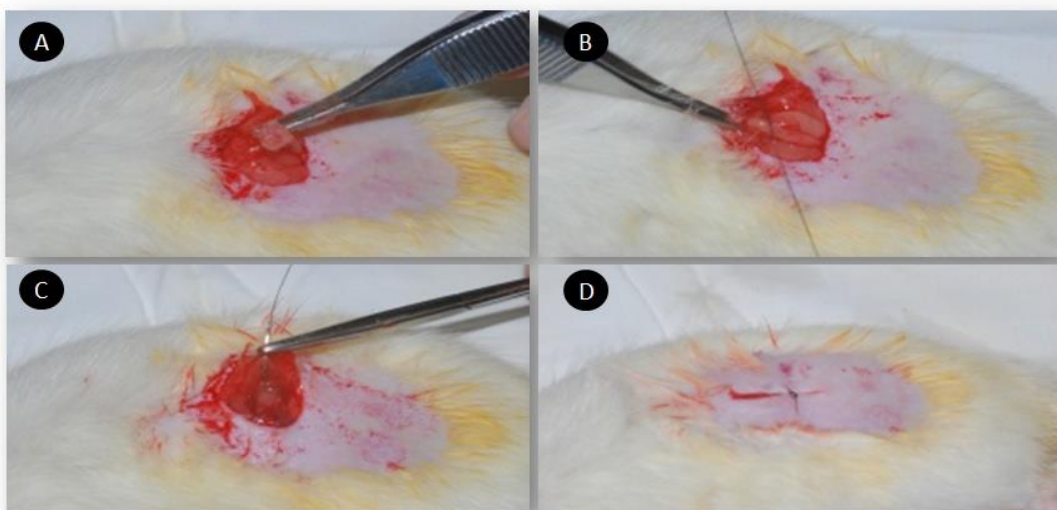
Figure 2 – Bilateral ovariectomy performed to induce osteopenic condition on animals. **(A)** Exposure of ovaries. **(B)** Lacquering of the region to avoid excessive bleeding. **(C)** Removal of both ovaries. **(D)** Suture of the planes with silk thread 4-0.

Drug treatment

Thirty days after bilateral ovariectomy, installed osteopenic condition, it was started drug treatment with strontium ranelate (Protos, Servier Ltd, Rio de Janeiro, RJ, Brazil). The animals from OVX-SR group received 625mg/kg/day of the drug dissolved in distilled water as vehicle (Bain et al. 2009, Zacchetti et al. 2014). It was administrated 0.2 ml of the solution by oral gavage up to final experiment. (Fig. 3).
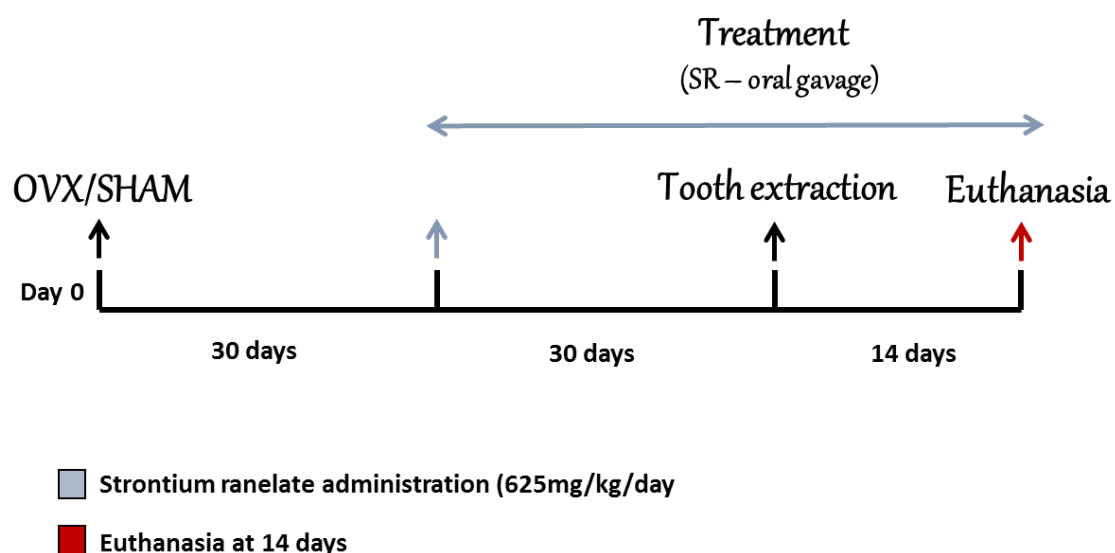
Figure 3 – Experimental design and time line correspondent to time of the treatment, surgeries procedures and period of euthanasia.

Tooth extraction

Thirty days after started drug treatment, all the animals underwent to upper right incisor extraction, creating a bone defect for further alveolar bone healing evaluation, according to previous studies (Okamoto and de Russo 1973). Animals were anesthetized with xylazine (10mg/kg; p.c., i.m. Coopazine; Coopers Brasil Ltda, Campinas, São Paulo, Brazil) and ketamine hydrochloride injection (80mg/kg; Vetaset; Fort Dodge Saúde

Animal Ltda, Campinas, São Paulo, Brazil) and, with proper instrumentation, the tooth was luxate followed by its removal (Fig. 4 A-D). All the animals received pentabiotic injection (0.1 ml/kg; Fort Dodge Saúde Animal Ltda) during the immediate postoperative period.
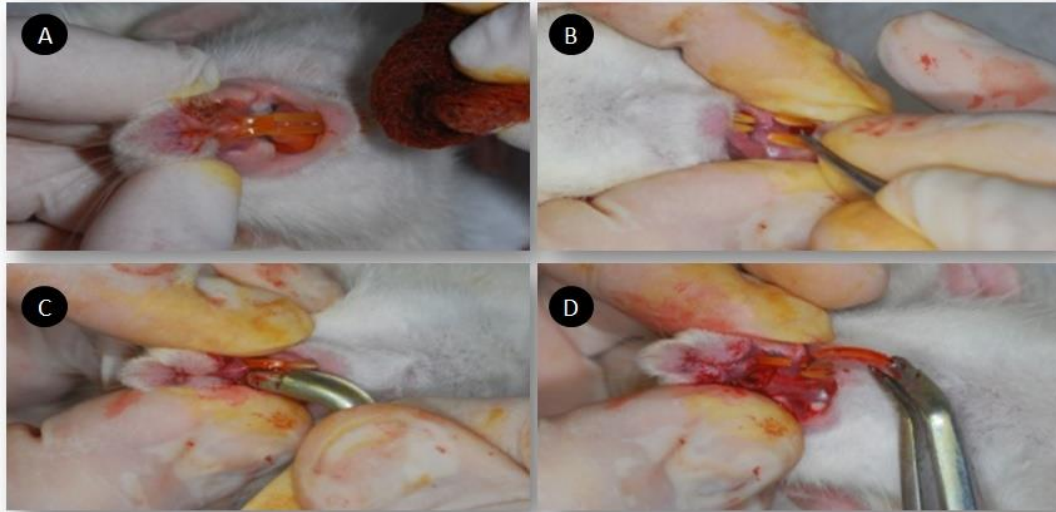
Figure 4 – Tooth extraction of right upper incisor. **(A)** Antisepsis of the region with iodine povidine. **(B)** Luxation of the right upper incisor with proper instrumental. **(C)** Movement of proper instrumental to perform tooth extraction. **(D)** Removal of the dental element followed by suture with silk thread 4-0.

Fluorochrome administration

Animals from group B underwent to intramuscular administration of 20 mg/kg of calcein (Sigma Chemical Company, St.Louis, Missouri, USA) at 14 days after tooth extraction to further evaluation of old bone on confocal microscopy, labeling by green color. Forty-two days after tooth extraction, it was performed 20 mg/kg of alizarin red administration via intramuscular (Sigma Chemical Company, St.Louis, Missouri) to further evaluation of new bone labeling by color red (Fig. 5). Fluorochromes were diluted to 1.5 mL in deionized water with a magnetic stirrer (Max Labor, Presidente Prudente, SP, Brazil).
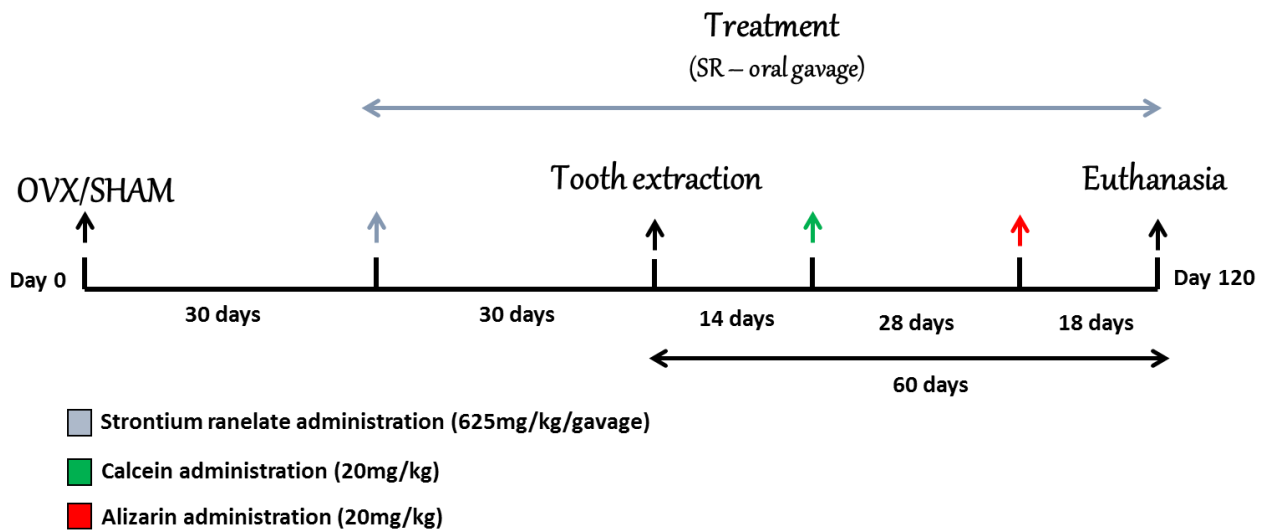
Figure 5 – Experimental design and time line correspondent to administration of fluorochromes regarding decalcified group.

Laboratory processing

Decalcified samples

Animals were euthanized at 14 after tooth extraction and it was collected the hemi-maxilla relative to alveolar healing site. The samples were destined to further histologic and immunohistochemistry analysis of the alveolar middle third. Samples were fixed in buffered 10% formalin (Analytical Reagents; Dynamic Dental-Hospital Ltd, Catanduva, SP, Brazil) for 48 h, soaked in water for 24 h and decalcified in ethylenediaminetetraacetic acid (EDTA, 10%), and then dehydrated using a series of ethanol concentrations. After these steps, the samples were diaphanous with xylol, embedded in paraffin, and sectioned to obtain five micrometers slices.

Histological analysis

The slices were mounted on slides subsequently stained with haematoxylin and eosin (H&E). After this, slices were evaluated through a light microscopy (LeicaR DMLB, Heerbrugg, Switzerland), in a qualitative way, observing the newly bone formation, spaces between trabeculae and presence of bone and connective tissue.

Immunohistochemistry analysis

For immunohistochemistry analysis, polyclonal goat antibodies (Santa Cruz Biotechnology, Dallas, Texas, USA) were used as primary antibodies against receptor activator of nuclear factor kappa-B ligand (RANKL; SC-7628) and osteoprotegerin (OPG; SC-8468) to characterize the osteoclastic activity. Immunostaining was visualized using the indirect immunoperoxidase detection method. Blocking of non-specific reactions was performed via the inactivation of endogenous peroxidase using a solution of 3% hydrogen peroxide (Merck, Kenilworth, NJ, USA), 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), and 20% of skim milk powder. Antigen retrieval was achieved using citrate phosphate buffer (pH 6.0) in the presence of moist heat.

The secondary antibody used was a biotinylated goat antibody produced in rabbit (Pierce Biotechnology, Rockford, IL, USA), which was treated with biotin and streptavidin (Dako, Glostrup, Denmark), Elite Kit, Avidin and Biotin (Vector Laboratories). Diaminobenzidine (Dako, Glostrup, Denmark) was used as the chromogen. Counterstaining was performed with Harris hematoxylin.

After this processing, the slices were evaluated using an ordinal qualitative analysis—the assignation of different "scores" (Manrique et al. 2015, Ramalho-Ferreira et al. 2016) to the presence of immunostained cells in the repaired region of the peri-implant bone. Analysis was performed using light microscopy (LeicaR DMLB, Heerbrugg, Switzerland) and assigned scores represented: no staining (0), mild staining (1), moderate staining (2), and intense staining (3). Higher scores reflected an increased area of diaminobenzidine-stained cells. The scores of the evaluator were subjected to the Kappa test, in which the index was adjusted to > 0.8, which indicates that the observed values were consistent. Absence of immunostaining was observed when the primary antibody was substituted with the serum of the host species, acting as a negative control for the secondary antibody.

Calcified tissues

Computerized tomography

Animals were euthanized at 60 days after tooth extraction and it was collected the right? maxilla relative to alveolar healing site. The samples were destined to further micro tomography and confocal microscopy analysis. Firstly, the samples were fixed in buffered 10% formalin (Analytical Reagents; Dynamic Dental-Hospital Ltd, Catanduva, SP, Brazil) for 48 h, soaked in water for 24 h and stored in alcohol 70%. After this, it was performed a scanning by SkyScan micro tomography (SkyScan 1176 Bruker MicroCT, Aatselaar, Belgium, 2003), using 9 µm thick slices (50Kv e 500µ) with copper and aluminum filter and rotation step of 0.3 mm. The images obtained by x-ray projections were stored and reconstituted considering the region of interest by software NRecon (SkyScan, 2011; Versão 1.6.6.0). After this, the software Data Viewer (SkyScan, Versão 1.4.4 64-bit) were used to reconstruct images to obtained proper position for all sample, observed into three planes (transversal, longitudinal and sagittal). By software CTAnalyser – CTAn (2003-11SkyScan, 2012 Bruker MicroCT Versão 1.12.4.0) it was possible to determine the region of interest (ROI), which was the alveolar third middle. This region was defined as total area and measured according to grayscales (thershould) of 25-90 shades, which made it possible to obtain the alveolar bone volume.

It was evaluated the parameters bone volume percent (BV/TV), trabecular thickness (Tb.Th), trabecular space (Tb.Sp), trabeculae number (Tb.N) and total porosity (Po.Tot), according to guidelines from JBMR (Bouxsein et al. 2010) All values obtained underwent to statistical analysis (Sigmaplot 12.3 software; Systat Software Inc., San Jose, CA, USA) by homoscedasticity test (Shapiro-Wilk test, $P = 0.351$) obtaining a normal distribution. Thus, a one factor (drug treatment) analysis of variance (ANOVA) was applied followed by Tukey post hoc test for significant results. For all data, a confidence level of 5% ($P < 0.05$) was elected.

Scanning Confocal Laser Microscopy

After micro tomography analysis, the same samples returned to the alcohol 70% and were dehydrated in a growing up sequence of alcohols (70-100%). After this, pieces

were embedded and infiltrated in a solution of acetone and methyl methacrylate slow (MMAL) (Clássico, Artigos Odontológicos Clássico, São Paulo, SP, Brazil) at a ratio of 1:1. This was followed by three MMAL baths. Benzoyl peroxide catalyst (1%, Riedel—de Haën AG, Seelze—Hannover, Germany) was added to the last bath. The specimens were placed in glass jars covered with a lid and were maintained in an oven at 37°C for 5 days until the resin polymerized.

After polymerization, the blocks containing the specimens were firstly mounted on acrylics blades and divided, specifically on the medium portion, parallel to the long axis of the maxilla (sagittal plane), through a mounted semi-precision saw (Exakt Advanced Technologies GmbH, Norderstedt, Germany). The sagittal cuts adhered to the acrylic blades were then mounted to histological blades through the "sandwich technique" and underwent to another sagittal cut. After that, it was obtained the slice mounted on the histological blade. The specimens underwent to a progressive manual wear with a polishing machine (Exakt Advanced Technologies GmbH, Norderstedt, Germany) with sandpaper (granulation of 120, 300, 400, 600, 800, and 1200; Exakt Advanced Technologies GmbH, Norderstedt, Germany) under fluorescent light, until a thickness of 80 μm was reached, as measured by a digital caliper (Exakt Advanced Technologies GmbH, Norderstedt, Germany).

The histological sections were mounted on slides with mineral oil (Petrolato Líquido, Mantecor, Taquara, RJ, Brazil) and fixed with coverslips and enamel to prevent oil leakage and section drying.

Longitudinal scans of interest area (Middle third of alveolar bone) were obtained, using a Leica CTR 4000 CS SPE microscope (Leica Microsystems, Heidelberg, Germany), using a ×10 objective (original amplification × 100). Images obtained by confocal microscopy were reconstructed through the stack of the software that is installed to manipulate the confocal microscope (Leica CTR 4000 CS SPE, Leica Microsystems, Heidelberg, Germany). It was possible observe two different colors that represented the precipitation of calcium after administration of calcein (green) at 14 days after tooth extraction (Fig. 6A) and alizarin (red) at 42 days after tooth extraction (Fig. 6B). Thus, the software made the overlap of both images and showed two overlapping fluorochromes (Fig. 6C). The predominance of color green represented a greater amount of old bone and

color red represented a greater amount of new bone (Ramalho-Ferreira, Faverani, Grossi-Oliveira, et al. 2015, Ramalho-Ferreira, Faverani, Prado, et al. 2015, Papalexiou et al. 2004).
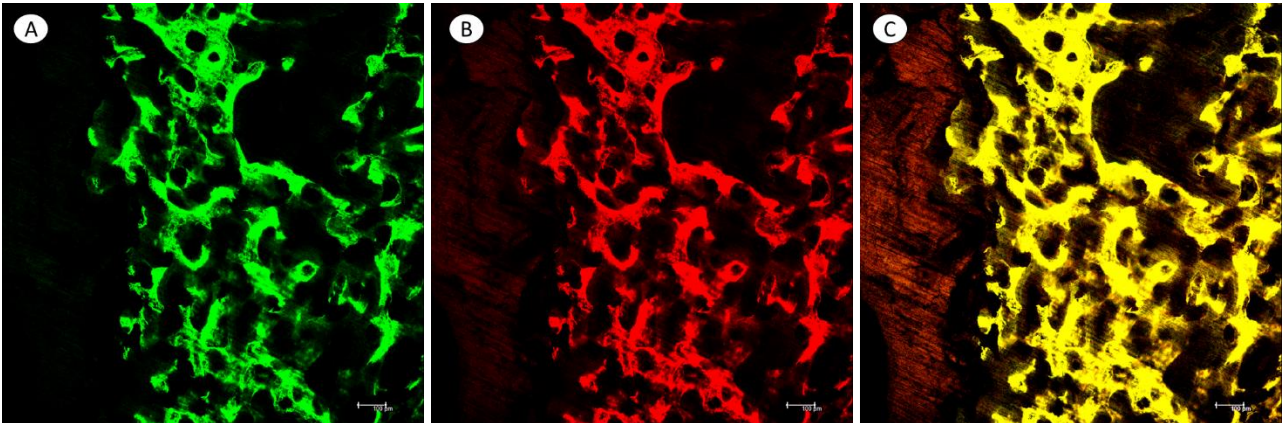
Figure 6 – Alveolar bone images obtained using confocal microscopy with overlapping of calcein (green) and alizarin (red) fluorochromes in the experimental groups. **(A)** Precipitation of calcein (green) in alveolar bone images by confocal microscopy. **(B)** Precipitation of alizarin (red) in alveolar bone images by confocal microscopy. **(C)** Overlapping of both fluorochromes obtained by software of confocal microscopy to evaluate bone dynamics.

Histometric analysis

These images were saved in TIFF format and transported to the ImageJ software (Processing Software and Image Analysis, Ontario, Canada). Using the "color threshold" tool, each image was standardized according to hue, saturation, and brightness to reveal the fluorochromes. Thus, "free hands" tool was selected and the calcein was highlighted and "measure" tool was used to provide the corresponding area in μm^2. The same procedure was performed for the alizarin, obtaining data related to the dynamics of the alveolar bone tissue (Fig. 7). Data obtained underwent a normality and homoscedasticity test (Shapiro-wilk test, $P < 0.05$), which were parametric (Sigmaplot 12.3 software; Systat Software Inc., San Jose, CA, USA). Thus, a two factor (group x fluorochromes) analysis of variance (ANOVA) was applied followed by the Tukey post hoc test for significance results. For all data, a confidence level of 5% ($P < 0.05$) was considered significant.

Figure 7 – Histometric analysis performed through the ImageJ software to evaluate quantity data. **(A) "**Freehand" tool selected to measure the fluorochrome area (µm²) on overlap of both alizarin (red) and calcein (green) fluorochromes.

Results

Results

Histologic analysis

It was obtained photomicrographs from 14 days histologic slices of all groups in a higher original objective (x25), whic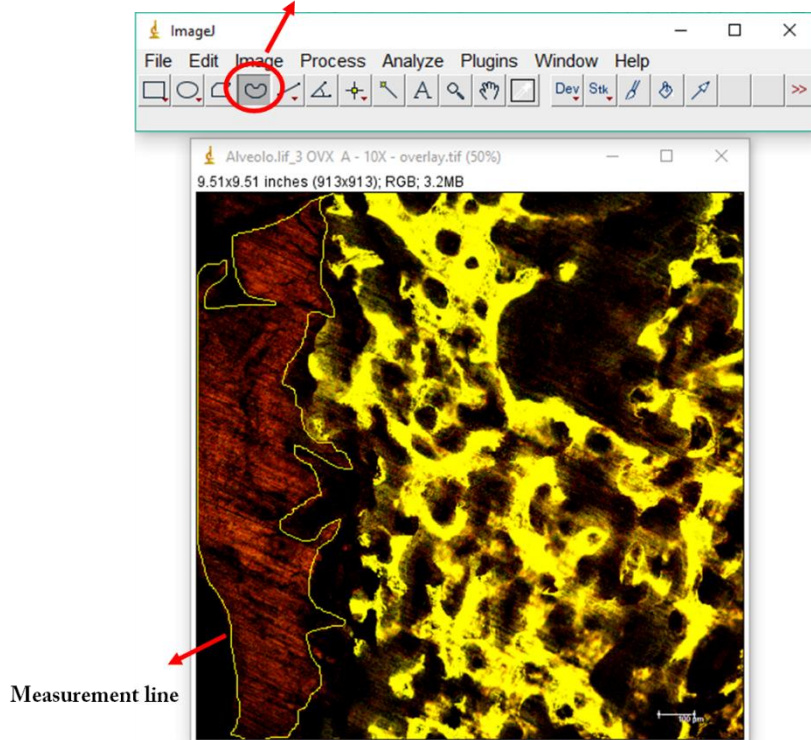h it was possible observed that SHAM group showed a great new bone formation, presence of considerable amount of connective tissue, characterizing smallest spaces between trabeculae (Fig. 8A). On the other hands, OVX group represented the worse results about histological finds, with poor new bone formation and great amount of connective tissue, characterizing greater spaces between trabeculae (Fig. 8B). Animals treated with strontium ranelate (OVX-SR) showed the best histological aspects with great amount of bone tissue against small areas composed of connective tissue (Fig. 8C).

Figure 8 – Photomicrographs in a higher original objective (x25) of histologic slices from alveolar bone healing 14 days after extraction of right upper incisor tooth. **(A)** SHAM group showing a balance amount of bone and connective tissue and few trabecular bone, characterizing a great alveolar bone healing. **(B)** OVX group showing a poor alveolar bone healing, with several and large trabecular bone and predominance of connective tissue. **(C)** OVX-SR group showing the best alveolar bone healing aspect, large amount of new bone formed, few number of trabecular bone e predominance of bone tissue against little amount of connective tissue. (*B.T*: Bone Tissue; *C.T*: Connective Tissue)

Immunohistochemistry analysis

Immunostaining was performed in all experimental groups at 14 day after incisor extraction, in order to evaluate the bone remodeling activity through the positive labeling for RANKL and OPG, the new members of tumoral necrosis fator, a family of proteins that are involved in the activation or inhibition of osteoclasts.

SHAM group:

OPG labeling: photomicrographs obtained at 14 days after tooth extraction showed a moderate staining (2) for this protein. The biomarker OPG showed greater labeling on extracellular matrix as well on trabecular bone (Fig. 9).

RANKL labeling: photomicrographs obtained at 14 days after tooth extraction showed a moderate staining for RANKL (2). Expression of the biomarker RANKL limited itself around extracellular matrix, with poor staining on trabecular bone (Fig. 9).

OVX group:

OPG labeling: photomicrographs obtained at 14 days after tooth extraction showed a moderate labeling (2) for this protein. OPG expression was most evident on extracellular matrix, with lower presence on trabecular bone (Fig. 9).

RANKL labeling: photomicrographs obtained at 14 days after tooth extraction showed an intense labeling for RANKL (3). Following the same pattern previously described, RANKL expression limited itself around extracellular matrix, with poor staining on trabecular bone (Fig. 9).

OVX-RE group

OPG labeling: photomicrographs obtained at 14 days after tooth extraction showed intense labeling (3) for this protein. Different to osteopenic rats, strontium ranelate increases OPG expression, which was present mostly on trabecular bone, as well discretely on connective tissue (Fig. 9).

RANKL labeling: On the other hands, photomicrographs of RANKL labeling from animals treated with strontium ranelate demonstrated a moderate expression (2), which was present mostly part on extracellular matrix and connective tissue with no labeling on trabecular bone (Fig. 9).

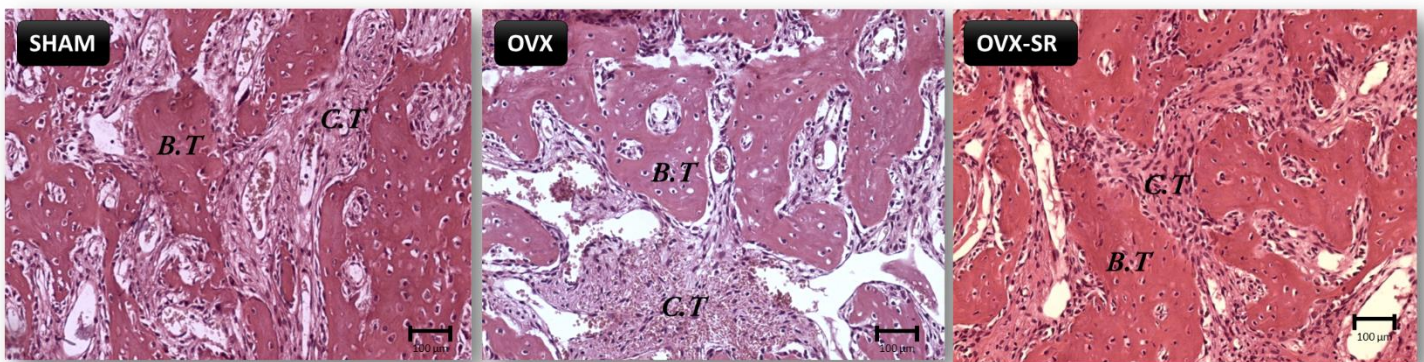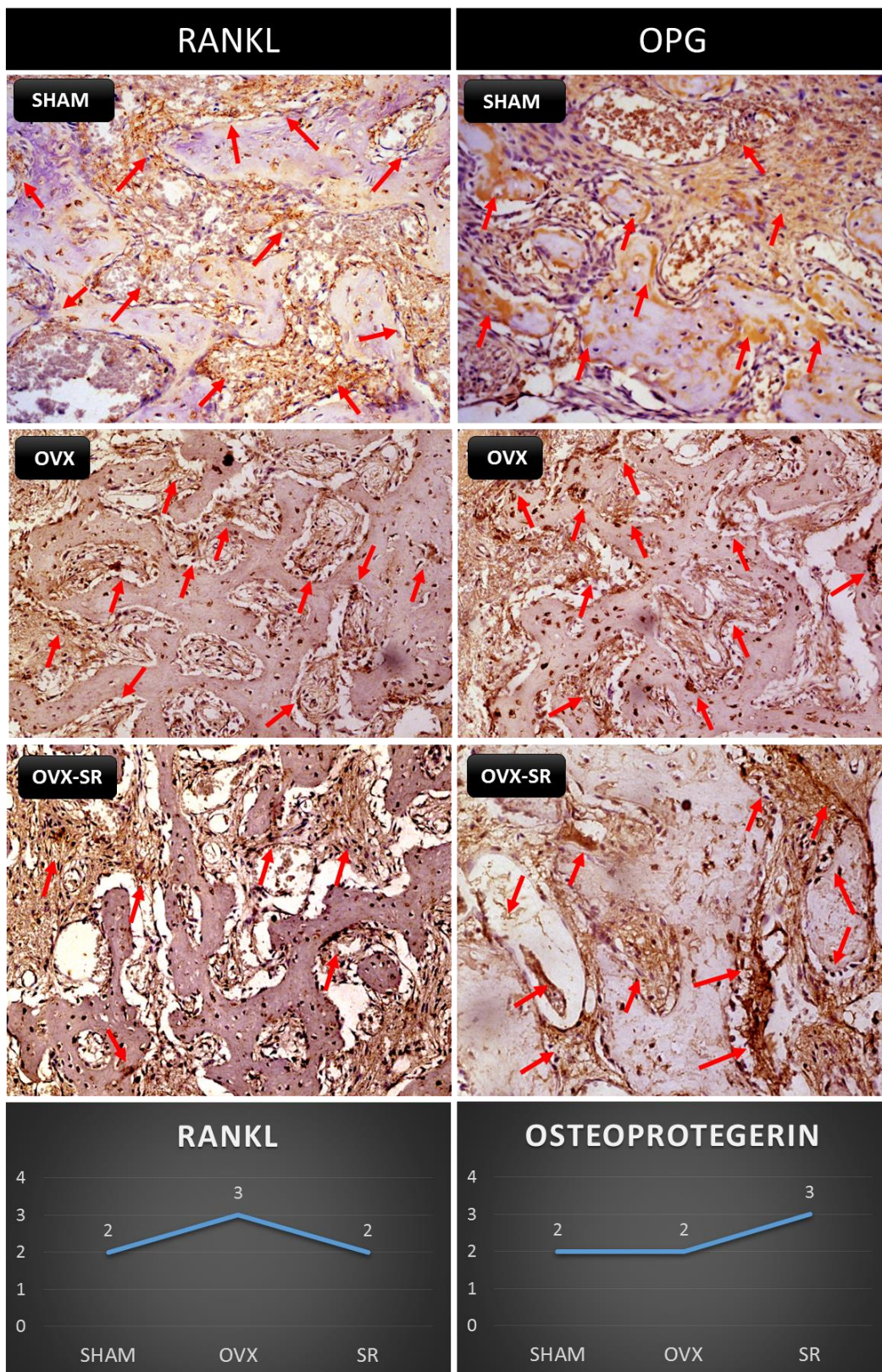**Figure 9 –** Photomicrographs in a higher original objective (x25) of histologic slices from alveolar bone healing of different experimental groups (SHAM, OVX and OVX-SR) at 14 days after tooth extraction. It was possible observed an increased area of diaminobenzidine-stained cells (brown areas) around alveolar trabecular bone where the biomarkers OPG and RANKL were intense, represented by red arrows. Representative scores about expression of the biomarker osteoprotegerin in different experimental groups, showing intense labeling for OVX-SR group and moderate labeling for SHAM and OVX group. Biomarker RANKL was intense to OVX group and moderate for SHAM and OVX-SR group.

Flurochrome area

The photomicrographs related to the maxillas from healthy animals (SHAM group) showed small amount of old bone, characterizing by lower precipitation of calcein at 14 days after tooth extraction, while precipitation of alizarin red was most presented on flurochromes overlapping, characterizing higher amount of new bone (Fig. 10A). Although, osteopenic animals (OVX group) showed a predominance of calcein precipitation (green), compared with alizarin red, prevailing the presence of old bone during alveolar healing (Fig. 10B). Treatment with strontium renalate provided a balance on fluorochromes overlapping, but with prevalence of alizarin precipitation (Fig. 10C).
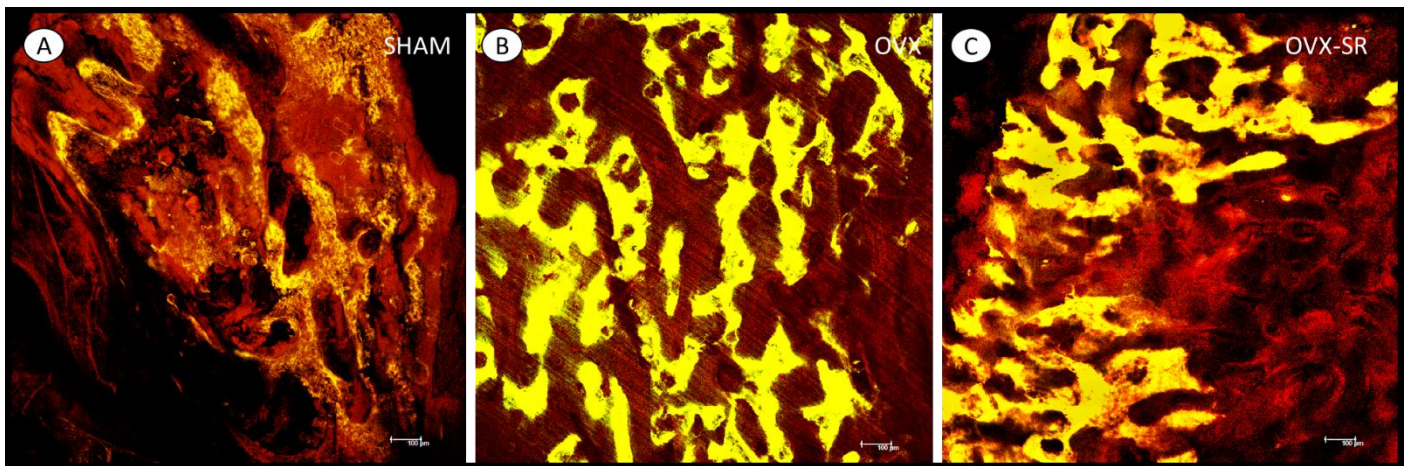
Figure 10 – Alveolar bone images obtained using confocal microscopy with overlapping of calcein (green) and alizarin (red) fluorochromes in the experimental groups. **(A)** Sham group showed a lower precipitation of calcium for calcein (green) administration with a little amount of old bone. **(B)** OVX group showed a large amount of old bone, labeling by the calcium precipitation in the calcein (green) administration. OVX-SR group showed a large amount of new bone, labeling by alizarin red and low amount of old bone (green).

The quantitative data about fluorochrome area showed that at 60 days after tooth extraction, there was a significance prevalence of areas with alizarin red precipitation compared to areas with calcein precipitation (green), for all experimental groups (Tukey test - P < 0.05). Regarding intergroups evaluation of each fluochrome, it was possible to observe that strontium ranelate demonstrated to increase significantly precipitation of alizarin red after 60 days of tooth extraction compared to SHAM (Tukey test – P = 0.008) and OVX (Tukey test – P = 0.013) groups, which showed no significant differences on precipitation of alizarin red between them (Tukey test – P > 0.05). There was a tendency of high values on precipitation of calcein (green) for OVX group, mainly compared to SHAM group (Tukey test – P > 0.05) and demonstrated to be closer to the values from OVX-SR group (Tukey test – P > 0.05) (Fig. 11).
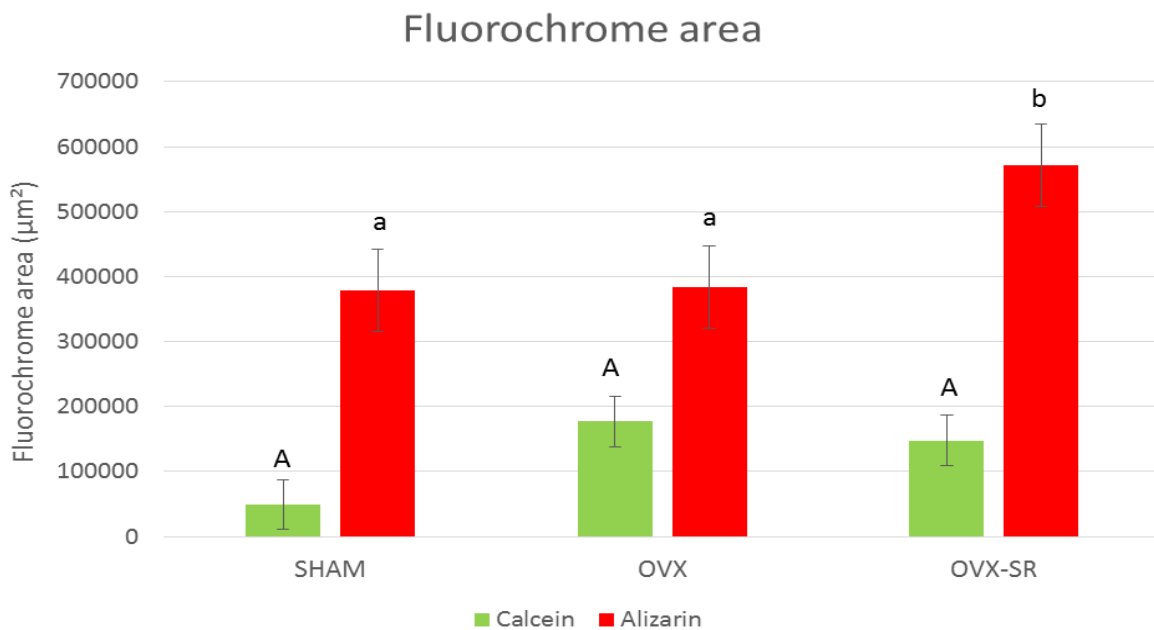
Fig. 11 - Avarage and standard deviation values of fluorochromes areas (Calcein and Alizarin red) in um² of experimental groups (SHAM, OVX and OVX-SR) at 60 days after tooth extraction. Different letters A/a or A/b show statistical significance difference between calcein and alizarin precipitation (P < 0.05) in the intragroup evaluation; uppercase letters represent similarity among groups (P > 0.05) in the intergroups evaluation; different lowercase letters (a/b) show statistical significance difference among groups (P < 0.05) in the intergroups evaluation.

Micro tomography analysis

Regarding morphologic parameters obtained through micro tomography scanning, it was possible observe that treatment with strontium ranelate seemed to increase values about bone volume percent (BV.TV) (Tukey test – P > 0.05) and trabecular thickness (Tb.Th) (Tukey test – P = 0.013) compared to osteopenic animals. On the other hands, the drug showed decreasing values about trabecular space (Tb.Sp) and total porosity (Po.Tot), contrary to OVX group results (Tukey test – P > 0.05). Regarding the trabecular number, despite osteopenic condition shows decreasing this parameter, strontium ranelate were not able to improve this condition, presents similar results to OVX group. Although, SHAM group demonstrated to increase these values, compared to both groups (Tukey test – P > 0.05) (Fig. 12 A-E)

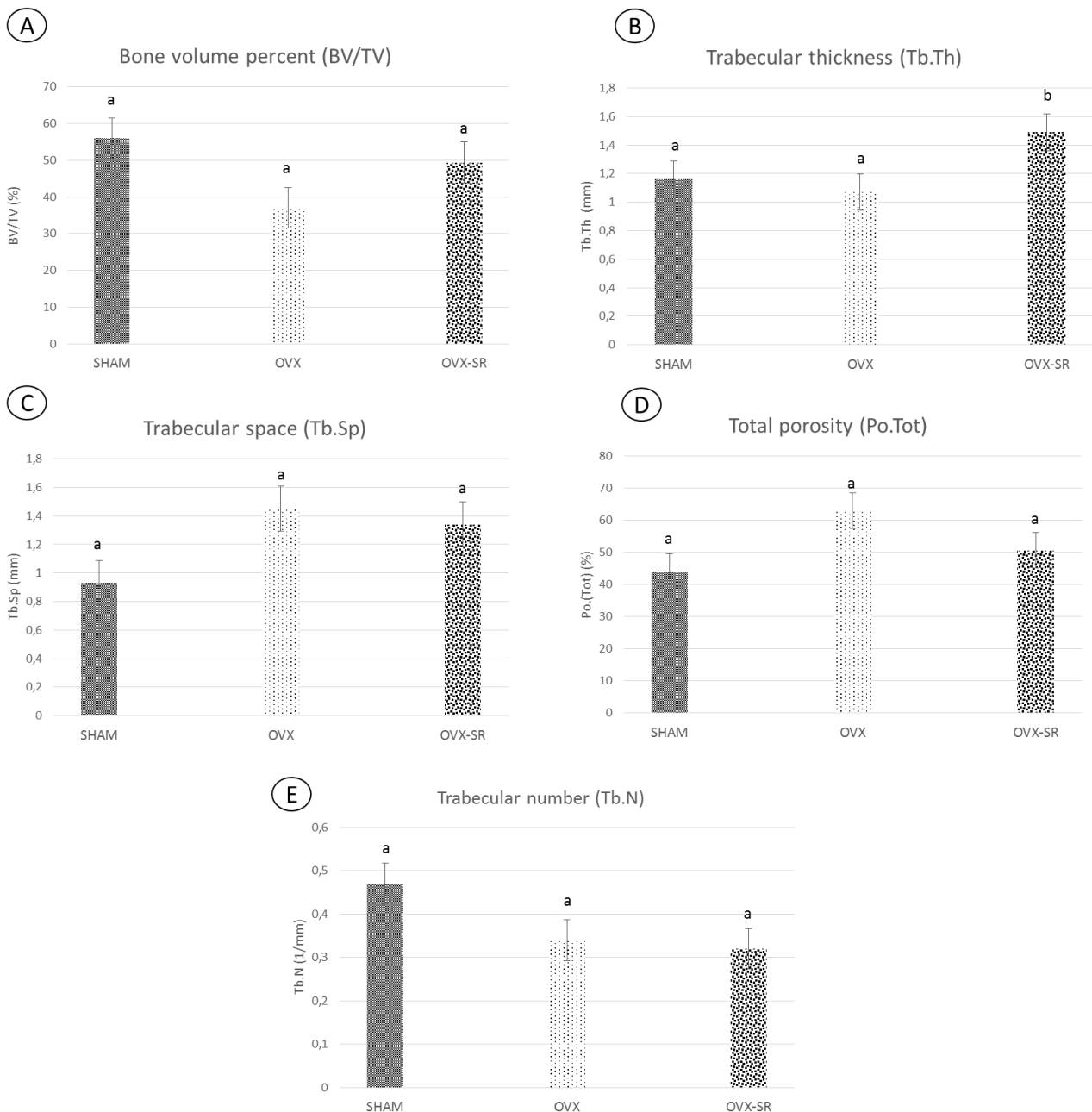**Fig. 12 -** Average and standard deviation values of micro tomographic parameters in the different experimental groups (SHAM, OVX and OVX-SR) at 60 days after tooth extraction. Different letters a or b show statistical significance difference between groups, according to analyzed parameters (P < 0.05); same letters represent similarity among groups (P > 0.05) for each parameter analyzed.

Discussion
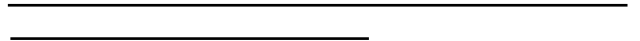
Discussion

This study indicated that six or twelve weeks of strontium ranelate treatment improves alveolar bone healing of osteopenic rats at 14 days after tooth extraction, which represents the period of greater cellular acitivity; and 60 days after tooth extraction, which represents final bone microstructure and its quality. SR treatment showed to be able to increases cellular activity related to bone formation and improves hsitologic characteristics when compared with non-treated osteopenic rats. Moreover, twelve weeks of treatment demonstread to upregulates bone turnover while improved bone microstructure parameters.

SR has been shown an effecitve drug on treatment of osteoporosis, reducing risk of vertebral and non-vertebral bone fractures and promotes enhance of bone mineral density (BMD) (Reginster et al. 2005, Seeman et al. 2006). Although, little is known about its effect on maxillofacial bone. When analyzed on alveolar bone healing of osteopenic rats in this study, it was possible observed that six weeks of SR treatment leads to an intense expression of OPG as decrease RANKL labeling of OVX rats after 14 days after tooth extraction. On the other hand, Non-treated OVX animals showed a moderate labeling for OPG and intense expression of RANKL, suggesting that SR simultaneously stimulates alveolar bone formation and inhibt its resorption. These data is consistent with previously studies that suggested an anabolic action of SR, inhibiting osteoclastogenesis through decreases of RANKL expression (Hofbauer et al. 2000, Marie et al. 2005) and stimulates osteoblastogenesis through activation of CaSR (Marie 2006, Bonnelye et al. 2008).

Moreover, long-term used of SR (twelve weeks) showed to improves alveolar bone *turnover* of osteopenic rats, sixty days after tooth extraction, supported by highest values of new bone area (alizarin red) to OVX-SR group compared to SHAM (Tukey test - P = 0.008) and OVX group (Tukey test - P = 0.013). Despite osteopenic animals showed a tendency to higher values for old bone area, there was no significant difference among groups and OVX-SR group showed near values to them. This might occurred due the anti-osteoclastic effetc of SR, which prevents this resorption activity responsible to replace the old bone remains. On the other hand, the greater OPG expression suggest do not compromising bone *turnover*.

It is already kown that quality of bone tissue is crucial for the success of oral rehabilitations (Jaffin and Berman 1991, Jemt et al. 1992). It has been suggested that bone density, bone volume and trabecular bone are fundamental parameters on implants survival rate (Shapurian et al. 2006, Parfitt et al. 1987). Micro-CT demonstrates to be the gold standard modality to evaluate these bone parameters, however is not applied in the clinic (Burghardt, Link, and Majumdar 2011). This currently study demonstreated an improvement on histomorphometric aspects of 14 days alveolar bone healing after six weeks of SR therapy on osteopenic rats. It was possible observed on histological slices the greater pattern of trabecular bone with minimally presence of connective tissue for OVX-SR group, showing to be superior even than healthy animals (SHAM group).

Indeed, tridimensional parameters evaluated by micro-CT about 60 days of alveolar healing showed that SR tratment was able to improve values for bone volume percent (BV/TV) and trabecular thickness (Tb.Th) (Tukey test – P <0.05), besides decreases bone porosity (Po.tot), similarity to the healthy animals. These data suggesting SR could improved bone microstruscture characteristics, as BMD and amount of bone formed supporting above mentioned data of this study, characterizing am greater alveolar bone quality. It is believed that activation of CaSR perfomed by SR also stimulates bone matrix mineralization (Marie, Felsenberg, and Brandi 2011). Futhermore, previously studies confirmed that SR treatment increases bone mineral density (Meunier et al. 2009, Morabito et al. 2016), which could explain these improvements on micro-CT data.

Due the high incidence of osteoporosis in worldwide population, several treatments have been proposed (Riggs, Khosla, and Melton 1998). However, little is dicussed about effects of these drugs on alveolar bone *turnover*. Is well known that biphosphonates, mainly alendronate, are the mostly drugs used for osteoporosis treatment and promotes great results regarding prevents vertebral bone and hip fracutres, as well as, increasing BMD (Wells et al. 2008). Although its long-term used could develop osteonecrosis of the jaw (Ruggiero and Drew 2007). Denosumab is a new therapy used on osteoporosis and it is also related to MRONJ development (Ruggiero et al. 2014). Raloxifene is another drug that has been widely studied. We previously demonstreated in pre-clinical studies that this drug has a greater effect on alveolar and peri-implantar bone healing of osteoporotic rats (Ramalho-Ferreira, Faverani, Prado, et al. 2015, Ramalho-Ferreira et al. 2016). However, this drug is not well accept on medical clinic,

due to the lack of effect to reduces non-vertebral fractures (Gallacher and Dixon 2010). On the other hands, it is well established that SR promotes a positive effect in reduces vertebral and non-vertebral fractures, such as increases BMD (Seeman et al. 2006, Reginster et al. 2005). It is believed that 2g of SR daily over a 3 to 4 years period could develop vascular and nervous system disorders, however these data is no consistent and further investigations are necessary (O'Donnell et al. 2006). Moreover, it was possible observed in this experimental study that this drug improves the quality and *turnover* of alvelolar bone tissue, which could represents a good choice, thinking about the medical-dentistry relationship.

It must be recognize that this pre-clinical study presents several limitations about effect of SR on alveolar bone. Other studies should be developed to find answers about this drug on peri-implant bone healing, its mechanism of action, mechanical properties and its effect on immediately loading implants. However, this is the first study to characterize the alveolar bone tissue of osteopenic rats under SR therapy.

Thus, it was concluded that SR treatment improves alveolar bone healing of osteopenic rats through an enchace of osteoblastic cellular activity and inhibit of osteclast activity, promoting a good quality of alveolar bone tissue.

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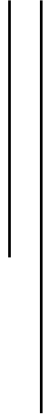
Attachments

ATTACHMENT A

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Avaliação do processo de reparo alveolar de ratas com deficiência de estrógeno tratadas com denosumab ou ranelato de estrôncio. Análise histométrica, imunoistoquímica, por microtomografia computadorizada e microscopia confocal"**, Processo FOA nº 00685-2015, sob responsabilidade de Roberta Okamoto apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 24 de março de 2016.

VALIDADE DESTE CERTIFICADO: 26 de Julho de 2017.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 26 de Agosto de 2017.

CERTIFICATE

We certify that the study entitled **"Evaluation of the wound healing process in estrogen deficiency rats treated with denosumab or strontim ranelate. Histometric, immunohistochemistry, computed microtomography and confocal microscopy analysis"**, Protocol FOA nº 00685-2015, under the supervision of Roberta Okamoto presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on March 24, 2016.

VALIDITY OF THIS CERTIFICATE: July 26, 2017.
DATE OF SUBMISSION OF THE FINAL REPORT: August 26, 2017.

Profa. Ass. Dra. Maria Gisela Laranjeira
Coordenadora da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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ATTACHMENT B

Revista proposta para publicação: *Journal of Dental Research*

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