Marcelo Tadahiro Wayama

Efeito do raloxifeno na lesão periapical em ratas ovariectomizadas.

ARAÇATUBA-SP

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DEDICATÓRIA

Dedico este trabalho,

À minha mãe Michiko, おかあさん、いつもかわいくって、ありがとう。いつもにこってわらっているおかあさんだいすきです。いっしょにがんばろ！

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Epígrafe

"Quanto maiores somos em humildade, tanto mais próximos estamos da grandeza."

Rabindranath Tagore

"Saber não basta, devemos aplicar.

Desejar não basta, devemos fazer."

Johann Wolfgang

Sumário

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Resumo

O objetivo deste estudo foi avaliar o efeito do raloxifeno RLX em lesões periapicais em ratas ovariectomizadas (OVX). Ratas Wistar (6 meses) foram distribuídas nos grupos: SHAM/VEI, OVX/VEI e OVX/RLX e receberam, por 60 dias, veículo (VEI) ou RLX, por gavagem. Durante o tratamento, a polpa do primeiro molar inferior direito e esquerdo foram expostas ao ambiente oral permitindo a análise da lesão aos 7 e 30 dias. Coleta sanguínea foi realizada para análise bioquímica. Logo após os animais foram eutanasiados e as mandíbulas separadas, radiografadas, processadas para análises histopatológica, histométrica e imunoistoquímica. Os dados foram submetidos ao teste de Tukey ou Dunn ($p<0,05$). A concentração plasmática de estradiol evidenciou o hipoestrogenismo em ratas OVX. A análise radiográfica mostrou diferença significativa apenas entre os períodos, as lesões com 7 dias apresentaram menor extensão que aquelas com 30 dias. Aos 7 dias, a atividade da fosfatase alcalina e o cálcio plasmático foram superiores em OVX/RLX em relação ao grupo SHAM/VEI. O fósforo plasmático foi maior no grupo OVX/RLX em ambos os períodos. O grupo OVX/VEI apresentou inflamação mais intensa com maior área de lesão e imunomarcação de RANKL, HIF1-

alfa e células TRAP-positivas que outros grupos em ambos os períodos. Enquanto o tratamento com RLX reverteu este quadro com padrão semelante à SHAM/VEI. Não houve diferença estatística na imunorreatividade de OPG e BALP entre os grupos em ambos sos períodos. Os resultados mostram que o hipoestrogenismo potencializa os efeitos da lesão periapical a qual é amenizada pela ação do RLX devido aos seus efeitos combinados em diminuir a atividade osteoclástica e amenizar o turnover ósseo.

Palavras-chave: Raloxifeno, estrogênios, periodontite apical, ovariectomia, remodelação óssea.

Wayama, MT. Effect of raloxifene on periapical lesion in ovariectomized rats. Araçatuba, 2014. 114p . Dissertation (Master in Endodontics) – Dental School of Araçatuba, São Paulo State University "Júlio de Mesquita Filho".

Abstract

The aim of this study was to evaluate the effect of raloxifene (RLX) on periapical lesions in ovarietomized rats (OVX). Wistar rats (6 months) were distributed into groups: SHAM/veh, OVX/veh and OVX/RLX that received for 60 days vehicle or RLX by gavage. During treatment, the pulp of lower first molar was exposed to the oral environment to enable lesion analysis on 7 and 30 days.Blood collection was performed for biochemical analysis. Soon after the animals were killed, the jaws separated, radiographed and processed for histological, histometric and immunohistochemical analyzes. Data were submitted to Tukey or Dunn test ($p < 0.05$). The plasma concentration of estradiol showed hypoestrogenism in OVX rats.Radiographically, the groups were similar but lesions on day 7 were smaller than lesions on day 30. On day 7, the alkaline phosphatase activity and plasma calcium were higher in OVX-RLX than the SHAM-veh. After 30 days of lesion, significant decrease was observed in calcium level in the raloxifene group. The alkaline phosphatase activity was higher in groups without RLX after 30 days oflesion. The plasma concentration of phosphorus was higher in RLX

group, in both time points. The OVX/veh group presented more intense inflammation, larger area of periapical lesion and immunostaining of RANKL, HIF-1alpha and TRAP-positive cells than other groups in both time points. While treatment with RLX reversed this condition and was similar to SHAM/veh. There was no statistical difference in immunoreactivity of OPG and BALP between groups in both time points. The results showed that hypoestrogenism potentiates the effects of periapical lesions, and this potentiation is diminished by RLX due its effects in decreasing osteoclast activity and soften bone turnover.

Keywords: Raloxifene, estrogens, apical periodontitis, ovariectomy, bone remodeling.

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Lista de siglas e abreviaturas

Abs = Absorbância

ANOVA = Análise de Variância

Al = Alumínio

Cat.= catálogo

d = dias

C = cemento

DR = Densitometria radiográfica

EDTA = Ácido etilenodiaminotetracético

IL = Interleucina

EPM = Erro Padrão de Média

ALP = Alkaline phosphatase

HE = hematoxicilina e eosina

i.m.= intramuscular

i.p. intraperitoneal

Kg = Kilograma

Kv = Kilovolt

M = Massa molar

mA = Miliampere

mmAl = Milímetro de alumínio

Mo = Mouse

MSRE = Moduladores seletivos do receptor de estrógeno

N^0 = Número

NaCl = Cloreto de Sódio

AO = osso alveolar

OPG = Osteoprotegerina

OVX = Ovariectomizada

P = Fósforo

p.c.= peso corporal

pH = Potencial hidrogeniônico

RANK = Receptor do fator ativador nuclear kappa-b

RANKL = Receptor ativador do fator nuclear kappa-b ligante

Rb = Rabbit

RLX = raloxifeno

RPM = Rotação por Minuto

TNF-alfa = Fator de necrose tumoral alfa

TRAP = Fosfatase ácida resistente ao tartrato

Sh = Sham

U/L = Unidade por Litro

Vei = Veículo

Vs = versus

Introdução

O aumento da expectativa de vida está fazendo com que a proporção de idosos cresça mais rapidamente do que qualquer outra faixa etária no mundo (Fabrício et al., 2008). Com o passar da idade, há maior vulnerabilidade e incidência de processos patológicos, como a osteoporose. Estas condições interferem na qualidade de vida e no aumento do índice de mortalidade, representando importante problema para a saúde das pessoas nesta faixa etária (Ferreira et al., 2012; Pinheiro et al., 2010). Neste contexto, há grande preocupação com a saúde dos idosos e com a prevenção de patologias decorrentes do processo de envelhecimento (Sousa et al., 2007), formulando novos estudos que possam melhorar a qualidade de vida destes indivíduos.

No indivíduo adulto, o esqueleto é mantido devido à sua regeneração contínua que ocorre pelo processo chamado remodelação óssea, isso ocorre através da remoção e posterior substituição do osso existente por tecido ósseo neoformado (Manolagas, 2000). A remodelação óssea é regulada por diferentes mecanismos, tais como os membros da superfamília do fator de necrose tumoral, o receptor ativador do fator nuclear kappa B/ligante (RANK/RANKL) e osteoprotegerina (OPG), que são essenciais para a osteoclastogênese (Honma et al., 2014). RANKL está expressa em osteoblastos, fibroblastos e células B e T, sendo uma citocina

essencial na formação de osteoclastos (Lerner, 2006). O RANKL se liga a RANK que está presente nos pré-osteoclastos e estimula a diferenciação em osteoclastos, enquanto que a ligação do RANKL com OPG inibe a sua diferenciação (Kotake et al., 2001).

O idoso pode apresentar diversas alterações neste processo que predispõem condições patológicas típicas do envelhecimento (Yazbek et al., 2008). A osteoporose é um processo patológico decorrente ao declínio na concentração plasmática de estrógeno, que ocorre em mulheres na menopausa (Bedell et al., 2012). Estas alterações resultam do aumento de cavidades de reabsorção, que por sua vez não são completamente preenchidas pela sua formação, resultando na diminuição da densidade óssea e consequente risco de fratura (Armas et al., 2012; Anbinder et al., 2006).

O estrógeno é um dos principais hormônios que participa do processo fisiológico do indivíduo, como crescimento e desenvolvimento celular, regulação do sistema reprodutor, neuronal, imune, cardiovascular e esquelético (Pettersson et al., 2001; Couse et al., 1999). Sobre o tecido esquelético o estrógeno promove a diminuição da reabsorção óssea, atuando diretamente ou indiretamente sobre células do metabolismo ósseo. Além disso, o estrógeno desempenha ação importante nas doenças inflamatórias, podendo interferir na produção de citocinas (Millán et al., 2013; Straub et al., 2007).

A deficiência do estrógeno pode influenciar regiões específicas como, por exemplo, os sítios locais de doenças periodontais e periapicopatias, fazendo com que o nível de reabsorção aumente. (Xiong et al., 2007). Nestes processos patológicos da região periodontal são envolvidas diversas células como osteoblastos e osteoclastos cujas funções determinam o desenvolvimento da perda da massa óssea (Zhang et al., 2007). Nas periapicopatias, além das células ósseas, citocinas como IL-1 e TNF-alfa também participam deste processo, os quais também são observados na osteoporose. Assim, os mecanismos celulares e moleculares que levam a perda óssea, são semelhantes entre o processo inflamatório e a osteoporose (Palomo et al., 2007). Estes dois quadros envolvem reabsorção óssea, entretanto a osteoporose não é a causa primordial da periodontite apical, mas que pode contribuir para o progresso da lesão (Xiong et al., 2007).

Diversos fármacos têm sido estudados como possíveis agentes terapêuticos para suprir a deficiência do estrógeno. Dentre estes, destacam-se os moduladores seletivos do receptor de estrógeno (MSRE) que são da classe de moléculas não hormonais e que dependendo do receptor de estrógeno que se liga, podem desencadear efeitos agonistas ou antagonistas sobre o receptor de estrógeno presente no tecido-alvo (Rossi et al., 2010). O cloridrato de raloxifeno (RLX), MSRE de segunda geração, mimetiza os efeitos benéficos do estrógeno sem estimular tecidos como mama e

endométrio (Lewis et al., 2005). Estudos mostram que a terapia com raloxifeno resulta no aumento da densidade mineral óssea, diminuindo significativamente a incidência de fraturas (Siris et al., 2005; Delmas et al., 2002; Ettinger et al., 1999).

O mecanismo de ação molecular deste MSRE envolve alta afinidade de ligação com o receptor de estrógeno, provocando alteração conformacional na estrutura do receptor, sua dimerização e associação com elementos resposta do DNA específicos para o RLX (Dutertre et al., 2000;). Devido a expressão dos receptores em órgãos distintos, há seleção de tecidos para sua atuação. Portanto dependendo de qual for receptor que se liga o efeito pode ser antagonista (como em mama e útero) não estimulando as vias estrogênicas, assim não causando tumores nestes tecidos, ou pode ter o efeito agonista exercendo sua função anti-reabsortiva (Perez, 2006; Sliwinski et al., 2005).

Diante do exposto e considerando a importância da terapêutica com MSRE é importante à realização de estudo experimental para análise da ação do raloxifeno sobre a lesão periapical em organismos com hipoestrogenismo.

Proposição

Objetivo geral:

- ✓ Analisar a atuação do raloxifeno na lesão periapical em ratas ovariectomizadas;

Objetivos específicos:

- ✓ Avaliar e comparar a lesão periapical de 7 e 30 dias em ratas adultas sham e ovariectomizadas (OVX) pela coloração de hematoxilina e eosina;
- ✓ Analisar e comparar a ação de raloxifeno na região periapical de ratas sham e OVX, utilizando a expressão das proteínas RANKL,TRAP, OPG, HIF-1α e BALP como indicadores celulares de predisposição à reabsorção ou formação de tecido ósseo;
- ✓ Analisar e comparar as concentrações dos marcadores de atividade celular do metabolismo ósseo (cálcio, fósforo e fosfatase alcalina) entre os grupos experimentais**.**

Artigo 1

Effect of raloxifene on periapical lesions in ovariectomized rats

Abstract

Introduction: The aim of this study was to evaluate the effect of raloxifene (RLX) on periapical lesions in ovariectomized (OVX) rats. Methods: Female Wistar rats were OVX or subjected to sham surgery and received vehicle or RLX by gavage for 60 days. The treatment groups were as follows: sham/vehicle, (SHAM-veh), OVX-veh and OVX-RLX. During treatment, the pulp of lower first molar was exposed to the oral environment allowing the lesions analysis on day 7 and 30. Blood samples were taken, the rats were killed, and the mandibles removed and prepared for radiographic, histopathological, histometric, and immunohistochemical analysis. Results: Estradiol plasma concentration showed hypoestrogenism in OVX rats. The OVX-veh group showed larger periapical lesions with more resorption lacunae and cells positive for tartrate-resistant acid phosphatase (TRAP). This condition was reverted in OVX/RLX group being similar to sham. Radiographically, the groups were similar but lesions on day 7 were smaller than lesions on day 30. Conclusion: The results showed that hypoestrogenism potentiates the progression of periapical lesions, and this potentiation is diminished by RLX due its effect in decreasing osteoclast activity.

Keywords: Raloxifene hydrochloride, periapical lesion, bone metabolism, ovariectomized.

Introduction

The growing elderly population has led to increasing interest in the treatment and prevention of diseases associated with the aging process (1). This interest has encouraged a large number of investigators to conduct new studies on animal models of the disease processes related to aging. In the current study, the effects of raloxifene (RLX) on periapical lesions in ovariectomized rats (OVX) were examined as a model of post-menopausal osteoporotic bone loss.

Periapical lesion starts with inflammation and necrosis of the tooth pulp. Bacterial growth reaches the channel system recruiting inflammatory cells, inducing osteoclasts and promoting bone resorption (2). Bone metabolism is also regulated by estrogen that interacts with cells involved in bone remodeling: osteocytes, osteoblasts, and osteoclasts (3). Therefore, these cells can be influenced by both systemic (e.g., estrogen) and local factors (e.g., inflammation and necrosis) (4).

Low estrogen concentration in plasma produces changes in many parts of the body, including oral tissues. The teeth and gums

are extremely susceptible to changes in estrogen levels (5). Estrogen has been shown to influence bone resorption and its deficiency aggravates osteopenia (6) and apical periodontitis (7). Thus, it is important to understand the mechanisms involved in the interaction between systemic and local factors.

Many therapies have been studied to treat and prevent the conditions resulting from menopause. However, some of these treatments have been associated with side effects like bloating and breast tenderness (8), or even breast and uterine tumors (9). RLX, a benzothiophene analogue, has been approved for the treatment and prevention of osteoporosis in postmenopausal women (10). RLX has been shown to increase bone mineral density and reduce the incidence of bone fractures (10-11); it has not been associated with breast and endometrial tumors due to its specific receptor activity; it does not activate estrogen receptors in breast or uterine tissues (12). Therefore, RLX is indicated in patients with a history of breast and/or endometrial neoplasms, or other factors that contraindicate hormone replacement therapy (13).

Although knowing the indication, there are no studies evaluating the effects of RLX on periapical lesions in animals with hypoestrogenism. Therefore, the aim of the present study was to evaluate periapical lesions in OVX rats treated with RLX.

Material and methods

Animals

Forty-eight female Wistar rats (six months of age) were used. The experimental procedures were approved by the institutional ethics committee (Ethics Committee on Animal Use – Univ Estadual Paulista - 00799-2012). The animals were distributed into 6 groups: sham surgery plus vehicle treatment with 7 days (SHAM-veh 7d) or 30 days of pulp exposure (SHAM-veh 30d); OVX plus vehicle treatment with 7 days (OVX-veh 7d) or 30 days of pulp exposure (OVX-veh 30d); and OVX plus RLX with 7 days (OVX-RLX 7d) or 30 days of pulp exposure (OVX-RLX 30d).

Estrous cycle

Determination of the estrous cycle status of the rats was performed by swab vaginal collection (9 am). The swabs were examined using an optical microscope, according the technique of Long and Evans (14). Females with regular cycle (12 days) were randomly distributed into the experimental groups. Females that did not have a regular estrous cycle were excluded from the study.

Ovariectomy

Rats were OVX or subjected to sham surgery. The animals were anesthetized with ketamine (75 mg/kg; Vetaset, Fort Dodge Animal

Health Ltd, São Paulo, Brazil) and xylazine (25 mg/kg; Coopazine, Coopers Ltd. Brazil, São Paulo, Brazil) by intraperitoneal injection. An abdominal incision was made to expose the distal portions of the fallopian tubes. Then the ovaries were removed in the OVX groups. In the sham groups, after the incision the ovaries were exposed but not removed. All animals had their incisions closed with sutures and received an intramuscular dose of antibiotics (1 mL/kg, Pentabiotic Veterinary, Fort Dodge Animal Health Ltd, São Paulo, Brazil).

Raloxifene or vehicle treatment

Ten days after OVX or sham surgery, vehicle (distilled water 0.3 mL) or raloxifene (Sigma-Aldrich, Munich, Germany) (1 mg/kg in 0.3 mL distilled water) was administered daily by gavage, for 60 days (15-17).

Periapical lesion induction

During the treatment it was performed the periapical lesion induction under general anesthesia. The right and left mandibular first molars had their pulp exposed to the oral environment with the aid of carbon drill bur (Drill Long Neck Ln, Maillefer, Dentsply, Catanduva, Brazil). The initial lesion was 0.1 mm in diameter.

Sample Collection

Under general anesthesia, blood was collected from the jugular vein (18), centrifuged (3,000 rpm for 20 min at 2°C) and the plasma stored in a freezer at -20°C for measurement of estradiol. The animals were killed by anesthetic overdose and the mandibles removed for radiographic, histopathological, histometric, and immunohistochemical analysis. The uteruses were also removed and weighed.

Plasma estradiol level measurement and uterus weight

Plasma estradiol concentration was measured in duplicate using a Biomedicals estradiol kit by radioimmunoassay (Costa Mesa, CA, USA). The minimum detectable dose of estradiol was 5.0 pg/mL and the intra-assay value was 3.9%. Uterus weights were determined on a precision balance (Mettler Toledo, Barueri, SP – Brazil).

Radiographic analysis

The mandibles were fixed in 4% formaldehyde for 24 hours. The radiographs were obtained using a digital X-ray machine (Dabi Atlante Spectro 70/10 ®, Ribeirão Preto, São Paulo, Brazil) with calibration at 70 kV and 10 mA, 12 pulses and 40 cm of focal length. The radiation incidence was focused perpendicular to the film-object plane. A phosphorus activated optical plate (Digora ®, Soredex,

Orion Corporation, Helsinki, Finland) and an aluminum penetrometer (6063 alloy) were used to capture images (24 bits in TIFF format (tagged image file format). The most central portion of the lesion was selected for analysis and ten repeated measurements were performed to determine the average bone densitometry (pixel) (19).

Histopathological and immunohistochemical analysis

Mandibles were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 60 days, subjected to conventional histological processing, embedded in paraffin, and cut into semi-serial sections. Sections were either stained with hematoxylin and eosin or submitted to immunohistochemistry using an indirect immunoperoxidase technique for tartrate-resistant acid phosphatase (TRAP) (primary antibody goat anti-TRAP SC 30832, Santa Cruz Biotechnology, Santa Cruz, CA) following previously described protocol (20).

Histological analysis was conducted by a certified histologist (EE) using the following parameters: nature and extension of inflammation, presence and extension of necrosis, vasculature state, and pattern of cellularity of dental and periodontal tissues.

For the histometric analysis of the periapical lesion, the distal root of the mandibular first molar was examined using Leica Microsystems software (Leica, Wetzlar, Germany). The limits of

periapical lesion were external cementum surface, periodontal ligament, and the external alveolar bone surface. The area of the periapical lesion in mm^2 was calculated at five equidistant sections and the widest area was selected.

Only mature osteoclasts were quantified as TRAP-positive multinucleated cells that were quantified in the perimeter of lesion. The results were expressed as multinucleated TRAP-positive cells per mm^2.

Statistical Analysis

Data were tabulated and statistically analyzed using analysis of variance (ANOVA) for multiple comparisons. Tukey's test was used for pairwise comparisons using Sigmaplot software (San Jose, CA – USA) at 5% of significance.

Results

Serum estradiol levels and uterus weight

At 7^{th} and 30^{th} days, OVX rats showed levels significantly lower than rats in the sham groups ($p<0.001$). Moreover, the uteruses of the OVX rats weighed less than the uteruses of sham rat ($p<0.001$). The SHAM-veh 30d group showed higher serum estradiol than SHAM-veh 7d group ($p<0.0001$). Estradiol level or uteruses weigh were not affected by RLX treatment in OVX rats ($p>0.05$) (Tab. 1).

Radiographic analysis

The radiographic density of the periapical lesion at 30 days (Fig.1 D-F) after exposure to the oral environment was significantly lower than after 7 days (Fig.1 A-C). However, there were no significant differences between groups at the same time point (Tab.1).

Histological analysis

Histology images of periapical lesions in the different experimental groups are shown in Figure 1 (G-R).

On day 7, SHAM-veh 7d and OVX-RLX 7d showed the root canal occupied by large amount of inflammatory cells and necrotic pulp

remnants (Fig.1 G-H, O-P) . The periapical region showed an inflammatory infiltrate composed predominantly by polymorphonuclear neutrophils. The surface of the alveolar bone demonstrates large numbers of active osteoclasts. The root canal was occupied by necrotic debris and the periapical region had an intense polymorphonuclear inflammatory infiltrate that reached the alveolar bone. The alveolar bone surface showed large number of active osteoclasts and resorption lacunae in almost of the whole extent of the lesion.

On day 30, the root canal was composed by necrotic tissue in most specimens. The inflammatory infiltrate was more intense with many lymphocytes and some neutrophils. On the surface of the alveolar bone, a large number of active osteoclasts were observed. The OVX-veh 30d group had necrotic pulp remnants in the root canal and a large periapical lesion (Fig.1 M-N). The inflammatory infiltrate was composed of lymphocytes and neutrophils and was larger area than the infiltrates in the other treatment groups. The surface of the alveolar bone was irregular due to the large number of resorption lacunae with active osteoclasts.

Histometric analysis

The periapical lesion areas were larger in OVX-veh groups when compared to SHAM-veh and OVX-RLX ($p<0.001$). The size of

periapical lesions in OVX-RLX groups was similar to that in SHAM-veh ($p>0.05$) (Tab 1). There was an increase in the size of periapical lesions in all experimental groups when comparing day 30 with day 7 of pulp exposure to the oral environment (Fig. 2A) (Tab.1).

TRAP immunohistochemistry

Immunohistochemical technique for TRAP was highly specific to osteoclasts. Labeling was confined to the cytosolic compartment of predominantly multinucleated cells. Immunostaining of TRAP is shown in Fig 2B-E.

The number of TRAP-positive multinucleated cells per millimeter on the periapical lesion perimeter in OVX-veh was higher than that observed in SHAM-veh and OVX-RLX ($p<0.001$) (Tab.1). The number of TRAP-positive cells in OVX-RLX groups was similar to that in SHAM-veh ($p>0.05$) (Tab.1). In OVX-veh, there was an increase in the number of TRAP-positive multinucleated cells on day 30 compared with day 7. This difference between the times points did not occur in the other groups.

Discussion

OVX rats are an experimental model used to simulate the bone loss observed in women with hypoestrogenism (21). Confirmation of

the efficacy of OVX in producing hypoestrogenism in this study can be seen in plasma levels of estradiol as OVX rats showed levels significantly lower than rats in the sham groups. Moreover, the uteruses of the OVX rats weighed less than the uteruses of sham rat, showing the decrease in serum estradiol concentration resulted in uterine atrophy.

Serum estradiol concentration of SHAM-veh 30d rats was higher than SHAM-veh 7d rats. This was probably due to the phase of the estrous cycle (22). The SHAM-veh 30d rats were in the proestrus phase and SHAM-veh 7d rats in diestrus. These results characterize the animals at different stages of the estrous cycle with serum estradiol very oscillating during the estrous cycle (23).

In the present study, the uteruses of rats in the OVX-RLX 7d and OVX-RLX 30d groups were not significantly different in weight when compared to the OVX-veh at both time points. This finding indicates that RLX had no effect on the uterus, which supports its reported estrogen antagonism action on the receptor present in this organ (24). This lack of effect on the uterus is an important aspect to be considered when selecting treatment with RLX for patients with a history of uterine cancer.

Radiographic analysis showed differences between groups at day 7 and day 30. However, there were no significant differences between them at the same time point. This may be due to

radiographic analysis techniques that are not sufficiently sensitive to detect differences. More sensitive methods (e.g., micro-or cone beam computerized tomography (25-26)) may be useful in future studies.

In this study, the data indicated an imbalance in bone remodeling after OVX, with exacerbation of the resorptive process due to a large number of active osteoclastic cells and resorption lacunae and the larger size of the periapical lesion when compared to sham rats. Some studies indicate that OVX may trigger changes in bone metabolism, increasing bone turnover as well as cytokines and osteoclast number (27-28). Studies with postmenopausal women have demonstrated that they have significantly lower bone mineral density when compared to premenopausal women (29) which could also indicate an exacerbation of bone resorption processes.

The reduction in estrogen plasma concentration during menopause, results in higher bone metabolic activity (30), which can influence the survival of osteoclasts (31). It was shown that the treatment with estrogen diminished bone resorption by promoting a reduction in the number of osteoclasts. Rats that did not receive estrogen therapy showed more TRAP-positive cells (32). This was in agreement with the results of the present study, in which immunostaining for TRAP showed fewer TRAP-positive cells in the sham group when compared with the OVX group.

Raloxifene in postmenopausal women increased bone density by its agonist effect, diminishing the action of osteoclasts (33-34). Osteoclast activity and lesion size was diminished with RLX treatment being similar to the SHAM group, demonstrating the role of RLX in decreasing resorptive activity and diminishing bone loss. These were similar effects to those seen in a previous study (35).

More studies are needed to better understand the mechanisms involved in the moderation of periapical lesions in animals with hypoestrogenism treated with RLX. Nevertheless, the results of the present study showed that RLX was effective in mimicking the protective effect of estrogen, preventing the deleterious effects of estrogen deficiency and decreasing the osteoclasts activity and bone turnover.

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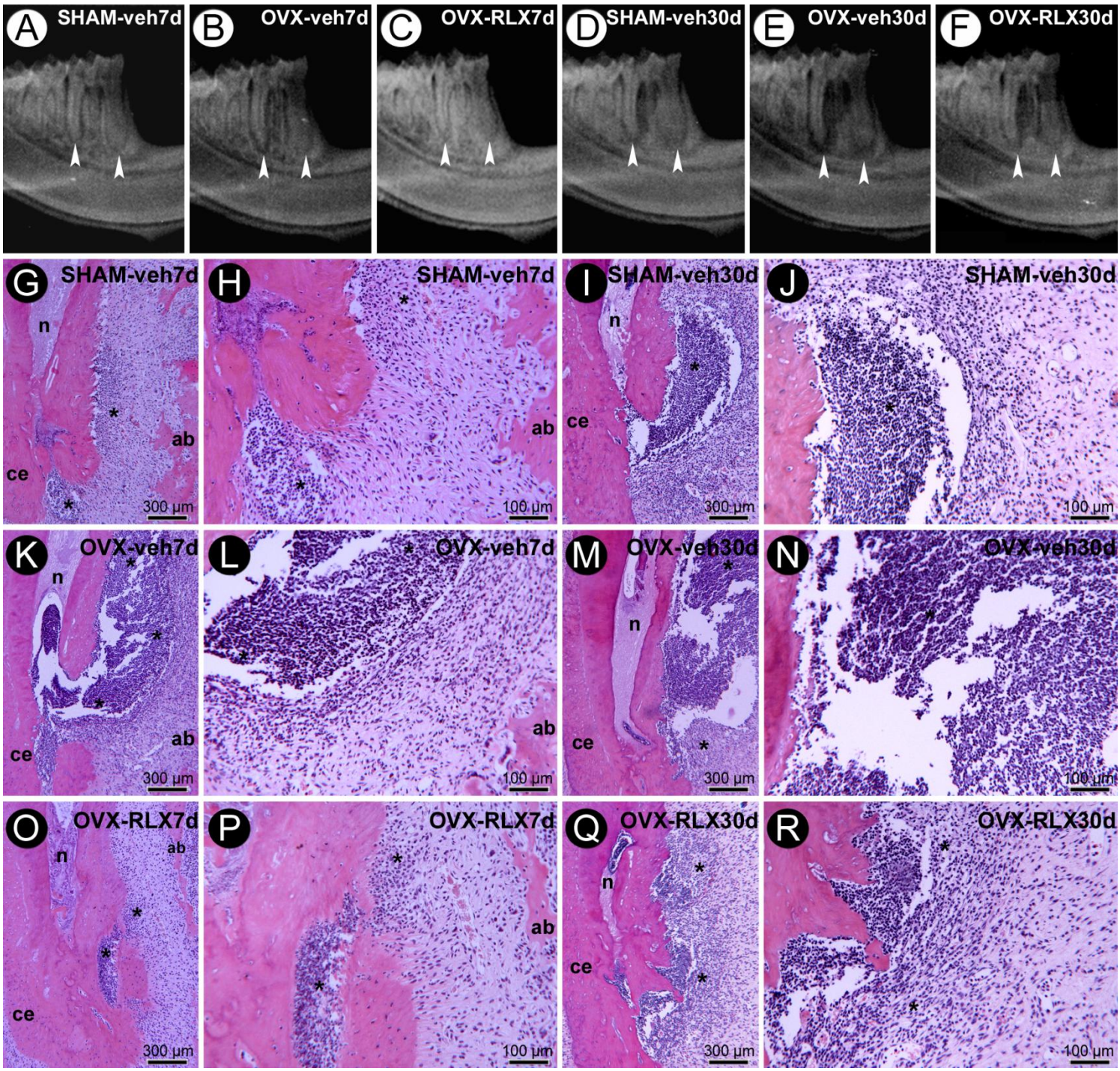
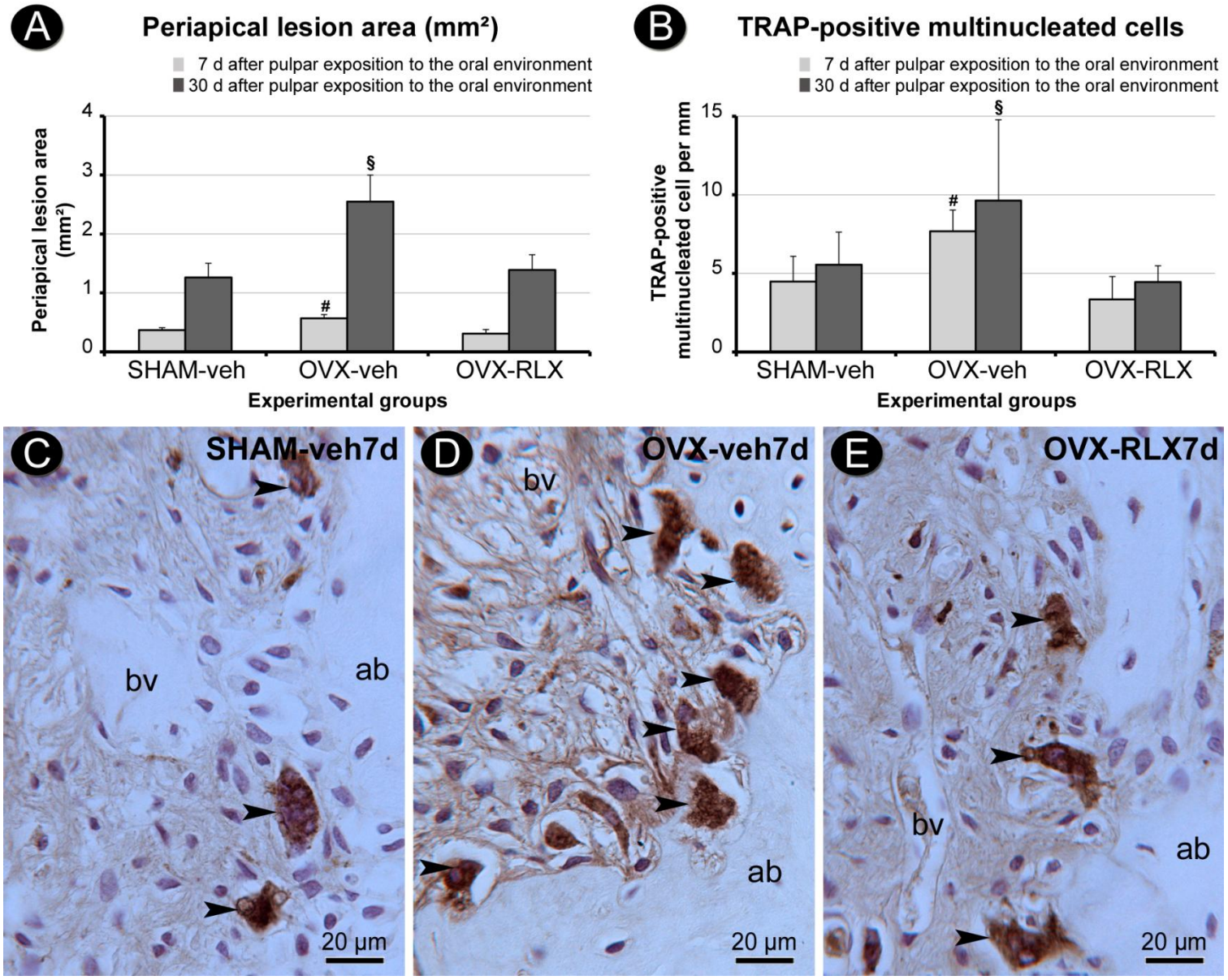
Figure 1



Radiographic and histologic aspects of periapical lesions at 7 and 30 days after pulp exposure to the oral environment. A – F: Periapicial radiographs of the mandibular first molar in groups SHAM-veh7d (A), OVX-veh7d (B), OVX-RLX7d (C), SHAM-veh30d (D), OVX-veh30d (E),

OVX-RLX30d (F). Periapical lesions of greater severity were observed in OVX-veh 7d rats (B) and OVX-veh 30d rats (E) than in SHAM or RLX treated. G − R: Photomicrographs showing the histological appearance and magnitude of inflammation in periapical lesion at 7 and 30 days after pulp exposure to the oral environment in groups SHAM-veh7d (G − H), SHAM-veh30d (I − J), OVX-veh7d (K − L), OVX-veh30d (M − N), OVX-RLX7d (O − P), OVX-RLX30d (Q − R). A greater magnitude of inflammatory response was seen in OVX-veh 7d (K − L) and OVX-veh 30d (M − N) compared to other treatment groups. Periapical inflammatory responses were similar in SHAM-veh (G − J) and OVX-RLX (O − R) groups at day 7 and day 30 days after pulp exposure to the oral environment. Abbreviations and symbols: *, inflammatory infiltrate; ab, alveolar bone; ce, cementum; n, necrotic pulp remnants. G − H: H&E staining; scale bars: G, I, K, M, O and Q, 300 μm; H, J, L, N, P, and R, 100 μm. Original magnification: G, I, K, M, O, and Q, x100; H, J, L, N, P, and R, x250.

Figure 2



A: Periapical lesion area (mm²) in distal root of the mandibular first molar in different treatment groups. B: The number of TRAP-positive multinucleated cells per mm in the perimeter of the periapical lesion of the distal root of the mandibular first molar in different treatment groups. C – E: Photomicrographs showing TRAP-positive multinucleated cells (black arrowheads) at 7 days after pulp exposure

to the oral environment in groups SHAM-veh7d (C), OVX-veh7d (D) and OVX-RLX7d (E). Abbreviation: ab, alveolar bone; bv, blood vessel. Hematoxylin counterstaining; scale bars: C – E, 20 μm. Original magnification: C – E, x1000. Symbols: $^{\#}p<0.05$vsSHAM-veh7d and OVX-RLX7d; $^{\S}p<0.05$ vs SHAM-veh30d and OVX-RLX30d ($p < 0.05$).

Table 1

Experimental groups	Serum estradiol levels (pg/mL)	Uterine weight (g)	Radiographic density (pixel)	Periapical lesion (mm^2)	TRAP ($cells/mm^2$)
SHAM-veh 7d	331.2 ± 146.9^{b}	0.54 ± 0.12^{b}	82.84 ± 4.49^{a}	0.37 ± 0.04^{a}	4.48 ± 1.60^{a}
OVX-veh 7d	146.9 ± 39.8^{a}	0.25 ± 0.15^{a}	82.58 ± 5.03^{a}	0.57 ± 0.06^{b}	7.68 ± 1.34^{b}
OVX-RLX 7d	136.0 ± 45.1^{a}	0.21 ± 0.06^{a}	83.24 ± 5.23^{a}	0.31 ± 0.07^{a}	$3.34 \pm 1,46^{a}$
SHAM-veh 30d	818.0 ± 159.9^{c}	0.59 ± 0.16^{b}	77.04 ± 5.24^{b}	1.26 ± 0.24^{c}	5.55 ± 2.07^{a}
OVX-veh 30d	125.8 ± 31.1^{a}	0.19 ± 0.14^{a}	77.05 ± 5.13^{b}	2.55 ± 0.45^{d}	9.63 ± 5.15^{c}
OVX-RLX 30d	119.9 ± 34.4^{a}	0.19 ± 0.06^{a}	78.82 ± 5.12^{b}	1.39 ± 0.26^{c}	4.45 ± 1.03^{a}

Serum estradiol levels, uterus weight, radiographic density, periapical lesion areas and TRAP-positice cells according to the groups. In a column, different letters indicate statistically different.

Artigo 2

Raloxifene decreases osteoclast activity in apical lesion

Abstract

In the present study, it was evaluated the raloxifene (RLX) action during the periapical lesions development in ovariectomized (OVX) rats. Wistar rats (6 months) were distributed into groups: SHAM-veh, OVX-VEI e OVX-RLX that received for 60 days vehicle or RLX by gavage. During treatment it was performed the exposition of the pulp of lower first molar to the oral environment on the scheduled day allowing lesion analysis after 7 and 30 days. Ninety days after the treatment, blood was collected for measurement of calcium, phosphorus, alkaline phosphatase and estradiol. The animals were killed and mandibles were submitted to immunohistochemical analysis. Estradiol plasma concentration showed hypoestrogenism in OVX rats. On day 7, the alkaline phosphatase activity and plasma calcium were higher in OVX-RLX than SHAM-veh group ($p<0.0002$). The plasma concentration of phosphorus was higher in RLX group, in both time points ($p<0.0001$). On day 7, OVX-veh group showed more immunostaining just for receptor activator of nuclear factor kappa-B ligand (RANKL) and hypoxia inducible factor-1 alpha (HIF1-alpha) than other groups ($p<0.05$). On 30 days, OVX-veh showed higher immunostaining just for RANKL than OVX-RLX ($p<0.05$). The RANKL and HIF1-alpha expression was diminished in OVX-RLX group being

similar to SHAM-veh in both time points. There was no statistical difference in immunoreactivity for osteoprogeterin (OPG) and bone-specific alkaline phosphatase (BALP) between all groups in both time points ($p>0.05$). The results showed that RLX therapy may be beneficial due decreasing osteoclast activity and bone turnover preventing the deleterious effects of estrogen deficiency.

Keywords: RLX hydrochloride, periapical lesion, bone metabolism, OVX.

Introduction

The number of elderly is increasing faster than any other age group in the world (Kinsella & He, 2008). Elderly presents several physiological changes that predispose pathological conditions of aging, such as osteoporosis (Christenson et al. 2012). Thus, studies are needed to improve the life quality of these individuals.

The skeleton is maintained by continuous regeneration that occurs by the bone remodeling process (Manolagas 2000). Bone remodeling is regulated by different mechanisms, such as members of the tumor necrosis factor superfamily, the receptor activator of nuclear factor kappa-B (RANK), RANKL and OPG, which are essential for osteoclastogenesis (Honma et al. 2014).

RANKL is expressed on osteoblasts, fibroblasts and B and T cells being a key cytokine in osteoclastogenesis (Teitelbaum 2000, Lerner 2006). The RANKL binds to RANK that is present in the pre-osteoclasts, and stimulates osteoclast differentiation, whereas the binding of RANKL to OPG inhibits osteoclastogenic activity (Yasuda et al 1998, Takayanagi et al. 2000, Kotake et al. 2001). Several systemic and local factors, such as hormone presence, influence the RANK/RANKL/ OPG system (Horowitz et al. 2001, Lerner 2004). The estrogen decrease bone resorption and increase bone formation by stimulating OPG secretion and RANKL inhibition (Kohli & Kohli 2011).

Other mediators participate in the regulation of bone metabolism such as HIF-1 alpha, tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase besides the TNF superfamily members (Wan et al. 2008, Bezerra et al. 2011, Press et al. 2014).

The bone metabolism is mainly regulated by estrogen, which is essential for the bone tissue maintenance because it interacts with cells involved in bone remodeling, as osteocytes, osteoblasts and osteoclasts (Khosla et al. 2012), which in turn are influenced by systemic and local factors (Raisz & Rodan 1998).

Osteoporosis is a pathological process resulting from the estrogen plasma concentration decrease, which occurs in menopausal women (Bedell et al. 2012). These changes increase resorption, which in turn are not completely filled by bone formation, resulting in

decreased bone density and subsequent fracture risk (Anbinder et al. 2006, Armas & Recker 2012). Osteoporosis interacts with local factors, such as periapical lesion, aggravating the bone loss (Pallos et al. 2006).

Estrogen deficiency affects specific regions such as apical periodontitis increasing the absorption sites (Xiong et al. 2007). During the periapical lesion formation, the bone cells and mediators such as RANK/RANKL/OPG participate in this process, which are also observed in osteoporosis (Zupan et al. 2012, Wan et al. 2014). Thus, the cellular and molecular mechanisms that lead to bone loss, are similar between inflammation process and osteoporosis (Palomo et al. 2007).

The RLX mimics the beneficial effects of estrogens without stimulating tissues such as breast and endometrium (Lewis & Jordan 2005) once it binds to different receptors (Rey et al., 2009). Studies demonstrate that RLX treatment results in increased bone mineral density and significantly reduce the fractures incidence (Carneiro et al. 2012, Mirkin et al. 2014, Pinkerton et al. 2014). Raloxifene influences bone remodeling cells as osteoblasts and osteoclast and inflammatory mediators as RANK/ RANKL/OPG (Mencej-Bedrač et al. 2014).

It was reported that the progression of periapical lesion was soothed after treatment of estrogen-deficient state with

bisphosphonates (Xiong et al. 2007). However, there is no study evaluating the effect of RLX on periapical lesion progression. This way, the aim of the present study was to evaluate the RLX action on periapical lesions in hypoestrogenic organisms.

Material and methods

Animals

For this study, 48 female Wistar rats (six months of age) from the Faculty of Dentistry of Araçatuba/UNESP were used. The experimental procedures proposed in this study were approved by institutional ethics committee (Ethics Committee on Animal Use – Univ Estadual Paulista - 00799-2012). The animals were distributed into 6 groups according to the pulp exposure time point, ovariectomy and treatment: sham surgery plus vehicle treatment with 7 days (SHAM-veh 7d) or 30 days of pulp exposure (SHAM-veh 30d); OVX plus vehicle treatment with 7 days (OVX-veh 7d) or 30 days of pulp exposure (OVX-veh 30d); and OVX plus RLX with 7 days (OVX-RLX 7d) or 30 days of pulp exposure (OVX-RLX 30d).

Estrous cycle

The Long and Evans technique (1922) was performed for the determination of estrous cycle status of the rats. The vaginal swab

collection was performed (9:00 am) and examined using an optical microscope. Rats presenting regular estrous cycle (twelve days) were selected and randomly divided into the experimental groups. Females showing irregular estrous cycle were excluded from the study.

Ovariectomy

The surgical procedure was performed under anesthesia with ketamine (75 mg/kg – by intraperitoneal; Vetaset - Fort Dodge Animal Health Ltd - São Paulo - Brazil) and xylazine (25 mg/kg – by intraperitoneal; Coopazine - Coopers Ltda Brazil - São Paulo - Brazil). In the ovariectomized group the distal portion of the fallopian tubes were exposed and the ovaries were removed. In the sham groups, the ovaries were exposed but not removed. After the surgery, all animals had their incisions closed with suture and received an intramuscular dose of antibiotics (1mL/kg, Pentabiotic Veterinary - Fort Dodge Animal Health Ltd - Sao Paulo - Brazil).

Raloxifene ou vehicle tratment

Ten days after ovariectomy or sham surgery, the rats received daily vehicle (distilled water - 0.3 mL) or RLX (Sigma-Aldrich, Muhich, Germany) (1 mg/kg in 0.3 mL distilled water) by gavage for 60 days (Luvizuto et al. 2010, Luvizuto et al. 2011).

Periapical lesion induction

During the treatment the pulp exposition to the oral environment was performed for lesion analysis on 7 and 30 days. All pulpal exposures of mandibular first molars were standardized with 0.1 mm diameter, with the aid of carbon drill bur (Drill Long Neck Ln - Maillefer, Dentsply - Catanduva - Brazil), under general anesthesia.

Sample Collection

Ninety days after the beginning of administration of RLX or vehicle, animals were anesthetized for blood collection from the jugular vein (Harms & Ojeda, 1974). The blood was centrifuged (3.000 rpm - 20 min − 20 C) and plasma stored in a freezer at -20 ºC for measurement of calcium, phosphorus, alkaline phosphatase and estradiol. The animals were killed by anesthetic overdose and the mandibles removed for immunohistochemical analysis. The uteruses were also removed used and weighed.

Plasma estradiol level and uterus weight

The estradiol measurement was performed according to Biomedicals estradiol kit protocol by radioimmunoassay (Costa Mesa, CA - USA). All samples were assayed in duplicate and in the same assay to avoid inter-assay error. The minimum detectable dose of

estradiol was 5.0 pg/mL and the intra-assay value was 3.9%. The uterus weights were determined on a precision balance (Mettler Toledo, Barueri, SP − Brazil).

Biochemical Analysis

Biochemical analysis of calcium, phosphorus and alkaline phosphatase were performed to analyze cellular activity of bone metabolism. Measurements were performed using commercial kits (Calcium: Labtest catalog 90 − Lagoa Santa/MG − Brazil; Phosphorus: Labtest Catalog 40 − Lagoa Santa/MG − Brazil; Alkaline phosphatase: Labtest Catalog 40 − Lagoa Santa/MG − Brazil).

Immunohistochemical processing

Mandibles were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 60 days, subjected to conventional histological processing, embedded in paraffin, and sliced into semi-serial sections. Sections were either stained with hematoxylin and eosin or submitted to immunohistochemistry using an indirect immunoperoxidase technique for OPG (Rabbit anti-opg − SC11383 − Santa Cruz Biotechnology, Santa Cruz, CA), RANKL (Goat anti-rankl − SC7627 - Santa Cruz Biotechnology, Santa Cruz, CA), HIF-1α (Rabbit − SC10790 - Santa Cruz Biotechnology, Santa Cruz, CA), bone

alkaline phosphatase (Goat − SC15065 - Santa Cruz Biotechnology, Santa Cruz, CA) following previously described protocol (Garcia et al. 2013).

RANKL, OPG, HIF-1α and BALP were analyzed in the proximity of the periapical lesion with a 400x magnification (Leica Microsystems, Wetzlar, Germany). A semi-quantitative immunolabeling analysis was performed. Three histologic sections from each animal was used and the criteria for establishing the immunoreactivity pattern was modified (Kim et al. 2007): score 0 (zero immunoreactivity pattern): total absence of cells immunoreactive (IR); score 1 (low immunoreactivity pattern): about 1/4 IR cells per area; score 2 (moderate immunoreactivity pattern): around 1/2 IR cells per area; score3 (high simmunoreactivity pattern): about 3/4 IR cells per area.

Statistical Analysis

Data were tabulated and analyzed statistically using Sigmaplot software (San Jose, CA − USA). Multiple comparisons were performed using Kruskal-wallis followed by Dunn test for nonparametric data. And analysis of variance (ANOVA) followed by Tukey test was performed for parametric data. The level of significance was 5%.

Results

Serum estradiol levels and uterus weight

In both time points, the sham rats showed levels significantly higher than in the OVX rats groups ($p<0.001$). In addition, the uteruses of the sham rats weighed more than the uteruses of OVX rats ($p<0.001$). The SHAM-veh 7d group showed lower serum estradiol than SHAM-veh 30d group ($p<0.0001$). Uteruses weigh and estradiol level were not affected by RLX treatment in OVX rats ($p>0.05$) (Tab. 1).

Biochemical analysis

The results obtained of plasma concentrations of calcium, phosphorus and alkaline phosphatase of the experimental animals are shown in Figure 1.

The plasma calcium concentration of OVX-RLX-7 d was higher than SHAM-veh 7d ($p=0.0002$). In the SHAM-veh 30d, the plasma concentration of calcium was higher than SHAM-veh 7 ($p=0.0002$). After 30 days, the plasma calcium concentration was lower in the OVX-RLX compared with SHAM-veh 30 d ($p=0.0002$) and OVX-RLX 7 d ($p=0.0002$).

In OVX-RLX group in both time points, the plasma phosphorus concentration was higher than SHAM-veh 7 d ($p<0.0001$), SHAM-veh-30 d ($p<0.0001$) and OVX-veh 30 d ($p<0.0001$).

The alkaline phosphatase activity was higher in OVX-RLX 7 d than SHAM-veh 7 d ($p<0.0001$). The enzyme activity was higher in SHAM-veh 30d and OVX-Veh 30 d when compared with the corresponding groups, with 7 days ($p<0.0001$).

Immunohistochemistry

Immunohistochemical technique for RANKL, OPG, BALP and HIF-1α was highly specific in the detection of these proteins. Labeling was confined to the cytosolic compartment of the immunoreactive cells and the extracellular matrix.

Labeling for RANKL, OPG and BALP was confined predominantly in osteoblasts, while HIF-1α was expressed in fibroblasts, endothelial cells and osteoblasts.

The photomicrographs showing the histological appearance of immunolabeling and the immunoreactivity pattern for RANKL, OPG, BALP and HIF-1α immunohistochemistry are shown in Fig 2 and 3.

SHAM-veh

On day 7 and 30, the immunostaining for OPG and BALP prevailed low. On day 7, the immunolabeling for RANKL prevailed low and for HIF-1α prevailed moderate . On day 30, the immunoreactivity pattern for RANKL prevailed moderate and HIF-1α was low to moderate.

OVX-veh

The immunostaining pattern for OPG and BALP prevailed low on day 7 and 30. It was noted immunostaining pattern high for HIF-1α and RANKL on day 7. On day 30, the immunoreactivity pattern for RANKL was moderate to high and for HIF-1α prevailed moderate.

OVX-RLX

The immunostaining for OPG and BALP prevailed low on day 7 and 30. On day 7, the immunolabeling pattern for RANKL prevailed low and for HIF-1α moderate. On day 30, the immunoreactivity pattern for RANKL prevailed low and for HIF-1α low to moderate.

Discussion

Conventionally, studies involving ovariectomy perform the determination of plasma estrogen and weighing of uterus for confirmatory procedure (García-Pérez et al. 2006). In the present study, the estrogen level in the OVX group was lower compared with sham rats, indicating that ovariectomy resulted in decrease in plasma estrogen. In addition, it was noted that the OVX rats uterus were also lighter than sham rats uterus, confirming again the efficacy of ovariectomy, since the absence of serum concentration of estrogen results in atrophy of this organ (Lopez-Belmonte et al. 2012). The uterus of rats treated with RLX did not suffer any modification compared to the sham rats, showing their antagonistic effect on the receptor present in this organ. Based on such observation, RLX has been indicated for patients with a history of uterine cancer due their antagonistic effect on this organ (Jolly et al. 2003).

The plasma concentration of estrogen in sham rats, after 30 days of pulpar exposition, was higher and corresponds to proestrus phase and the animals of the 7 days in diestrus. These results characterize the animals at different stages of the estrous cycle with plasma level of estrogen is very oscillating during the estrous cycle (Marcondes et al. 2002).

It could be observed that serum calcium level in OVX-RLX was the highest suggesting that RLX increased calcium absorption when

compared to untreated group. The bone matrix is composed of organic and inorganic components whose composition is given mostly by calcium and phosphate ions to form hydroxyapatite crystals (Prentice 2004, Bauer 2013). Some authors suggest that RLX increases gastrointestinal absorption of calcium, indirectly benefiting the bone tissue (Naves-Díaz et al. 2010). It was also observed that OVX-RLX group showed higher plasma concentration of alkaline fosfatase and phosphorus than sham rats that is according to previous studies (Canpolat et al. 2010, Tasci et al. 2010).

The present study observed higher expression of RANKL in the OVX-veh group when compared to sham. RANKL is a cytokine present in osteoblasts and its connection to RANK results in the osteoclasts differentiation (Silva et al. 2012). Estrogen deficiency results in bone loss affecting the expression of RANKL and OPG (Michael et al. 2005). It was shown that RANKL expression in bone marrow cells in postmenopausal women is higher than premenopausal (Eghbali-Fatourechi et al. 2003). Another study observed that there was decreased RANKL expression in periodontal ligament cells of humans after treatment with estrogen, indicating antireabsortive effect of this hormone (Liang et al. 2008). Moreover, when RLX was used, the immunostaining of RANKL was lower than OVX-veh, suggesting lower osteoclast activity, which is according to previous study (Bitto et al. 2008). The effect of RLX may be related to the suppression of RANK expression diminishing osteoclast formation from estrogen-deficient

animals (Shevde et al. 2000). Moreover, RLX can mimic the bone protective effects of estrogen suppressing primary monocytic cell differentiation into osteoclasts (Black et al. 1994, Cosman & Lindsay 1999).

In the present study, it was not observed difference in the levels of OPG between the SHAM-veh and OVX-veh that is according to previous study that showed no difference on OPG levels between postmenopausal women treated with hormone replacement therapy and untreated groups (Rahnama et al. 2013). The levels of OPG was not altered even with RLX treatment similar to previous study on human osteoblasts cultures (Giner et al. 2010).

Although no significant difference in OPG level had been observed, the osteoclastogenesis was considered affected once RANKL was increased in OVX-veh group. Based on these data, it appears that the therapeutic effect of RLX on bone loss is independent on OPG but dependent on RANKL, which is according to previous report that showed the RLX action is independent of OPG (Yan et al. 2010).

Alkaline phosphatase immunostaining of periapical lesion evidenced no significant difference between the groups, on both 7 and 30 days. Such result can be due to the progression of periapical lesion involves bone destruction resulting from the osteoclasts activity but not significant bone formation.

Regarding to HIF−1alpha, OVX-veh group had a higher level compared with other groups at 7 days. HIF-1alpha is one of the oxygen-dependent inflammatory mediators (Wan et al. 2008). Its interaction with the estrogen is still unclear but a study observed that estrogen deficiency in ovariectomized rats results the increase of HIF-1alpha in osteoclasts promoting their resorptive activity leading consequently increased bone mass loss (Miyauchi et al. 2013). The increased presence of HIF-1alpha in the OVX-veh group may have led to increased osteoclasts activity via RANKL. This correlation between HIF-1alpha and RANKL has also been demonstread by some authors (Trebec-Reynolds et al. 2010, Dandajena et al. 2012). On the other hand, OVX-RLX group showed immunostaining of HIF-1alpha similar to SHAM-veh, probably because raloxifene mimicked the beneficial effects of estrogen. It was shown that estrogen inhibits over-expression of HIF-1alpha, probably for this reason the SHAM-veh and OVX-RLX groups showed lower expression of HIF-1alpha than OVX-veh (Jia & Liu, 2010).

At 30 days of periapical lesion, there was no significant difference between groups in the immunostaining of HIF1-alpha, maybe due to the chronicity pattern at this time point. The highest recruitment of inflammatory cells and mediators occurs in the initial period, in active phase. Moreover, the bone turnover is also smaller at chronic stage (Kawashima et al. 2007, Teixeira et al. 2011).

According to this study, it was noted that the RLX was effective on bone resorption modulation resulted by ovariectomy. Therefore, these results indicate that the RLX was effect in mimicking the beneficial action of estrogen diminishing the osteoclast activity and preventing the deleterious effects of estrogen deficiency. Nevertheless, further studies should be considered to clinically evaluate the relevance of these findings.

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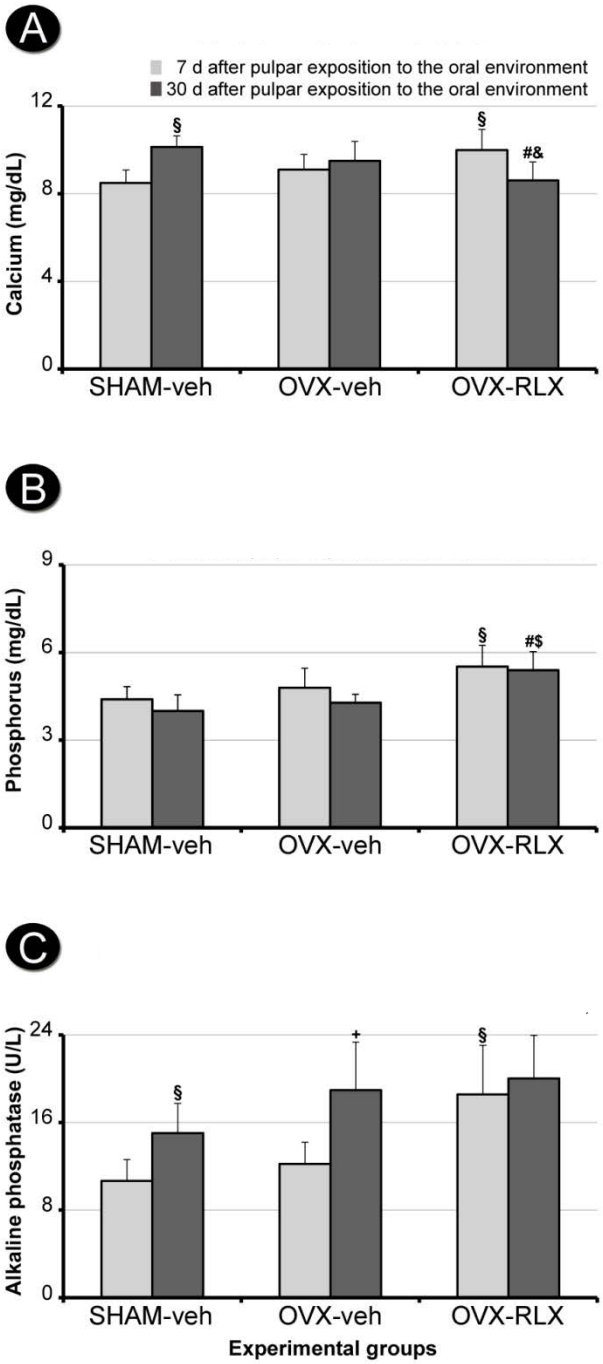
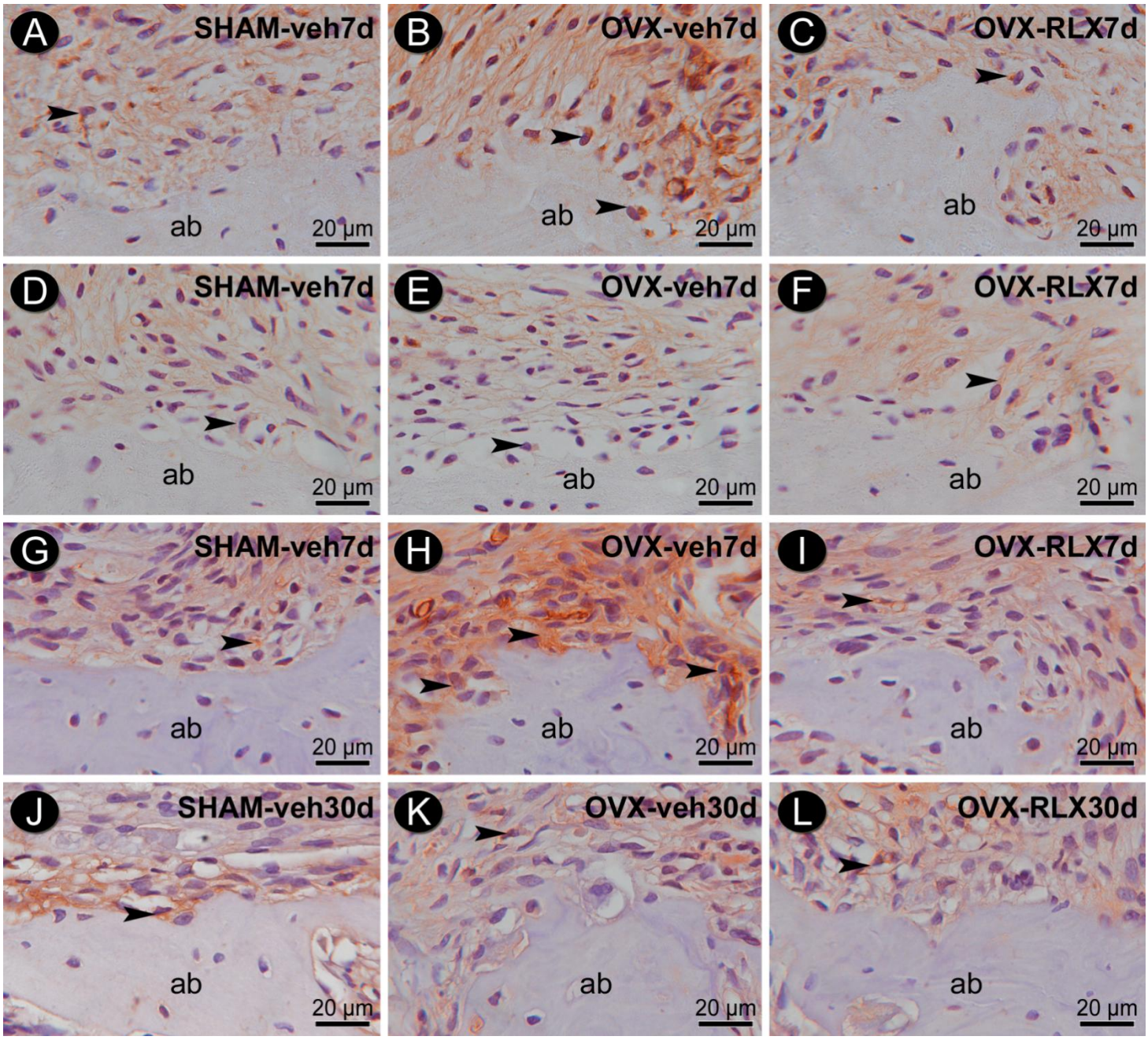
Figure 1



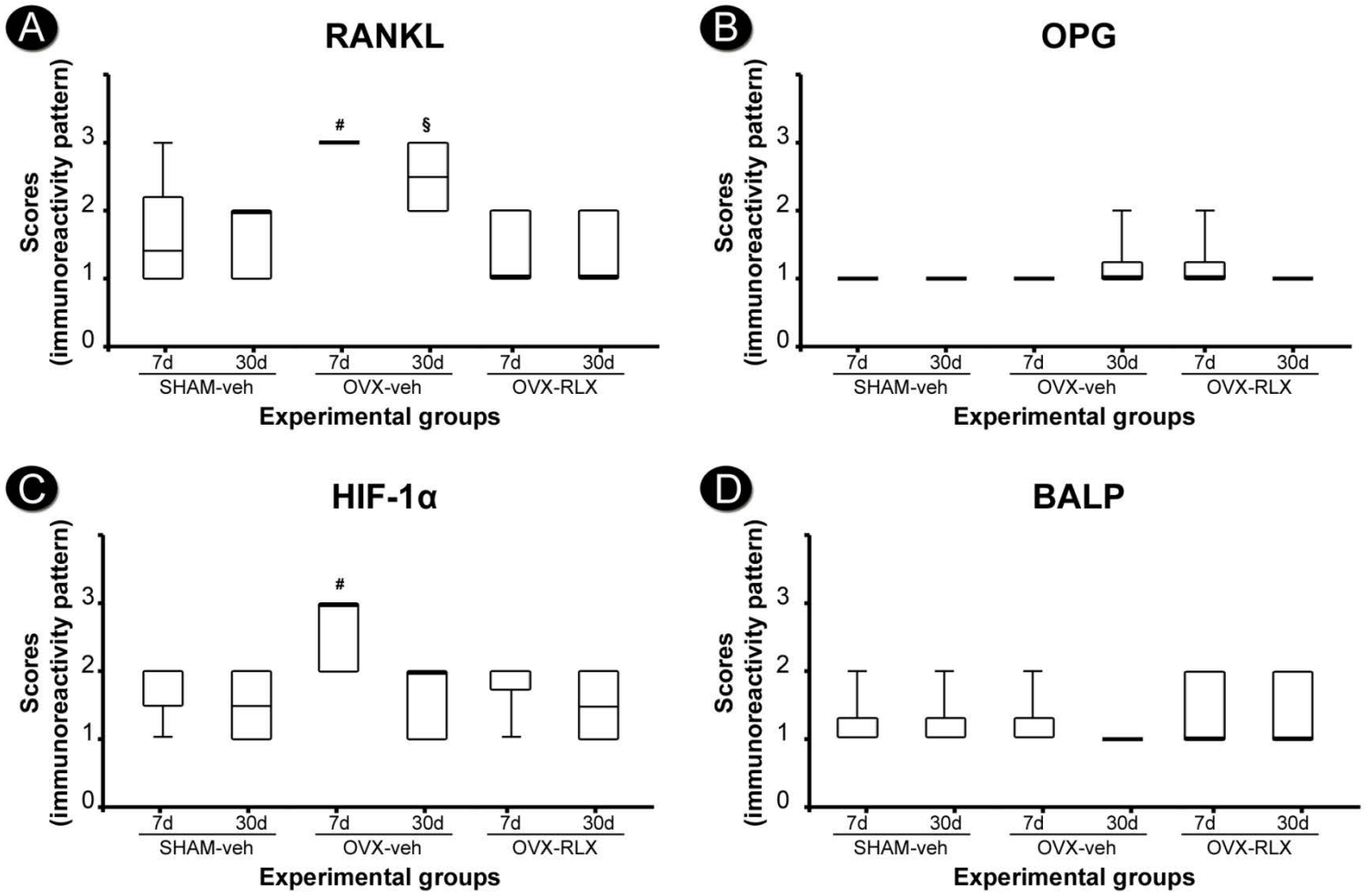
Graphic showing calcium (A), phosphurus (B) and alkaline phosphatase (C) levels in blood in different treatment groups. Symbols: $^{\S}p < 0.05$ vs SHAM-veh7d; $^{\#}p < 0.05$ vs SHAM-veh30d; $^{\&}p < 0.05$ vs OVX-RLX7d; $^{\$}p < 0.05$ vs OVX-veh30d; $^{+}p < 0.05$ vs OVX-veh7d.

Figure 2



Photomicrographs showing the histological appearance of immunolabeling for RANKL (A-C), OPG (D-F), HIF-1α (G-I) and BALP (J-L). Note greater immunolabeling for RANKL and HIF-1α. Abbreviations and symbols: ab, alveolar bone; black arrowheads, immunolabeling cells. Hematoxylin counterstaining. Scale bars: 20 µm. Original magnification: x1000.

Figure 3



Graphic showing the immunoreactivity pattern for RANKL (A), OPG (B), HIF-1α (C) and BALP (D) of periapical lesions at 7 and 30 days after pulp exposure to the oral environment. Symbols: #, statistically significant difference between the indicated group and SHAM-veh7d and OVX-RLX7d ($p < 0.05$); §, statistically significant difference between the indicated group and OVX-RLX30d ($p < 0.05$).

Table 1

Experimental groups	Serum estradiol levels (pg/mL)	Uterine weight (g)
SHAM-veh 7d	355.5 ± 146.2[b]	0.56 ± 0.12[b]
OVX-veh 7d	140.3 ± 42.8[a]	0.21 ± 0.13[a]
OVX-RLX 7d	124.3 ± 38.9[a]	0.19 ± 0.03[a]
SHAM-veh 30d	884.2 ± 69.8[c]	0.63 ± 0.12[b]
OVX-veh 30d	118.4 ± 30.5[a]	0.14 ± 0.03[a]
OVX-RLX 30d	109.2 ± 28.7[a]	0.18 ± 0.05[a]

Serum estradiol levels, uterus weight according to the groups. In a column, different letters indicate statistically different.

Conclusão

Os resultados mostram que o RLX foi eficaz em mimetizar o efeito do estrógeno, prevenindo os efeitos deletérios da deficiência deste hormônio e diminuindo a atividade dos osteoclastos e turnover ósseo. No entanto, mais estudos devem ser realizados para avaliar clinicamente a relevância destes achados.

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Anexos

Anexo 1

Comitê de Ética no Uso de Animais

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Araçatuba

Comitê de Ética no Uso de Animais (CEUA)
Committee for Ethical Use of Animals (CEUA)

CERTIFICADO

Certificamos que o Projeto **"Efeito do raloxifeno na lesão periapical em ratas ovariectomizadas"** sob responsabilidade do Pesquisador **JOÃO EDUARDO GOMES FILHO** e colaboração de Rita Cássia Menegati Dornelles, Marcelo Tadahiro Wayama e Simone Watanabe está de acordo com os Princípios Éticos da Experimentação Animal (COBEA) e foi aprovado pelo CEUA, de acordo com o processo **00799-2012**.

CERTIFICATE

We certify that the research **"Effect of raloxifen on periapical lesions in ovarectomized rats"**, process number **00799-2012**, under responsibility of **JOÃO EDUARDO GOMES GILHO** and with collaboration of Rita Cássia Menegati Dornelles, Marcelo Tadahiro Wayama and Simone Watanabe agree with Ethical Principles in Animal Research (COBEA) and was approved by CEUA.

Prof. Dr. Edilson Ervolino
CEUA Vice-Coordinador

Faculdade de Odontologia e Faculdade de Medicina Veterinária – **Departamento de Clínica, Cirurgia e Reprodução Animal** – Rua Clóvis Pestana, 793 CEP 16050-680 Araçatuba – SP
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Anexo 2

Diretrizes para publicação de trabalhos no Journal ofEndodontics

Writing an effective article is a challenging assignment. The following guidelines are provided to assist authors in submitting manuscripts.

The JOE publishes original and review articles related to the scientific and applied aspects of endodontics. Moreover, the JOE has a diverse readership that includes full-time clinicians, full-time academicians, residents, students and scientists. Effective communication with this diverse readership requires careful attention to writing style.

1. Organization of Original Research Manuscripts

Please Note: All abstracts should be organized into sections that start with a one-word title (in bold), i.e., Introduction, Methods, Results, Conclusions, etc., and should not exceed more than 250 words in length.

1. **Title Page:** The title should describe the major emphasis of the paper. It should be as short as possible without loss of clarity. Remember that the title is your advertising billboard—it represents your major opportunity to solicit readers to spend

the time to read your paper. It is best not to use abbreviations in the title since this may lead to imprecise coding by electronic citation programs such as PubMed (e.g., use "sodium hypochlorite" rather than NaOCl). The author list must conform to published standards on authorship (see authorship criteria in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals at www.icmje.org). The manuscript title, name and address (including email) of one author designated as the corresponding author. This author will be responsible for editing proofs and ordering reprints when applicable. The contribution of each author should also be highlighted in the cover letter.

2. **Abstract:** The abstract should concisely describe the purpose of the study, the hypothesis, methods, major findings and conclusions. The abstract should describe the new contributions made by this study. The word limitations (250 words) and the wide distribution of the abstract (e.g., PubMed) make this section challenging to write clearly. This section often is written last by many authors since they can draw on the rest of the manuscript. Write the abstract in past tense since the study has been completed. Three to ten keywords should be listed below the abstract.
3. **Introduction:** The introduction should briefly review the pertinent literature in order to identify the gap in knowledge

that the study is intended to address and the limitations of previous studies in the area. The purpose of the study, the tested hypothesis and its scope should be clearly described. Authors should realize that this section of the paper is their primary opportunity to establish communication with the diverse readership of the JOE. Readers who are not expert in the topic of the manuscript are likely to skip the paper if the introduction fails to succinctly summarize the gap in knowledge that the study addresses. It is important to note that many successful manuscripts require no more than a few paragraphs to accomplish these goals. Therefore, authors should refrain from performing extensive review or the literature, and discussing the results of the study in this section.

4. **Materials and Methods:** The objective of the materials and methods section is to permit other investigators to repeat your experiments. The four components to this section are the detailed description of the materials used and their components, the experimental design, the procedures employed, and the statistical tests used to analyze the results. The vast majority of manuscripts should cite prior studies using similar methods and succinctly describe the essential aspects used in the present study. Thus, the reader should still be able to understand the method used in the experimental approach and concentration of the main reagents (e.g., antibodies, drugs,

etc.) even when citing a previously published method. The inclusion of a "methods figure" will be rejected unless the procedure is novel and requires an illustration for comprehension. If the method is novel, then the authors should carefully describe the method and include validation experiments. If the study utilized a commercial product, the manuscript must state that they either followed manufacturer's protocol or specify any changes made to the protocol. If the study used an in vitro model to simulate a clinical outcome, the authors must describe experiments made to validate the model, or previous literature that proved the clinical relevance of the model. Studies on humans must conform to the Helsinki Declaration of 1975 and state that the institutional IRB/equivalent committee(s) approved the protocol and that informed consent was obtained after the risks and benefits of participation were described to the subjects or patients recruited. Studies involving animals must state that the institutional animal care and use committee approved the protocol. The statistical analysis section should describe which tests were used to analyze which dependent measures; p-values should be specified. Additional details may include randomization scheme, stratification (if any), power analysis as a basis for sample size computation, drop-outs from clinical

trials, the effects of important confounding variables, and bivariate versus multivariate analysis.

5. **Results:** Only experimental results are appropriate in this section (i.e., neither methods, discussion, nor conclusions should be in this section). Include only those data that are critical for the study, as defined by the aim(s). Do not include all available data without justification; any repetitive findings will be rejected from publication. All Figures, Charts and Tables should be described in their order of numbering with a brief description of the major findings. Author may consider the use of supplemental figures, tables or video clips that will be published online. Supplemental material is often used to provide additional information or control experiments that support the results section (e.g., microarray data).
6. **Figures:** There are two general types of figures. The first type of figures includes photographs, radiographs or micrographs. Include only essential figures, and even if essential, the use of composite figures containing several panels of photographs is encouraged. For example, most photo-, radio- or micrographs take up one column-width, or about 185 mm wide X 185 mm tall. If instead, you construct a two columns-width figure (i.e., about 175 mm wide X 125 mm high when published in the JOE), you would be able to place about 12 panels of photomicrographs (or radiographs, etc.) as an array of four

columns across and three rows down (with each panel about 40 X 40 mm). This will require some editing to emphasize the most important feature of each photomicrograph, but it greatly increases the total number of illustrations that you can present in your paper. Remember that each panel must be clearly identified with a letter (e.g., "A," "B," etc.), in order for the reader to understand each individual panel. Several nice examples of composite figures are seen in recent articles by Jeger et al (J Endod 2012;38:884−888); Olivieri et al., (J Endod 2012;38:1007 1011); Tsai et al (J Endod 2012;38:965−970). Please note that color figures may be published at no cost to the authors and authors are encouraged to use color to enhance the value of the illustration. Please note that a multipanel, composite figure only counts as one figure when considering the total number of figures in a manuscript (see section 3, below, for maximum number of allowable figures).

The second type of figures are graphs (i.e., line drawings including bar graphs) that plot a dependent measure (on the Y axis) as a function of an independent measure (usually plotted on the X axis). Examples include a graph depicting pain scores over time, etc. Graphs should be used when the overall trend of the results are more important than the exact numerical values

of the results. For example, a graph is a convenient way of reporting that an ibuprofen-treated group reported less pain than a placebo group over the first 24 hours, but was the same as the placebo group for the next 96 hours. In this case, the trend of the results is the primary finding; the actual pain scores are not as critical as the relative differences between the NSAID and placebo groups.

7. **Tables:** Tables are appropriate when it is critical to present exact numerical values. However, not all results need be placed in either a table or figure. For example, the following table may not be necessary:

% NaOCl	N/Group	% Inhibition of Growth
0.001	5	0
0.003	5	0
0.01	5	0
0.03	5	0
0.1	5	100
0.3	5	100
0.001	5	0
0.003	5	0

Instead, the results could simply state that there was no inhibition of growth from 0.001-0.03% NaOCl, and a 100% inhibition of growth from 0.03-3% NaOCl (N=5/group). Similarly, if the results are not significant, then it is probably not necessary to include the results in either a table or as a

figure. These and many other suggestions on figure and table construction are described in additional detail in Day (1998).

8. **Discussion:** This section should be used to interpret and explain the results. Both the strengths and weaknesses of the observations should be discussed. How do these findings compare to the published literature? What are the clinical implications? Although this last section might be tentative given the nature of a particular study, the authors should realize that even preliminary clinical implications might have value for the clinical readership. Ideally, a review of the potential clinical significance is the last section of the discussion. What are the major conclusions of the study? How does the data support these conclusions Acknowledgments: All authors must affirm that they have no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years. Any other potential conflict of interest should be disclosed. Any author for whom this statement is not true must append a paragraph to the manuscript that fully discloses any financial or other interest that poses a conflict. Likewise the sources and correct

attributions of all other grants, contracts or donations that funded the study must be disclosed.

9. **References:** The reference style follows Index Medicus and can be easily learned from reading past issues of the JOE. The JOE uses the Vancouver reference style, which can be found in most citation management software products. Citations are placed in parentheses at the end of a sentence or at the end of a clause that requires a literature citation. Do not use superscript for references. Original reports are limited to 35 references. There are no limits in the number of references for review articles.

Anexo 3

Diretrizes para publicação de trabalhos no International Endodontic Journal

The journal to which you are submitting your manuscript employs a plagiarism detection system. By submitting your manuscript to this journal you accept that your manuscript may be screened for plagiarism against previously published works.

Organization of Original Research Manuscripts

1.1. Format

Language: The language of publication is English. It is preferred that manuscript is professionally edited. A list of independent suppliers of

editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication

Presentation: Authors should pay special attention to the presentation of their research findings or clinical reports so that they may be communicated clearly. Technical jargon should be avoided as much as possible and clearly explained where its use is unavoidable. Abbreviations should also be kept to a minimum, particularly those that are not standard. The background and hypotheses underlying the study, as well as its main conclusions, should be clearly explained. Titles and abstracts especially should be written in language that will be readily intelligible to any scientist.

Abbreviations: International Endodontic Journal adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

1.2. Structure

All manuscripts submitted to International Endodontic Journal should include Title Page, Abstract, Main Text, References and

Acknowledgements, Tables, Figures and Figure Legends as appropriate

Title Page: The title page should bear: (i) Title, which should be concise as well as descriptive; (ii) Initial(s) and last (family) name of each author; (iii) Name and address of department, hospital or institution to which work should be attributed; (iv) Running title (no more than 30 letters and spaces); (v) No more than six keywords (in alphabetical order); (vi) Name, full postal address, telephone, fax number and e-mail address of author responsible for correspondence.

Abstract for Original Scientific Articles should be no more than 250 words giving details of what was done using the following structure:

- Aim: Give a clear statement of the main aim of the study and the main hypothesis tested, if any.
- Methodology: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and statistical tests.
- Results: Give the main results of the study, including the outcome of any statistical analysis.
- Conclusions: State the primary conclusions of the study and their implications. Suggest areas for further research, if appropriate.

Abstract for Review Articles should be non-structured of no more than 250 words giving details of what was done including the literature search strategy.

Abstract for Mini Review Articles should be non-structured of no more than 250 words, including a clear research question, details of the literature search strategy and clear conclusions.

Abstract for Case Reports should be no more than 250 words using the following structure:

- Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.
- Summary: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and analysis if any.
- Key learning points: Provide up to 5 short, bullet-pointed statements to highlight the key messages of the report. All points must be fully justified by material presented in the report.

Abstract for Clinical Articles should be no more than 250 words using the following structure:

- Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.

• Methodology: Describe the methods adopted.

• Results: Give the main results of the study.

• Conclusions: State the primary conclusions of the study.

Main Text of Original Scientific Article should include Introduction, Materials and Methods, Results, Discussion and Conclusion

Introduction: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested.

Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced.

(i) Clinical Trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material.

(ii) Experimental Subjects: experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been

carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study, if applicable. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

(iii) Suppliers: Suppliers of materials should be named and their location (Company, town/city, state, country) included.

Results: should present the observations with minimal reference to earlier literature or to possible interpretations. Data should not be duplicated in Tables and Figures.

Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The Discussion section should progress with a review of the methodology before discussing the results in light of previous work in the field. The Discussion should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.

Conclusion: should contain a summary of the findings.

2.1. References

It is the policy of the Journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting. The EndNote reference style can be obtained upon request to the editorial office (iejeditor@cardiff.ac.uk). Reference Manager reference styles can be searched for here: www.refman.com/support/rmstyles.asp

In the text: single or double authors should be acknowledged together with the year of publication, e.g. (Pitt Ford & Roberts 1990). If more than two authors the first author followed by et al. is sufficient, e.g. (Tobias et al. 1991). If more than 1 paper is cited the references should be in year order and separated by "," e.g. (Pitt Ford & Roberts 1990, Tobias et al. 1991).

Reference list: All references should be brought together at the end of the paper in alphabetical order and should be in the following form.

3.1. Tables, Figures and Figure Legends

Tables: Tables should be double-spaced with no vertical rulings, with a single bold ruling beneath the column titles. Units of measurements must be included in the column title.

Figures: All figures should be planned to fit within either 1 column width (8.0 cm), 1.5 column widths (13.0 cm) or 2 column widths (17.0 cm), and must be suitable for photocopy reproduction from the printed version of the manuscript. Lettering on figures should be in a clear, sans serif typeface (e.g. Helvetica); if possible, the same typeface should be used for all figures in a paper. After reduction for publication, upper-case text and numbers should be at least 1.5-2.0 mm high (10 point Helvetica). After reduction, symbols should be at least 2.0-3.0 mm high (10 point). All half-tone photographs should be submitted at final reproduction size. In general, multi-part figures should be arranged as they would appear in the final version.

Reduction to the scale that will be used on the page is not necessary, but any special requirements (such as the separation distance of stereo pairs) should be clearly specified.

Unnecessary figures and parts (panels) of figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected; each panel of a multipart figure should be sized so that the whole figure can be reduced by the same amount and reproduced on the printed page at the smallest size at which essential details are visible.

Figures should be on a white background, and should avoid excessive boxing, unnecessary colour, shading and/or decorative effects (e.g. 3-dimensional skyscraper histograms) and highly pixelated computer drawings. The vertical axis of histograms should not be truncated to exaggerate small differences. The line spacing should be wide enough to remain clear on reduction to the minimum acceptable printed size.

Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same typesize as used elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field. Thousands should be separated by a thin space (1 000). Unusual units or abbreviations

should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general, visual cues (on the figures themselves) are preferred to verbal explanations in the legend (e.g. broken line, open red triangles etc.)

Figure legends: Figure legends should begin with a brief title for the whole figure and continue with a short description of each panel and the symbols used; they should not contain any details of methods.

Anexo 4 – Instruções para realização da dosagem hormonal de estrógeno por radioimunoensaio utilizando o kit Estradiol MP Biomedicals (Costa Mesa, CA – USA).

Primeiramente deve-se formular uma planilha para organizar os primeiros tubos (tubo 5 ao tubo 18) para fazer a curva padrão (veja abaixo). E em seguida enumerar os tubos correspondentes às amostras a serem dosadas. O procedimento é realizado de acordo com sequência como mostra a tabela abaixo da esquerda para direita.

Tubo	Descrição (pg/mL)	Diluente	Padrão ou Amostras	E_2*	Anti-anti-E_2	INCUBAÇÃO 90 MIN A 37° C	Precipitante	*CENTRIFUGAR/ASPIRAR/CONTAR
1	TC (anti E2 marcado)	-	-	500 µL	-		-	
2	TC	-	-	500 µL	-		-	
3	(NSB) (iodo, P0, tampão ou diluente)	100 µL	**50 µL**	500 µL	-		500 µL	
4	(NSB)	100 µL	**50 µL**	500 µL	-		500 µL	
5	P0 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
6	P0 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
7	P10 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
8	P10 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
9	P30 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
10	P30 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
11	P100 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
12	P100 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
13	P300 pg/ mL	-	**50 µL**	500 µL	500 µL		500 µL	
14	P300 pg/ mL	-	**50 µL**	500 µL	500 µL		500 µL	
15	P1000 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
16	P1000 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
17	P3000 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
18	P3000 pg/mL	-	**50 µL**	500 µL	500 µL		500 µL	
19	CB (controle baixo)	-	**P30**	500 µL	500 µL		500 µL	
20	CB (controle baixo)	-	**P30**	500 µL	500 µL		500 µL	
21	CB (controle alto)	-	**P1000**	500 µL	500 µL		500 µL	
22	CB (controle alto)	-	**P1000**	500 µL	500 µL		500 µL	

As amostras sanguíneas de todos os animais experimentais utilizados são centrifugadas (3000 rpm; temperatura de 2° C; 15 min). O plasma é aliquotado em eppendorfs (500 µL) e estocado em freezer (-20° C) para dosagem posterior de estradiol (E2), por radioimunoensaio (RIE).

As concentrações plasmáticas de E2 são determinadas pelo método de duplo anticorpo, utilizando-se kit específico da MP Biomedicals (Costa Mesa, CA − USA). A dose mínima detectável de estradiol é de 0,3 ng/mL e o erro intra-ensaio é de 4,3%. Todas as amostras são dosadas em duplicata no mesmo ensaio, para evitar erro inter-ensaio.

Anexo 5 − Instruções para realização da dosagem de cálcio utilizando o kit Cálcio Liquiform Labtest cat. 90

O kit Cálcio Liquiform Labtest cat. 90 contém:

Reagentes do kit (100 testes)

R1 − Reagente 1 (tampão)

R2- Reagente 2 (cuidado, contém HCl)

R3- Padrão (10mg/dL) (Contém formol, cuidado com evaporação)

Material necessário

Pipeta automática que libere 20µL

Pipeta automática que libere 1mL ou repipetador automático

Ponteiras

Placa para leitura na leitora Elisa (template)

Béqueres para colocar os reagentes a serem pipetados

Amostra (plasma, que não seja coletado com EDTA ou fluoreto)

Tubos de ensaio em duplicata para as amostras

Tubo para padrão e branco

Estante para tubos

Obs: é importante que os tubos estejam bem limpos, de preferência lavados com HCl e muito bem enxaguados com água destilada.

Procedimento

Primeiramente deve-se preparar o reagente de trabalho, onde se mistura 3 volumes do reagente 1 (R1) com 1 volume do reagente 2 (R2), calcular a quantidade de reagente que será necessário já que se usa 1mL do mesmo por amostra. No kit a proporção do R1 para o R2 já está certa, então caso vá fazer 60 testes (incluindo padrão e branco), é só misturar o conteúdo todo dos dois reagentes. Toma-se cuidado para não preparar o reagente de trabalho em excesso, pois sua estabilidade é de apenas 8 horas, tendo que ser descartado caso não utilizado.

Nos tubos de teste adicionar 20µL do plasma e o padrão no tubo correspondente e em todos os tubos pipetar 1mL do reagente de

trabalho. Agitar a estante levemente para misturar o reagente com a amostra. A estabilidade da cor não é citada na bula, porém recomenda-se fazer a leitura o mais rápido possível, para evitar alguma perda.

Se a leitura for feita no espectrofotômetro manual (570nm) adicionar o branco na cubeta e zerar o aparelho. Proceder as leituras das amostras.

Caso a leitura for feita na leitora Elisa, deve-se pipetar 300µL da reação em cada poço, de acordo com a ordem definida (A1- Branco/ A2-Padrão/ A3-Padrão/ A4-amostra). Ligar o computador, entrar no programa KC Junior, open protocol, Cálcio labtest, modify protocol e arrumar o template de acordo com o que pipetou na placa. Não esquecerde conferir o comprimento de onda (570nm). Fazer uma leitura sem placa, só para calibrar o aparelho (read plate). Depois fechar os resultados (file- close results). Colocar a placa na leitora e realizar a leitura (read plate), salvando com ID (Cálcioseunome/orientador- dd/mm/AA). Após abrirá a leitura das absorbâncias, então abra o Excel e copie as informações (template e absorbâncias menos o branco).

Cálculos

Cálcio (mg/dL) = Abs teste (amostras)/abs padrão x 10

Pode-se fazer o cálculo usando o fator, onde: fator=10/abs padrão

Cálcio (mg/dL)= abs teste x fator

Caso deseje pode se realizar o cálculo no Excel, recomenda-se usar o fator, multiplicando-o pelas absorbâncias das amostras.

Exemplos de cálculos:

Fator=10/1,346 =7,43

Cálcio = 1,270 x 7,43 = 9,4mg/dL

Anexo 6 – Instruções para realização da dosagem de fósforo utilizando o kit Fósforo Liquiform Labtest cat. 12

O kit Fósforo Liquiform Labtest cat. 12 contém:

Reagentes do kit (100 testes)

R1 – Reagente de cor (cuidado, cáustico)

R2- Padrão (5,0mg/dL)

Material necessário

Pipeta automática que libere 10µL

Pipeta automática que libere 1mL ou repipetador automático

Ponteiras

Cronômetro

Banho Maria (37°C)

Placa para leitura na leitora Elisa (template)

Béqueres para colocar os reagentes a serem pipetados

Amostra (plasma)

Tubos de ensaio em duplicata para as amostras

Tubo para padrão e branco

Estante para tubos

Obs: é importante que os tubos estejam bem limpos, sem resíduos de detergentes, uma vez que o fósforo inorgânico é componente comum da maioria dos detergentes.

Procedimento

Nos tubos de teste adicionar 10µL do plasma e em todos os tubos pipetar 1mL do reagente de cor (R1), de preferência com o repipetador pelo tempo de estabiliade. Agitar a estante levemente para misturar o reagente com a amostra. Levar ao banho maria que já deve estar na temperatura (37°C), deixar por 5 minutos. Após deve-se retirar a estante do banho e secá-la. A absorbância é estável somente por 30 minutos, recomendando-se leitura imediata.

Se a leitura for feita no espectrofotômetro manual (340nm) adicionar o branco na cubeta e zerar o aparelho. Proceder as leituras das amostras.

Caso a leitura for feita na leitora Elisa, deve-se pipetar 300µL da reação em cada poço, de acordo com a ordem definida (A1- Branco/ A2-Padrão/ A3-Padrão/ A4-amostra). Ligar o computador, entrar no programa KC Junior, open protocol, Fósforo labtest, modify protocol e arrumar o template de acordo com o que pipetou na placa. Não esquecer de conferir o comprimento de onda (340nm). Fazer uma leitura sem placa, só para calibrar o aparelho (read plate). Depois fechar os resultados (file- close results). Colocara placa na leitora e realizar a leitura (read plate), salvando com ID (Fósforoseunome/orientador- dd/mm/AA). Após abrirá a leitura das absorbâncias, então abra o Excel e copie as informações (template e absorbâncias menos o branco).

Cálculos

Fósforo inorgânico (mg/dL) = Abs teste (amostras)/abs padrão x 5

Pode-se fazer o cálculo usando o fator, onde: fator=5/abs padrão

Fósforo inorgânico(mg/dL)= abs teste x fator

Caso deseje pode se realizar o cálculo no Excel, recomenda-se usar o fator, multiplicando-o pelas absorbâncias das amostras.

Exemplos de cálculos:

Fator=5/0,247 =20,2

Fósforo inorgânico = 0,190 x 20,2 = 3,8mg/dL

Anexo 7 – Instruções para realização da dosagem de fosfatase alcalina utilizando o kit Fosfatase Alcalina Liquiform Labtest cat. 40

O kit Fosfatase Alcalina Liquiform Labtest cat. 40 contém:

Reagentes do kit (100 testes)

R1 – Substrato

R2 – Tampão (pH 10,1- básico "cuidado")

R3- Reagente de cor (reagente de parada- também alcalino "cuidado")

R4- Padrão 45U/L

Material necessário

Pipeta automática que libere 50µL

Pipeta automática que libere 500 µL e 2mL ou repipetador automático (duas ponteiras)

Ponteiras

Cronômetro

Banho Maria (37°C)

Placa para leitura na leitora Elisa (template)

3 béqueres para colocar os reagentes a serem pipetados

Amostra (plasma)

Tubos de ensaio em duplicata para as amostras

Tubo para padrão e branco

Estante para tubos

Procedimento

Em todos os tubos pipetar 0,5mL (500µL) do tampão (R2) e 50 µL do substrato (R1). Levar ao banho maria que já deve estar na temperatura (37°C), deixar por 2 minutos. Os passos seguintes sugere-se fazer em duas pessoas, pois a reação exige extrema atenção quanto ao tempo. A adição da amostra (50µL) deve ser feita com intervalos (de 20 em 20 segundos) de acordo com a prática de pipetar de quem manuseia a amostra, importante que seja calculado de forma que a última amostra seja pipetada antes de completar o tempo da reação (10 minutos) não se esquecer de pipetar 50µL padrão (R4) no tubo correspondente. Após dos 10 minutos da adição da primeira amostra deve-se adicionar 2mL do reagente de parada (R3) , procedendo sua adição de acordo com o tempo em que foram adicionadas as amostras (de 20 em 20 segundos), desta forma a reação em todos os tubos ocorrerá no tempo de 10 minutos. A reação toda ocorre dentro do banho maria. Após a adição do R3 em todos os tubos, deve-se retirar a estante do banho e secá-la. A cor é estável durante 120 minutos.

Se a leitura for feita no espectrofotômetro manual (590nm) adicionar o branco na cubeta e zerar o aparelho. Proceder as leituras das amostras.

Caso a leitura for feita na leitora Elisa, deve-se pipetar 300µL da reação em cada poço, de acordo com a ordem definida (A1- Branco/ A2-Padrão/ A3-Padrão/ A4-amostra). Ligar o computador, entrar no programa KC Junior, open protocol, fosfatase alcalina labtest, modify protocol e arrumar o template de acordo com o que pipetou na placa. Não esquecer de conferir o comprimento de onda (590nm). Fazer uma leitura sem placa, só para calibrar o aparelho (read plate). Depois fechar os resultados (file- close results). Colocara placa na leitora e realizar a leitura (read plate), salvando com ID (FAseunome/orientador- dd/mm/AA). Após abrirá a leitura das absorbâncias, então abra o Excel e copie as informações (template e absorbâncias menos o branco).

Cálculos

Fosfatase alcalina (U/L) = Abs teste (amostras)/abs padrão x 45

Pode-se fazer o cálculo usando o fator, onde: fator=45/abs padrão

Fosfatase alcalina (U/L)= abs teste x fator

Caso deseje pode se realizar o cálculo no Excel, recomenda-se usar o fator, multiplicando-o pelas absorbâncias da amostras.

Exemplos de cálculos:

Fator=45/0,360 =125

Fosfatase alcalina = 0,295 x 125 = 37 U/L

Anexo 8 – Instruções para realização da coloração de hematoxilina e eosina.

Procedimento para o preoparo da HEMATOXILINA DE HARRIS:

Hematoxilina em cristais	5g
Alúmen de amônia ou de potássio	20g
Álcool 95%	50ml
Sulfato de Alumínio e Potássio	100g
Água Destilada	1000ml
Óxido de Mercúrio	2,5g

- Dissolver a Hematoxilina no álcool e o Alúmen na água destilada com o auxílio de calor.
- Misturar as duas soluções e ferver o mais rápido possível.
- Remover da fonte de calor e juntar o Óxido de Mercúrio aos poucos com cuidado, reaquecer até a fervura e contar um minuto.
- Afastar da fonte de calor e mergulhar em uma bacia com água e gelo imediatamente.

- Adicionar 4 ml de Ácido Acético, quando o corante estiver resfriado.

Procedimento para o preoparo de EOSINA:

Eosina 2,5g

Água destilada 50 ml

Álcool 95% 200 ml

Álcool 80% 750 ml

- Dissolver a EOSINA na água destilada e acrescentar o álcool 95%
- Juntar os 750 ml de álcool 80% e mais 5 ml de Ácido Acético.

Após preparar as soluções acima, montar a bateria de álcool e xilol para o procedimento da coloração propriamente dito.

As etapas da coloração são realizadas com o tempo exato em uma solução como mostra abaixo:

Xilol I.....5 min

Xilol II.....5 min

Álcool absoluto...1 min

Álcool 95%.....1 min

Alcool 70%.....1 min

Hematoxilina de Harris.....5 min

Água Corrente.....Lave até a água se tornar límpida

Álcool ácido.....0,2 segundos

Água.....3 min

Eosina.....40 segundos

Álcool 95%.....1 min

Álcool 95%.....1 min

Álcool Absoluto.....1 min

Alcool Absoluto.....1 min

Álcool Absoluto.....1 min

Xilol I.....1 min

Xilol II.....1 min

Xilol III.....5 min

Montar as lâminas em balsamo ou entelan

Anexo 9 – Instruções para realização da coloração imunohistoquímica

Foram selecionados 3cortes histológicos de cada espécime que foram desparafinizados em xilol e hidratados em série decrescente de etanol (100º - 100º - 100º - 90º - 70° GL). A recuperação antigênica foi realizada através da imersão das lâminas histológicas em tampão citrato de sódio 0,1 M, pH 6,0. em câmara pressurizada (Decloaking chamber®, Biocare Medical, Concord, CA, EUA) a 95°C, por 20 minutos. No final de cada etapa da reação imunoistoquímica, as lâminas histológicas foram lavadas em PBS (tampão fosfato salino) 0,1 M, pH 7,4. Posteriormente, as lâminas foram imersas em 3% de peróxido de hidrogênio por 1 hora e 1% de soro albumina bovino por 12 horas para bloqueio da peroxidase endógena e bloqueio dos sítios inespecíficos, respectivamente. As lâminas contendo amostras de cada grupo experimental foram divididas em três lotes, e cada lote foi incubado com um dos seguintes anticorpos primários: anti-TRAP do rato gerado em cabra (SC-30833, Santa Cruz Biotechnology, Santa Cruz, CA, EUA), anti-RANKL do rato gerado em cabra (SC-7628, Santa Cruz Biotechnology, Santa Cruz, CA, EUA), anti-OPG do rato gerado em cabra (SC-8468, Santa Cruz Biotechnology, Santa Cruz, CA, EUA), HIF1-alfa (Rabbit – SC10790 - Santa Cruz Biotechnology, Santa Cruz, CA) e fosfatase alcalina óssea (Goat – SC15065 - Santa Cruz Biotechnology. Os cortes histológicos foram incubados com anticorpo secundário biotinilado por 2 horas e

subsequentemente tratados com estreptavidina conjugada com a peroxidase da raiz forte - HRP por 1 hora (Universal Dako Labeled HRP Streptavidin-Biotin Kit®, Dako Laboratories, CA, EUA). Procedeu-se a revelação utilizando como cromógeno o 3,3'- tetracloridrato de diaminobenzidina (DAB chromogen Kit®, Dako Laboratories, CA, USA) e a contracoloração com hematoxilina de Harris. Como controle negativo, os espécimes foram submetidos aos procedimentos descritos anteriormente suprimindo-se a utilização dos anticorpos primários.