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INFLUÊNCIA DO ÁCIDO ZOLEDRÔNICO NA PROGRESSÃO DE PERIODONTITE APICAL EM RATAS COM OSTEOPOROSE E ASSOCIAÇÃO DO ESTRESSE OXIDATIVO COM CONTEÚDO MINERAL ÓSSEO

Santa Maria, RS

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Odontológicas, Área de Concentração em Odontologia, Ênfase em Endodontia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de Doutora **em Ciências Odontológicas**.

Orientador: Prof. Dr. Carlos Alexandre Souza Bier

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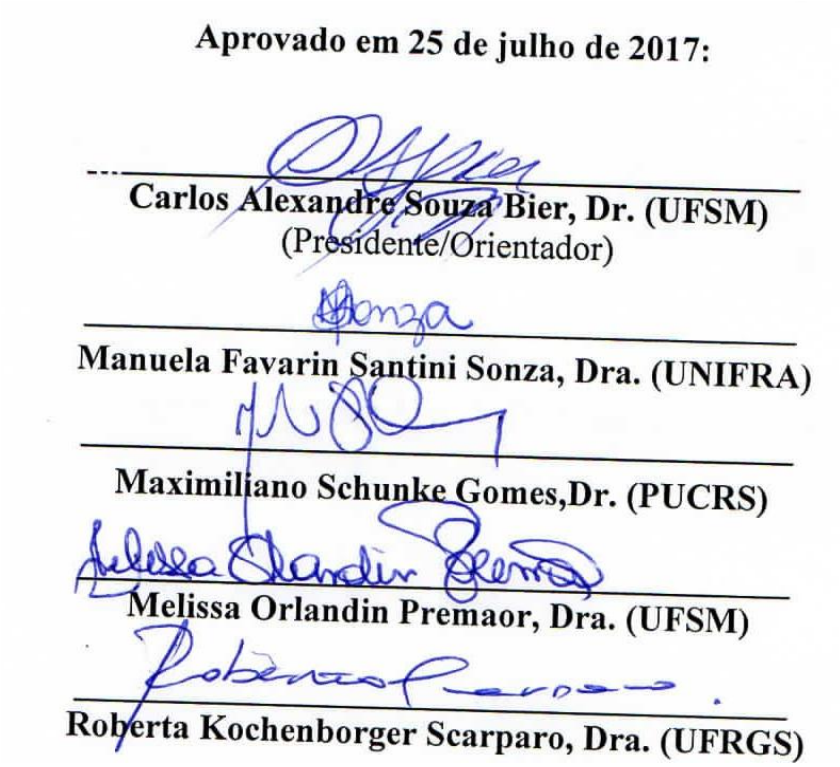

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DEDICATÓRIA

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"Ando devagar, porque já tive pressa e levo esse sorriso, porque já chorei demais,

Hoje me sinto mais forte, mais feliz, quem sabe,

Só levo a certeza de que muito pouco sei ou nada sei ...,

É preciso amor para poder pulsar,

É preciso paz para poder sorrir,

É preciso a chuva para florir...

Todo mundo ama um dia, todo mundo chora,

Um dia a gente chega e no outro vai embora,

Cada um de nós compõe a sua história,

Cada ser em si carrega o dom de ser capaz e ser feliz"

Almir Sater

RESUMO

INFLUÊNCIA DO ÁCIDO ZOLEDRÔNICO NO DESENVOLVIMENTO DE PERIODONTITE APICAL EM RATAS COM OSTEOPOROSE E ASSOCIAÇÃO DO ESTRESSE OXIDATIVO COM CONTEÚDO MINERAL ÓSSEO

AUTORA: Mariana De Carlo Bello
ORIENTADOR: Carlos Alexandre Souza Bier

A presente tese foi estruturada em dois estudos, apresentados em forma de artigos, que investigaram a associação entre os parâmetros de Estresse Oxidativo (OS) e osteoporose; e os efeitos sistêmicos e locais do tratamento da osteoporose com Ácido Zoledrônico (ZOL). O primeiro artigo avaliou a associação entre os marcadores de OS e o Conteúdo Mineral Ósseo (BMC) do fêmur, bem como determinou a presença destes marcadores no fígado de ratas com osteoporose tratadas e não tratadas com ZOL. Ratas da linhagem Wistar foram divididas em 4 grupos (n=10): OVX – cirurgia de ovariectomia e tratamento com veículo; OVX + ZOL - cirurgia de ovariectomia e tratamento com ZOL; SHAM – cirurgia sham e tratamento com veículo; SHAM + ZOL - cirurgia sham e tratamento com ZOL. Vinte e um dias depois da cirurgia, veículo ou ZOL foram administrados em uma única aplicação. Após 35 dias, os danos oxidativos e as defesas antioxidantes no fígado foram avaliados pela mensuração dos níveis de Peroxidação Lipídica (LP), Espécies Reativas de Oxigênio (ROS), Óxido Nítrico (NOx), grupos tiois não protéicos e Vitamina C. A partir da regressão multivariada foi observado que os marcadores de dano oxidativo (LP, ROS, NOx) foram associados com redução do BMC tanto no osso esponjoso quanto no osso cortical ($P < 0,05$). Na análise ajustada, ROS e ZOL apresentaram correlação negativa e positiva, respectivamente, com BMC tanto no osso esponjoso quanto no osso cortical ($P < 0,05$). Concluiu-se que os marcadores de OS estão correlacionados com a ocorrência da osteoporose e o tratamento com ZOL auxilia na manutenção da massa óssea. Já o segundo artigo avaliou a influência da administração de uma única dose de ZOL no tamanho de Lesões Periapicais (PL) de origem endodôntica em ratas ovariectomizadas e controle; adicionalmente, avaliou parâmetros sistêmicos, tais como, ROS, contagem de células sanguíneas brancas e BMC no fêmur e mandíbula. O segundo estudo apresentou os mesmos grupos descritos acima. Vinte e um dias depois da cirurgia, foi realizada abertura coronária e exposição do tecido pulpar do primeiro molar mandibular direito de todos animais a fim de induzir periodontite apical. Neste mesmo dia foi administrado, em uma única aplicação, veículo ou ZOL. Após 35 dias, a área da PL foi mensurada pela análise histométrica. O infiltrado inflamatório local e sistêmico foi avaliado pela análise histológica e hematológica, respectivamente. Os níveis de ROS foram quantificados para estimar o dano oxidativo. Foi observado que o tamanho da PL foi similar entre os grupos ($P > 0,05$). O infiltrado inflamatório local e sistêmico não foram afetados pelo tratamento com ZOL ($P > 0,05$). Os grupos OVX e OVX + ZOL apresentaram maiores níveis de ROS que os grupos SHAM ($P < 0,05$). O ZOL diminuiu os níveis de ROS nas ratas ovariectomizadas ($P < 0,05$). Concluiu-se que a terapia com ZOL não interfere na perda óssea periapical e nos parâmetros inflamatórios. Contudo, o ZOL reduziu um marcador de dano oxidativo.

Palavras-chave: Bisfosfonatos. Defesa antioxidante. Estresse oxidativo. Hipoestrogenismo. Osteoporose. Periodontite apical.

ABSTRACT

INFLUENCE OF ZOLEDRONIC ACID IN THE DEVELOPMENT OF APICAL PERIODONTITIS IN RATS WITH OSTEOPOROSIS AND ASSOCIATION OF OXIDATIVE STRESS WITH BONE MINERAL CONTENT

AUTHOR: Mariana De Carlo Bello
ADVISOR: Carlos Alexandre Souza Bier

The present thesis was structured in two studies presented as two articles, that investigated the association between the parameters of Oxidative Stress (OS) and osteoporosis and the systemic and local effects of the osteoporosis treatment with Zoledronic Acid (ZOL). The first paper evaluated the association between OS markers and Bone Mmineral Content (BMC) of the femur, as well as determined the presence of these markers in the liver of rats with osteoporosis treated and untreated with ZOL. Wistar rats were divided into 4 groups (n = 10): OVX - ovariectomy surgery and vehicle treatment; OVX + ZOL - ovariectomy surgery and ZOL treatment; SHAM - sham surgery and vehicle treatment; SHAM + ZOL - sham surgery and ZOL treatment. Twenty-one days after surgery, vehicle or ZOL were administered in a single application. After 35 days the animals were euthanized. Oxidative damage and antioxidant defenses in the liver were evaluated by measuring the levels of Lipid Peroxidation (LP), Reactive Oxygen Species (ROS), Nitric Oxide (NOx), non-protein thiol groups and vitamin C. From multivariate regression it was observed that markers of oxidative damage (LP, ROS, NOx) were associated with reduction of BMC in both cancellous and cortical bone ($P < .05$). In the adjusted analysis, ROS and ZOL presented negative and positive association, respectively, with BMC in both cancellous and cortical bone ($P < .05$). It was concluded that OS markers were associated with the occurrence of osteoporosis and ZOL treatment helps in maintaining bone mass. The second paper evaluated the influence of a single dose of ZOL on the size of Periapical Lesions (PL) in ovariectomized and control rats; additionally, it was evaluated systemic parameters such as ROS, white blood cell count and BMC in the femur and mandible. The second paper used the same groups described above. Twenty-one days after surgery, pulp exposure of the first right mandibular molar of all animals was performed in order to induce apical periodontitis. On the same day, vehicle or ZOL was administered in a single application. After 35 days, the PL area was measured by histometric analysis. Local and systemic inflammatory infiltrate were evaluated by histological and hematological analyses, respectively. ROS levels were quantified to estimate oxidative damage. It was observed that the PL size was similar between the groups ($P > .05$). Local and systemic inflammatory infiltrate were not affected by treatment with ZOL ($P > .05$). The OVX and OVX + ZOL groups presented higher levels of ROS than SHAM groups ($P < .05$). ZOL decreased ROS levels in ovariectomized rats ($P < .05$). It was concluded that ZOL therapy does not interfere in the periapical bone loss and in the inflammatory parameters. However, ZOL reduced a marker of oxidative damage.

Keywords: Antioxidant defense. Apical periodontitis. Bisphosphonates. Hypoestrogenism. Osteoporosis. Oxidative stress.

SUMÁRIO

1 INTRODUÇÃO

A osteoporose é um distúrbio osteometabólico caracterizado pela redução da densidade mineral óssea e pela deterioração da microarquitetura do tecido ósseo, levando a um aumento da fragilidade esquelética e ao risco de fraturas (CONSENSUS DEVELOPMENT CONFERENCE ON OSTEOPOROSIS, 2014). Esta doença sistêmica e silenciosa atinge principalmente mulheres após a menopausa, devido à perda da função ovariana e consequente redução dos níveis de estrogênio. A deficiência desse hormônio resulta em altas taxas de remodelação óssea, sendo que a reabsorção excede a formação, levando, dessa forma, à perda de massa óssea corporal (RIGGS et al., 1998; MANOLAGAS, 2000).

O tratamento farmacológico de primeira escolha para doenças osteometabólicas é a administração de bisfosfonatos, os quais fazem parte de uma classe terapêutica denominada de drogas anti-reabsortivas, uma vez que são capazes de preservar a densidade mineral óssea pela inibição da reabsorção óssea (CHRISTENSON et al., 2012; DHILLON, 2016). O Ácido Zoledrônico (ZOL) é um potente bisfosfonato de terceira geração que tem sido amplamente usado para tratamento da osteoporose (BLACK et al., 2007; LAMBRINOUDAKI et al., 2008; REID et al., 2009; KHAJURIA; RAZDAN; MAHAPATRA, 2011). Este medicamento apresenta grande afinidade aos tecidos mineralizados (NANCOLLAS et al., 2006), perimitindo ser administrado uma vez ao ano e garantindo boa adesão dos pacientes (DHILLON, 2016). Seu mecanismo de ação baseia-se na inibição da osteoclastogênese e na supressão da função e sobrevivência dos osteoclastos (NIELSEN et al., 1991; COXON et al., 2000; BENFORD et al., 2001). Pacientes que fazem o uso desse medicamento uma vez ao ano apresentam aumento da densidade mineral óssea (BROWN; ZACHARIN, 2009; ALMEIDA; UENIS; GUARNIERO, 2010), menor susceptibilidade a fraturas e redução dos marcadores de reabsorção óssea (BLACK et al., 2007). A osteonecrose dos maxilares é o efeito adverso mais associado à administração de altas e frequentes doses de bisfosfonatos para o tratamento de neoplasias (PEREIRA et al., 2004; MARTINS et al., 2004). Entretanto, quando esses medicamentos são utilizados na posologia para o tratamento da osteoporose, a incidência de osteonecrose é extremamente baixa (KHAN et al., 2015).

A perda óssea decorrente da osteoporose pós menopausa também vem sendo relacionada ao estresse oxidativo (SENDUR et al., 2009; BAEK et al., 2010; MANOLAGAS, 2010). O estresse oxidativo é consequência do desequilíbrio entre a formação e a remoção de radicais livres no organismo (HALLIWELL; WHITEMAN, 2004), favorecendo a ocorrência

de danos à estrutura e/ou à função biológica das células e induzindo a efeitos deletérios (BARBOSA et al., 2008). Os radicais livres parecem ter um importante impacto na patogênese da perda óssea pela ativação do Ligante do Receptor Ativador do Fator Nuclear Kappa (RANKL - do inglês, *receptor activator of nuclear factor kappa*- ß *ligand*) (BAI et al., 2005). O RANKL presente na membrana dos osteoblastos liga-se ao seu receptor RANK (RANK - do inglês, *receptor activator of nuclear factor kappa*- ß), localizado na membrana dos pré-osteoclastos (ROUX et al., 2002), estimulando a diferenciação do precursor do osteoclasto em osteoclasto maduro (NAKAGAWA et al., 1998; TAKAMI et al., 1999; CLARKE, 2008). Em contraste, a Osteoprotegerina (OPG) produzida pelo osteoblasto, bloqueia a formação de osteoclastos, ligando-se ao RANKL (SIMONET et al., 1997), com o qual tem grande afinidade, impedindo que RANK se ligue ao RANKL e inibindo a osteoclastogênese (SIMONET et al., 1997; ROUX et al., 2002). O balanço entre a produção de RANKL e de OPG determina a quantidade de osso que é reabsorvida (NAKAGAWA et al., 1998; ROUX et al., 2002). Desta forma, o estresse oxidativo influencia na diferenciação e função dos osteoclastos (GARRETT et al., 1990; BAX et al., 1992). Além disso, uma vez que o estrogênio apresenta atividade antioxidante (LEAN et al., 2003), a deficiência deste hormônio provoca acúmulo de radicais livres, o quais estimulam a formação de osteoclastos (GARRETT et al., 1990; BAX et al., 1992). Estudos clínicos em mulheres com osteoporose mostraram associação entre o aumento de marcadores de dano oxidativo e a redução dos níveis plasmáticos de antioxidantes e diminuição da densidade mineral óssea (BASU et al., 2001; MAGGIO et al., 2003; OZGOCMEN et al., 2007; SÁNCHEZ-RODRÍGUEZ et al., 2007; BAEK et al., 2010; CERVELLATI et al., 2014).

Os efeitos da osteoporose pós menopausa, causada pela deficiência de estrogênio, são maiores nos ossos longos como no fêmur, e também nas vértebras (MANAGEMENT OF OSTEOPOROSIS IN POSTMENOPAUSAL WOMEN, 2010). Entretanto, a literatura sugere uma relação positiva entre a perda de massa óssea sistêmica e o comprometimento ósseo da cavidade oral (KRIBBS et al., 1983; BINTE et al., 2007; RICHA et al., 2016). A deficiência de estrogênio pode afetar o padrão de reabsorção óssea em lesões periapicais decorrentes da infecção microbiana do canal radicular (GILLES et al., 1997; XIONG et al., 2007; WAYAMA et al., 2015; BRASIL et al., 2017). Entretanto, existem controvérsias sobre o real impacto do hipoestrogenismo no desenvolvimento da periodontite apical, pois alguns autores não demonstraram tal relação (LIU et al., 2010; Zhang et al., 2011).

Gilles et al. (1997) avaliaram a perda óssea periapical na região de dentes molares de ratas ovariectomizadas e não ovariectomizadas. Foram administrados localmente interleucina-

1 e *Campylobacter rects*, um patógeno periodontal, no conduto radicular e feita avaliação radiográfica da evolução da perda óssea na lesão periapical. Os autores observaram que houve uma perda óssea maior nas ratas ovariectomizadas em relação às não ovariectomizadas, tanto no grupo controle, como nos grupos que utilizaram interleucina-1 e *Campylobacter rectus* para acelerar a perda óssea. Brasil et al. (2017) avaliaram o efeito de longos períodos de deficiência de estrogênio no desenvolvimento de periodontite apical em ratas. Os animais foram submetidos a cirurgia de ovariectomia (grupo OVX) ou a simulação da ovariectomia (grupo controle) e somente após 120 dias as lesões periapicais foram induzidas. O desenvolvimento das lesões periapicais foi avaliado em 21 e 40 dias. Os autores observaram que em 21 dias de indução da lesão periapical não houve diferença estatisticamente significante entre o grupo OVX e o grupo controle. Já em 40 dias, o tamanho da lesão foi maior nas ratas ovariectomizadas. O longo período de deficiência de estrogênio provavelmente agravou as consequências da osteoporose no osso mandibular. Liu et al. (2010) também avaliaram a perda óssea periapical em ratas ovariectomizadas (OVX) e observaram que o tamanho da lesão foi similar nos grupos OVX e controle. Zhang et al. (2011) observaram que os grupos submetidos a cirurgia de remoção bilateral dos ovários apresentaram maior concentração sistêmica de citocinas inflamatórias que os grupos em que foi realizado apenas a simulação da ovariectomia, porém, o tamanho da lesão periapical entre os grupos foi similar.

Da mesma forma que a osteoporose parece afetar o metabolismo ósseo mandibular, acelerando a progressão da periodontite apical, o tratamento sistêmico desta desordem óssea com bisfosfonatos pode influenciar o desenvolvimento de patologias periapicais. Xiong et al. (2007) verificaram que o tamanho das lesões periapicais em ratas ovariectomizadas, tanto na análise radiográfica quanto na histométrica, foi maior que em ratas com níveis de estrogênio normais. Já, quando as ratas ovariectomizadas foram tratadas com alendronato por 40 dias (0,25mg/kg/dia), a perda óssea na região periapical foi reduzida. Wayama et al. (2015) investigaram pela primeira vez o efeito da administração sistêmica do ZOL, 1 vez por semana durante 4 semanas (0,04 mg/kg), na progressão de lesões periapicais em ratas ovariectomizadas. Os autores observaram que a deficiência de estrogênio agrava a progressão das lesões e que a terapia com ZOL pode conter a destruição óssea na região periapical. Entretanto, a frequência de administração e a alta dose utilizada não caracteriza o protocolo para tratamento da osteoporose em humanos.

Estudos clínicos são escassos e os resultados são preliminares. Lopes-Lopez et al. (2015) observaram que baixa densidade mineral óssea foi correlacionada com a presença de

lesões periapicais, entretanto, nenhuma diferença estatística foi detectada na presença de radiolucidez periapical de origem endodôntica em mulheres saudáveis, osteopênicas ou osteoporóticas.

Portanto, os objetivos do primeiro artigo apresentado neste trabalho foram avaliar a associação entre os marcadores de estresse oxidativo e o Conteúdo Mineral Ósseo (BMC – do inglês, *bone mineral content*) do fêmur, bem como determinar a presença destes marcadores em ratas com osteoporose tratadas e não tratadas com ZOL. Já os objetivos do segundo artigo foram avaliar a influência da administração de uma dose única de ZOL no tamanho de lesões periapicais de origem endodôntica em ratas ovariectomizadas; adicionalmente, parâmetros sistêmicos, tais como, formação de Espécies Reativa de Oxigênio (ROS – do inglês, *reactive oxygen species*), contagem de células sanguíneas brancas e BMC do fêmur e mandíbula foram avaliados.

2 ARTIGO 1 – OSTEOPOROSIS AND SYSTEMIC TREATMENT WITH ZOLEDRONIC ACID IN RATS: OXIDATIVE STRESS PARAMETERS

Este artigo será submetido à publicação no periódico *Bone*, Fator de impacto = 3.736; Qualis A2. As normas para publicação estão descritas no Anexo A.

OSTEOPOROSIS AND SYSTEMIC TREATMENT WITH ZOLEDRONIC ACID IN RATS: OXIDATIVE STRESS PARAMETERS

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Abbreviations: BMC, bone mineral content; CT, multislice computed tomography; DCF, fluorescent dichlorofluorescein; DCHF-DA, 2,7-dichlorofluorescein diacetate; HU, hounsfield units; IP, intraperitoneal; GSH, glutathione; LP, lipid peroxidation; MDA, malondialdehyde; NOx, nitric oxide; NPSH, non-protein thiols; OPG, osteoprotegerin; OS, oxidative stress; OVX, ovariectomy; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; ROS, reactive oxygen species; SHAM, simulation of ovariectomy surgery; TBA, thiobarbituric acid; Vit C, Vitamin C; ZOL, Zoledronic acid.

ABSTRACT

Introduction: The aim of this study was to assess the association between bone mineral content (BMC) and oxidative stress (OS) markers, as well as to quantify OS markers in rats with osteoporosis treated with zoledronic acid (ZOL). **Methods:** 40 female Wistar rats were divided into the following groups (n = 10): OVX - ovariectomy plus saline; OVX + ZOL - ovariectomy plus ZOL; SHAM - sham surgery plus saline; SHAM + ZOL - sham surgery plus ZOL. Twenty-one days after surgery, saline or ZOL (100 μg/kg) were administered intraperitoneally in a single application. After 35 days, oxidative damage and antioxidant defenses in the liver were evaluated by measuring the levels of lipid peroxidation (LP), reactive oxygen species (ROS), nitric oxide (NOx), non-protein thiols (NPSH) and vitamin C (Vit C). Data were analyzed by multivariate linear regression, Pearson correlation, two-way ANOVA and Bonferroni *posthoc* test ($P < .05$). **Results:** Multivariate regression showed that oxidative damage markers (ROS, LP, NOx) were associated with BMC reduction in both cancellous and cortical bone ($P < .05$). In the adjusted analysis, ROS and ZOL maintained negative and positive association, respectively, with BMC in both cancellous and cortical bone ($P < .05$). Positive correlation coefficients were observed both between ROS generation and LP levels and between ROS and NOx levels ($P < .05$). **Conclusions:** OS markers are associated with occurrence of osteoporosis and treatment with ZOL assists to maintain bone mass.

Keywords: Oxidative stress. Bisphosphonate. Osteoporosis. Estrogen deficiency.

1. INTRODUCTION

Osteoporosis is a systemic disorder associated with aging (1). This bone disease progresses slowly and it is characterized by low bone strength and microarchitectural deterioration leading to increased bone fragility, and consequently it increases the risk of fractures (2).

This bone loss can be related to estrogen deficiency that occurs with aging, in postmenopausal women, due to the decline in the production of ovarian hormones (3). Estrogen plays a major role in maintaining bone homeostasis through the balance between bone formation by osteoblasts and bone resorption by osteoclasts. This hormone influences in the receptor activator of nuclear factor kappa-B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) system (4). RANKL, which is expressed mainly in osteoblast lineage cells, binds to RANK present in the osteoclast lineage cells, stimulates osteoclastogenesis, increases osteoclastic activity and inhibits mature osteoclast apoptosis. These events are blocked when OPG, also produced predominantly by the osteoblasts, binds to RANKL, preventing the interaction between RANK and its ligand (3).

Recent evidence has taken changes this paradigm. Postmenopausal osteoporosis is not justified solely by estrogen deficiency. Oxidative stress (OS) is related to age and has also been associated with a key pathogenic mechanism for the development of osteoporosis (3, 5). OS is defined as an imbalance between oxidizing and antioxidant mechanisms of the body, with excessive production of free radicals, which results in cellular damage (6). The reduction of estrogen implies in the generation of reactive oxygen species (ROS) and changes antioxidant defense capacity, leading to an accumulation of oxidative species, which are capable of stimulating the formation of osteoclasts and the resorption activity (7). ROS affect the genesis and the lifespan of osteoclasts by influencing the RANK/RANKL/OPG system by stimulation of RANKL expression (3). Studies suggest that the anti-osteoporotic effects of estrogen result, at least in part, from their ability to protect bone against oxidative stress. In fact, estrogen loss accelerates the aging effect on bone by reducing antioxidant defenses (3).

Zoledronic acid (ZOL) is a potent bisphosphonate used for the treatment of osteoporosis (8). This drug inhibits osteoclast-mediated bone resorption, since it affects the RANK/RANKL/OPG system, stimulating the production of OPG and reducing the production of RANKL (9). Bisphosphonates have antioxidant properties (10); however, if the mechanism of bone loss prevention is, in part, attributed to ZOL action on the OS, this is not known yet.

Considering the above, it is not yet clear the relationship of OS with osteoporosis and the effect of ZOL on different oxidant and antioxidant parameters. The primary objective of

this study was to evaluate the association between bone mineral content (BMC) of femoral with estradiol level, ZOL and OS markers; the secondary objective was to quantify OS markers in rats with osteoporosis treated with ZOL.

2. MATERIALS AND METHODS

2.1 Animals

Forty female Wistar rats, weighing between 150 and 200 grams and aged approximately 70 days from the Center for Biological Models and Experimental (CeMBE) were used. The animals were kept under controlled conditions in ventilated cages (temperature: 22 ± 1ºC, 70% humidity, and a cycle of 12h light and 12h dark). Standard rodent chow and tap water were provided *ad libitum*, except during the experimental sessions. All animals were allowed to acclimate to the laboratory environment for one week before surgical procedures were carried out. The weight of animals was measured to allow calculation of the anesthetic and analgesic substances.

The experimental protocols followed the current Brazilian guidelines for the care and use of animals for scientific and didactic procedures, from the National Council for the Control of Animal Experimentation (CONCEA, Brazil), which are according to the Guide for the Care and Use of Laboratory Animals published by National Institutes of Health. The local Animal Ethics Committee evaluated and approved all the protocols (CEUA 15/00436).

2.2 Experimental Design and Distribution of Groups

Animals were randomly distributed into four groups (n=10): OVX – ovariectomy and treatment with saline; OVX + ZOL – ovariectomy and treatment with ZOL; SHAM – simulation of ovariectomy (sham surgery) and treatment with saline; SHAM + ZOL – sham surgery and treatment with ZOL.

2.3 Ovariectomy

Animals were anesthetized intraperitoneally (IP) with a mixture of xylazine (10mg/kg; Anasedan®, Sespo industry, and LTD Trade, Paulinia, São Paulo, Brazil) and ketamine (100 mg/kg; Dopalen, Sespo industry and LTD Trade, Paulinia, São Paulo, Brazil). To induce the systemic condition of osteoporosis, bilateral ovariectomy was performed in twenty animals (11). The remaining rats were subjected to sham surgery. Paracetamol was administered (80mg/kg) by gavage for 48h after surgery, aiming to eliminate any discomfort (Cembe/PUCRS protocol).

2.4 Zoledronic Acid

Twenty-one days after ovariectomy, ZOL (Zometa®, Novartis, Basel, Switzerland) was administered IP in a single application (100 μg/kg body weight) (12). As a control, an IP dose of saline was administered to the animals that no received the drug. The period between the administration of ZOL and euthanasia was 35 days (13).

2.5 Sample Collection

Fifty-six days after the start of the experimental phase, the animals were euthanized by deep anesthesia with sevoflurane (Sevorane®, Abbott Laboratories Ltda Brazil, São Paulo, Brazil). The uterus of the rats was removed and weighed. Blood samples were obtained for analysis of estradiol level. Samples of liver were collected for evaluation of indicators of OS. Femur was removed, dissected, placed in formalin solution (10%) for tissue fixation for performing multislice computed tomography (CT).

2.6 Analysis of Uterus Weight and Estrogen Level

Weight of the uterus was measured on a precision scale (g). Blood samples were collected by cardiac puncture vacutainer device (BD Vacutainer® Blood Collection Eclipse ™ Needle) and centrifuged for 12 min to obtain the serum. Estrogen level was verified by chemiluminescence (Estradiol IMMULITE, Diagnostic Products Corp., Los Angeles, California). All samples were assayed in duplicate and in the same assay to avoid interassay error.

2.7 Bone Mineral Content Analysis by Multislice Computed Tomography

Femur was submitted to CT (BrightSpeed, GE Healthcare, Little Chalfont, *United Kingdom)* to determine BMC of the cortical and cancellous bone. The images were analyzed by a calibrated and blinded radiologist to the comparison groups, with the auxiliary of the OsiriX software (Prixmeo SARL, Geneva, Switzerland). The measurement of BMC in the femur was obtained in the condyle 2 mm from the growth plate, in Hounsfield units (HU), based on the study of Kuroda et al. (2003) (14), with minor adaptations.

2.8 Estimation of Oxidative Damage in the Liver

Lipid peroxidation (LP): LP levels were estimated through the pink chromogen produced by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) formed

during lipid oxidation of skin homogenates, and measured spectrophotometrically at 535nm (15). Results were expressed as nmol MDA/g liver.

Reactive oxygen species (ROS): ROS levels were quantified using the oxidant-sensing fluorescent probe, 2,7-dichlorofluorescein diacetate (DCHF-DA) (16). The oxidation (DCHF-DA) to fluorescent dichlorofluorescein (DCF) was determined at 488nm for excitation and 525nm for emission. Liver samples (20μL) were added to a medium containing TrisHCl buffer (0.01mM; pH 7.4) and DCFH-DA (10μM). After DCFH-DA addition, the medium was incubated in the dark for 1h until fluorescence measurement procedure. DCF-RS levels were corrected by the protein content (17) and expressed as a percentage of values from control.

Nitric oxide (NOx): For NOx levels, the liver homogenized with ZnSO4 (200mM) and acetonitrile (96%), centrifuged at 16.000g at 4ºC for 30min and the supernatant was collected. The supernatant was used to determine the nitrate and nitrite content, an indicator of NOx production (18). Nitrate/nitrite content was estimated in a medium containing 300mL of 2% VCl_3 (in 5% HCl), 200mL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 200mL of 2% sulfanilamide (in 5% HCl). After incubating at 37ºC for 60min, nitrite levels were determined spectrophotometrically at 540nm, based on the reduction of nitrate to nitrite by VCl_3. Tissue nitrate/nitrite levels were expressed as nmol of NOx/g of liver.

2.9 Estimation Antioxidant Defenses in the Liver

Non-protein thiols (NPSH): NPSH levels were determined after the reaction of liver homogenates with 5,5´-dithiobis-(2-nitrobenzoic acid). The yellow color formed was read at 412nm, in accordance with Boyne and Ellman (1972) (19) protocol. A standard curve using GSH was plotted in order to calculate GSH content, expressed as μmol NPSH/g of liver.

Vitamin C (Vit C): Liver's Vit C was estimated as described by Galley et al. (1996) (20). This method produces an orange chromogen by the reaction with dinitrophenylhydrazine at 37°C, measured spectrophotometrically at 520nm. A standard curve using ascorbic acid was used to calculate the content of Vit C and expressed as mg Vit C/g of liver.

2.10 Statistical Analysis

All data were submitted to the Shapiro-Wilk normality test. Comparative analysis of the data among groups was performed by the parametric two-way ANOVA and Bonferroni multiple comparison test. Multivariate linear regression analysis was performed to evaluate the association between the outcome (cortical and cancellous BMC) and predictors (estradiol,

ZOL, LP, ROS, NOx, NPSH and Vit C). Pearson correlation was applied to the data of oxidative stress. Data analyses were performed using STATA 12.0 software (Stata Corp. College Station, TX, USA). The level of statistical significance was set at 5% ($P < .05$).

3. RESULTS

3.1 Serum Estradiol Levels and Uterus Weight

For serum estradiol levels, two-way ANOVA revealed a significant main effect of ovariectomy, treatment and ovariectomy × treatment interaction [$F(1, 27) = 136.71$, $P < .0001$; 77.45, $P < .0001$; and 55.46, $P < .0001$, respectively]. *Post hoc* test revealed that OVX and OVX + ZOL groups demonstrated significantly lower levels of estradiol when compared with SHAM groups ($P < .05$) (Table 1).

For uterus weight, two-way ANOVA also revealed a significant main effect of ovariectomy, treatment, and ovariectomy × treatment interaction [$F(1, 27) = 195.47$, $P = .00$; 10.02, $P = .00$; and 9.88, $P = .00$, respectively]. Bonferroni' test showed that the uterus of the OVX groups weighed less than SHAM groups ($P < .05$) (Table 1).

Additionally, *post hoc* comparisons revealed that uterus weight ($P < .05$) and estradiol level ($P < .05$) were affected by ZOL treatment in SHAM groups (Table 1).

3.2 Bone Mineral Content Analysis

Two-way ANOVA revealed a significant main effect of treatment and ovariectomy × treatment interaction [$F(1, 27) = 13.17$, $P = .00$; 8.74, $P < .0001$, respectively]. *The post hoc* test revealed that OVX group showed less bone content than the other groups in the condyle of the femur, in both cortical ($P < .05$) and cancellous bone ($P < .05$). The groups treated with ZOL showed a tendency to be associated with a higher mineral content in both cancellous and cortical bone of the femur (Table 1).

Table 1. Mean ± standard error mean (SE) of the uterus weight, serum estradiol levels, and femoral mineral content.

Groups	Uterus weight (g)	Serum estradiol levels (pg/mL)	Cortical Bone (HU)	Cancellous Bone (HU)
SHAM	0.080 ± 0.006^{b}	17.28 ± 2.64^{b}	1476.39 ± 63.10^{a}	875.68 ± 95.02^{a}
SHAM + ZOL	0.113 ± 0.010^{a}	39.08 ± 1.06^{a}	1520.25 ± 48.35^{a}	1153.12 ± 62.55^{ac}
OVX	0.024 ± 0.001^{c}	11.59 ± 0.53^{c}	1211.80 ± 70.61^{b}	498.50 ± 52.40^{b}
OVX + ZOL	0.024 ± 0.001^{c}	13.40 ± 1.48^{c}	1640.64 ± 60.62^{a}	1174.69 ± 48.94^{c}

Different letters in the same column indicate significant difference among groups. $P < .05$ (two-way ANOVA plus Bonferroni test).

3.3 Oxidant and Antioxidant Parameters

ROS*:* Two-way ANOVA revealed a significant main effect of ovariectomy and treatment [F(1, 27) = 68.05, *P* = .0000; 11.42, *P* = .0022]. *Post hoc* test revealed that OVX groups showed higher levels of ROS than SHAM groups (*P* < .05) and the OVX + ZOL group showed lower ROS level in comparison with OVX group (*P* < .05) (Figure 1).

NOx: Two-way ANOVA revealed a significant main effect of treatment and ovariectomy × treatment interaction [F(1, 27) = 55.96, *P* < .0001 and 16.77, *P* = .0003, respectively]. *Post hoc* Bonferroni revealed that OVX group showed the highest NOx levels than other groups (*P* < .05). ZOL treatment in ovariectomized rats decreased NOx levels in comparison with OVX group (*P* < .05) (Figure 1).

LP: Two-way ANOVA revealed a significant main effect of ovariectomy, treatment, and ovariectomy × treatment interaction [F(1, 27) = 23.94, *P* = .0000; 8.014, *P* = .0086; 7,399, *P* = .0112, respectively]. *Post hoc* Bonferroni revealed that OVX group showed higher levels of LP than SHAM group (*P* < .05), while ZOL treatment decreased these levels in rats with osteoporosis (*P* < .05) (Figure 1).

NPSH: Two-way ANOVA revealed a significant main effect of ovariectomy and ovariectomy × treatment interaction [F(1, 27) = 75.40, *P* < .0001; 6.95, *P* = .0136]. *Post hoc* test revealed that OVX groups showed lower levels of NPSH than SHAM groups (*P* < .05). When the rats with osteoporosis were treated with ZOL, NPSH levels decreased, compared to untreated rats (*P* < .05) (Figure 2).

Vit C: Two-way ANOVA revealed a significant main effect of ovariectomy [F(1, 27) = 33.50, *P* < .0001]. *Post hoc* test showed lower levels of Vit C in OVX groups when compared to the SHAM groups (*P* < .05) (Figure 2).

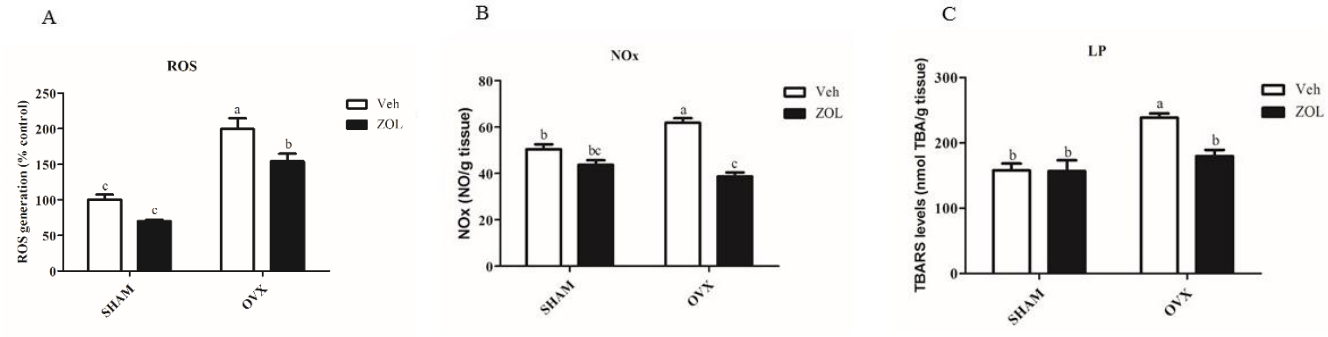

Figure 1. Influence of ZOL treatment on hepatic oxidative status in liver of ovariectomized rats: (A) RS level, (B) NOx activy and (C) LP level activity. Values are shown as mean±SE (n = 5-9). Different lowercase letters indicate significant difference among groups (*P* < .05).

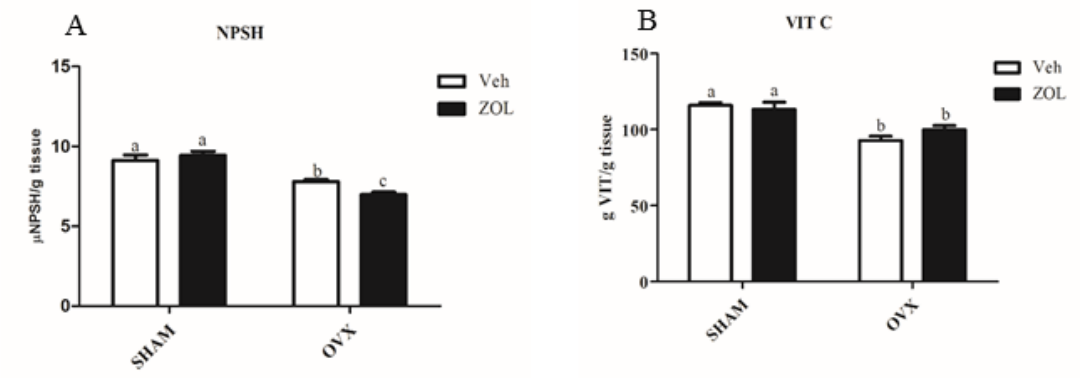

Figure 2. Influence of ZOL treatment on antioxidant defences in liver of ovariectomized rats: (A) NPSH and (B) Vit C levels. Values are shown as mean±SE (n = 5-9) Different lowercase letter indicate significant difference among groups ($P < .05$).

3.4 Multivariate Linear Regression

Non-adjusted linear regression analysis for cortical bone showed a positive significant correlation between BMC of femur and ZOL ($P < .05$), and a negative significant correlation between BMC of femur and ROS, NOx, and LP levels ($P < .05$). However, when all variables were included in the adjusted model, no statistically significant difference was observed. When the analysis was adjusted for ZOL treatment and ROS, both showed a significant correlation with BMC ($R^2 = 0.32$; F = 6.81) (Table 2).

Table 2. Linear regression of BMC femoral cortical bone and associated factors.

	Cortical Bone			
Variable	**Non-adjusted 95% CI**	***P***	**Adjusted 95% CI**	***P***
ZOL	(116.09 – 413.74)	*0.001*	(69.27 – 365.02)	*0.006*
ESTRADIOL	(-4.13 – 12.28)	*0.319*	---	---
ROS	(-3.32 – -0.55)	*0.008*	(-2.64 – -0.05)	*0.041*
NOx	(-20.04 – -6.19)	*0.001*	---	---
LP	(-4.61 – -1.10)	*0.002*	---	---
NPSH	(-96.21 – 62.08)	*0.663*	---	---
Vit C	(-2.90 – 11.15)	*0.240*	---	---

Non-adjusted linear regression analysis for cancellous bone showed a significant positive correlation between BMC of femur and ZOL, Vit C and estradiol levels ($P < .05$), as also significant negative correlation between femoral BMC and ROS, NOx and LP ($P < .05$). However, when all variables were included in the model, significant difference was observed

only for the ZOL variable. When the analysis was adjusted for NOx and estradiol, the variables ZOL and ROS remained statistically significant (R^2=0.69; F=17.14) (Table 3).

Table 3. Linear regression of BMC femoral cancellous bone and associated factors.

	Cancellous Bone			
Variable	**Non-adjusted 95% CI**	***P***	**Adjusted 95% CI**	***P***
ZOL	(339.40 – 665.69)	*0.000*	(122.82 – 618.03)	*0.005**
ESTRADIOL	(1.67 – 23.05)	*0.025*	---	---
ROS	(-5.05 – -1.41)	*0.001*	(-4.13 – -0.62)	0.010*
NOx	(-31.34 – -15.43)	*0.000*	---	---
LP	(-6.51 – -1.64)	*0.002*	---	---
NPSH	(-132.24 – 89.15)	*0.694*	---	---
VIT C	(1.61 – 19.98)	*0.023*	---	---

* Adjusted for NOx and estradiol.

3.5 Pearson's correlation

Interestingly, both LP (r^2=0.3026; *P* = .0013) and NOx (r^2=0.1708; *P* = .02) levels displayed a significant positive correlation with ROS generations in the liver of rats (Figure 3).

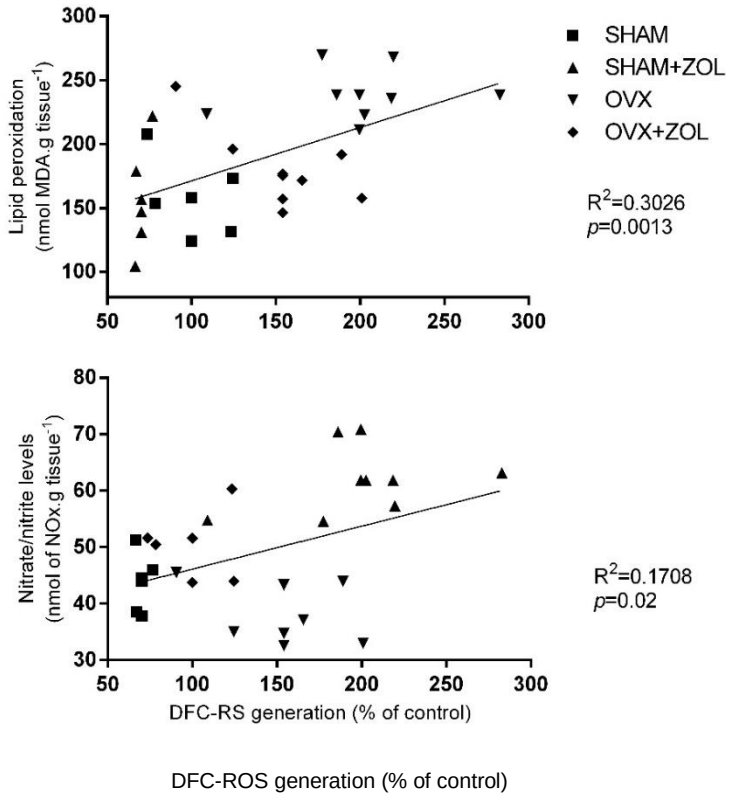

Figure 3. Linear regression between ROS generation and LP levels (A) and NOx levels (B) in the liver of OVX/SHAM surgery rats, treated or not with ZOL. Linear regression was evidenced by Pearson's correlation coefficients (n = 6).

4. DISCUSSION

Postmenopausal osteoporosis is an osteometabolic disease that occurs mainly by estrogen deficiency. However, free radicals can also play an important role on bone metabolism, thus contributing to osteoporosis (21). Excessive generation of free radicals, that exceeds the endogenous antioxidant capacity, results in OS that could damage all cellular components and compromise their viability and function (6). The literature has shown that previous reports about the association between BMC and OS are inconclusive. In the present study, it was observed a negative association between OS markers (ROS, NOx, and LP) and BMC, which is in agreement with the previous literature, which showed a positive correlation between OS and osteoporotic status (22, 23). Some evidence suggests that OS is an important pathogenic mechanism related to physiological organismal aging that contributes to progressive loss of bone strength and mass (24-28). Clinical studies showed that OS has been positively correlated with bone loss in osteoporosis patients (3, 5).

Our results from the adjusted analysis showed that ZOL treatment has a significant association with BMC, both for the cortical and cancellous bone. The positive relationship between ZOL and BMC evidenced by the present study is already consolidated in literature. This drug increases bone mineral density, decreases the risk of fracture and it is considered efficient and safe for treatment of osteoporosis (29-32). In a clinical study where postmenopausal women underwent a treatment with annual dose of ZOL, the risk of fracture was significantly reduced (33). Besides, greater bone mineral density was observed in the hip, femur and lumbar vertebrae of those patients (34).

ROS, which is an important marker of bone resorption, also showed a significant association with BMC in the adjusted regression, both for the cortical and cancellous bone. The increase of ROS levels was negatively correlated with less BMC, which is in agreement with other studies (24, 28, 35, 36, 37). Hydrogen peroxide is a ROS, whose generation has been involved in the bone loss pathogenesis by stimulating differentiation and function of osteoclasts (38, 39). Thus, findings of our current study reiterate the notion that an estrogen deficiency activates ROS generation that is pivotal to bone loss, since it stimulates RANKL expression (3).

Interestingly, our study also showed a positive correlation between ROS generation and LP development and increased NOx levels, reinforcing the coordinated action of

ovariectomy-induced ROS generation in a causal relationship with the oxidative damages observed in the OVX group. In fact, the increased ROS production may have led to development of LP in ovariectomized rats, as also seen in the study of Muthusami et al. (2005) (40).

The OVX group presented higher oxidative damages, which were represented by increased levels of ROS, LP, NOx levels, and lower BMC. These findings are in agreement with previous studies, which showed that the increased oxidant status is inversely related to BMC, thus accelerating the effects of osteoporosis (24, 25, 28, 35, 36; 37, 40, 41).

Considering the antioxidant defense system, the reduced activity of antioxidants may also be related to bone loss. Vit C is an antioxidant that promotes collagen genesis (42, 43), stimulating differentiacion of osteoblasts and formation of bone (44). In fact, the reduction of this antioxidant is associated with estrogen deficiency and decreased capability to scavenge free radicals (45, 46). In our study, ovariectomized rats showed lower levels of Vit C in comparison with healthy animals, which may also have contributed to the lower BMC observed in OVX group. Clinical studies have demonstrated that antioxidant defenses are markedly decreased in osteoporotic women (25, 36, 47), while the oxidant activity is significantly increased (36, 47). The reduction of antioxidant capacity associated to increased ROS generation, LP and NOx levels, as observed in our study, clearly suggest that the OS is a factor that contribute to bone loss in estrogen deficienty rats aggravating the osteoporotic status.

In the current study, it was observed that the ZOL administration in ovariectomized rats increased BMC, reducing both prooxidants and antioxidant molecule NPSH. These findings suggest that the antioxidant defense may have been consumed in order to reduce the activity of prooxidant molecules and consequently, minimizing bone loss (48). ZOL is an intravenous bisphosphonate approved worldwide for treating osteoporosis and cancerous diseases (49, 50). Its high affinity to bone and long duration of action, allow the once-yearly administration for treatment of osteoporosis (51). Our outcomes are showing a drecreased oxidative damage in liver of ovariectomized and ZOL treated animals, indicating the antioxidant properties of this compound. This finding is in agreement with the literature, which showed that bisphosphonates reduces LP and NOx levels (36), which radical scavenging property was also observed *in vitro* (10). It was detected that malondialdehyde levels, an end product of LP, returned to normal by administration of risedronate, other bisphosphonate compound, in ovariectomized rats (36). In addition, Koçer et al. (2014) (49) observed that administration of bisphosphonates for 6 weeks was able to reduce OS induced

by cancer. Inversely, other studies have related high and frequent doses of ZOL with oxidative damages and development and impairment of antioxidant defense system (52, 53).

In our study, osteoporosis was induced by ovariectomy surgery. The bilateral removal of ovaries results in the suppression of estrogen levels and consequently the reduction in uterine weight (54) and influence the maintenance of bone homeostasis (55, 56). The deficiency of this ovarian hormone is the pivotal cause identified of postmenopausal bone loss (57). In the present study, OVX groups presented lower estrogen levels, uterine weight and BMC than SHAM groups, indicating the effectiveness of the experimental model used.

5. CONCLUSION

Our results support the idea that an imbalance between oxidant molecules and antioxidant defenses leads to oxidative damage and consequently bone loss associated with osteoporosis. In the other hand, ZOL seems to have an important effect on OS, suggesting that it is a protective factor for maintenance of bone homeostasis. Nevertheless, further research may be of value to better clarify the role of anti-osteoporotic drugs, especially ZOL, in the OS markers related to the regulation of bone mass.

HIGHLIGHTS

- Faced with estrogen deficiency, the cancellous bone is more susceptible to bone loss;
- OS markers play a key role in the outcome of osteoporosis;
- ZOL treatment is associated with maintenance of bone mass;
- ROS generation and LP levels presented positive correlation, as well as NOx levels.

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3 ARTIGO 2 - HISTOLOGICAL, HEMATOLOGICAL, AND OXIDATIVE STRESS FINDINGS OF ZOLEDRONIC ACID IN OSTEOPOROTIC RATS WITH APICAL PERIODONTITIS

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HISTOLOGICAL, HEMATOLOGICAL, AND OXIDATIVE STRESS FINDINGS OF ZOLEDRONIC ACID IN OSTEOPOROTIC RATS WITH APICAL PERIODONTITIS

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ABSTRACT

Introduction: The aim of this study was to evaluate the influence of zoledronic acid (ZOL) on the progression of periapical lesions in estrogen-deficient rats. **Methods:** Female Wistar rats were divided into the following groups: OVX, ovariectomy and treatment with saline; OVX + ZOL, ovariectomy and treatment with ZOL; SHAM, sham surgery and treatment with saline; SHAM + ZOL, sham surgery and treatment with ZOL. Vehicle or ZOL (100 μg/kg) were administered in a single intraperitoneal application on the day of the induction of the periapical lesion. Twenty-one days after surgery, pulps of the right mandibular first molar of all the rats were exposed to the oral environment to induce a periapical lesion. After 35 days, the area of the lesion was measured by histomorphometric analysis. The local and systemic inflammatory infiltrates were evaluated by histological and hematological analyses, respectively. The levels of reactive oxygen species (ROS) were quantified to estimate oxidative damage. Data were analyzed by Kruskal-Wallis test, two-way ANOVA and the Bonferroni post-hoc test ($P < .05$). **Results:** The size of the periapical lesion was similar among the groups ($P > .05$). The local and systemic inflammatory infiltrates were not affected by treatment with ZOL ($P > .05$). The OVX and OVX + ZOL groups showed higher ROS levels than SHAM groups ($P < .05$). Administration of ZOL decreased the ROS levels in the ovariectomized rats ($P < .05$). **Conclusions:** Therapy with ZOL does not affect the bone loss resulting from apical periodontitis or inflammatory parameters. However, ZOL reduced a marker of oxidative damage.

Keywords: Apical periodontitis. Osteoporosis. Bisphosphonate. Inflammation. Blood cell. Oxidative stress.

INTRODUCTION

Estrogen is an important hormone that modulates the bone remodeling process. Postmenopausal women have a reduced production of this hormone, which has implications for progressive alterations in the structure, quality, and density of bone. These bone alterations are characteristic of osteoporosis and can lead to bone fractures and consequently an increase in morbidity and mortality (1). Besides systemic changes, it has also been reported that osteoporosis can adversely affect certain oral conditions (2). Osteoporosis is considered a risk factor for periodontal disease (3), negatively affects the osseointegration of implants in rats (4), and is marginally associated with the of periapical lesions in postmenopausal women (5).

Apical periodontitis results from an inflammatory process generated when bacteria from the root canal system reach the periapical support tissues, inducing host defense mediators and recruitment of inflammatory cells to contain the spread of infection (6). However, clastic cells are also activated, promoting apical bone resorption (6). Because estrogen has an effect on the bone-resorption process and on the production of inflammatory cytokines (7), the deficiency of this hormone could be an aggravating factor for the development of apical periodontitis. Most cells and molecules that signal the pathogenesis of periapical lesions also participate in the bone-resorption process that occurs in osteoporosis (7, 8). Thus, because of the common points between these pathologies, osteoporosis may exert a potentiating effect on the progression of apical periodontitis (9). Studies in animal models using ovariectomized rats have been used to evaluate the effect of osteoporosis on the progression of periapical lesions (10, 11); however, the results are still inconclusive.

Zolendronic acid (ZOL) is a potent bisphosphonate used for the systemic treatment of bone diseases, due to its ability to reduce bone resorption (12). This drug acts directly and indirectly on osteoclasts and osteoblasts, suppressing bone remodeling (12). Yearly intravenous dosing with ZOL is considered a safe and effective treatment of postmenopausal osteoporosis, as it increases bone mineral density and reduces fracture rate (13). The alveolar bone can also be potentially affected by the use of this bisphosphonate (14, 15). However, little information is available for the effect of ZOL on the progression of apical periodontitis under conditions of estrogen deficiency.

Therefore, the aim of this study was to evaluate the influence of ZOL on the extent of apical periodontitis, on markers of oxidative stress, and on local and systemic inflammatory parameters in rats with estrogen deficiency.

MATERIALS AND METHODS

Animals

Female Wistar rats (aged 70 days, weighing 150 to 200 g) were obtained from the Central Animal House of the Pontifical Catholic University of Rio Grande do Sul (CeMBE; PUCRS; Brazil). The animals were housed under standard conditions of temperature (22° ± 2 °C), light (12-hour light-dark cycle), and humidity (50%–70%), in ventilated cages, with autoclaved wood chip bedding. Standard rodent chow and tap water were provided ad libitum, except during the experimental sessions. The surgical dental procedures and the sample collection were carried between 8:00 AM and 5:00 PM. The weight of animals was measured weekly to allow calculation of the anesthetic and analgesic medications.

The experimental protocols followed the current Brazilian guidelines for the care and use of animals for scientific and didactic procedures, from the National Council for the Control of Animal Experimentation (CONCEA, Brazil). The local Animal Ethics Committee evaluated and approved all the protocols (CEUA 15/00436).

Experimental Draw and Distribution of Groups

Forty female Wistar rats were randomly distributed into 4 groups (n=10): OVX, ovariectomy and treatment with vehicle; OVX + ZOL, ovariectomy and treatment with ZOL; SHAM, simulation of ovariectomy (sham surgery) and treatment with vehicle; SHAM + ZOL, sham surgery and treatment with ZOL. The induction of tooth pulp exposure of the right mandibular first molar was performed in all animals.

Ovariectomy

The animals were anesthetized by intraperitoneal (IP) injection of xylazine (10 mg/kg) and ketamine (100 mg/kg). Bilateral ovariectomy was performed in 20 animals (16). The other rats underwent sham surgery. To control discomfort, paracetamol (80 mg/kg) was administered by gavage for 48 hours after surgery (CeMBE/ PUCRS protocol).

Induction of Periapical Lesion

Twenty-one days after ovariectomy and sham surgery (17), all animals were anesthetized as previously described. An endodontic access of the right mandibular first molar was performed to induce a periapical lesion. Dental pulps were exposed on the central portion of the oclusal surface with the aid of 1011 HL round bur at high speed (KG Sorensen, Cotia, São Paulo, Brazil) to a depth nearly equal to the half diameter of the bur (1/2 mm) (18). A

#10 endodontic file (Dentsply-Maillefer, Ballaigues, Switzerland) was used to remove remnants of pulp tissue and the pulpal chamber was left exposed to the oral environment.

Zoledronic Acid

The dose of ZOL (Zometa, Novartis, Basel, Switzerland) was administered IP in a single application (100 µg/kg) (19) on the same day as the induction of the periapical lesion. As a control, an IP dose of saline was administered to the animals that did not receive the drug (19). The period between the administration of ZOL and euthanasia was 35 days.

Sample Collection

Euthanasia was performed by deep anesthesia with sevoflurane (Sevorane, Abbott Laboratories Ltda Brazil, São Paulo). The uterus of the rats was removed and weighed. Blood samples were obtained for the analysis of estradiol and levels of systemic inflammation. Liver samples were collected for the evaluation of oxidative stress indicators. The mandible and femur were removed, dissected, and placed in formalin solution (10%) for tissue fixation. The mandible was used for histological analysis and multislice computed tomography (CT). The femur was evaluated by CT.

Uterus Weight and Estradiol Level

Uterus weight was determined using a precision scale (Mettler Toledo, Barueri, São Paulo, Brazil). Blood samples were collected by cardiac puncture vacutainer device (BD Vacutainer® Blood Collection Eclipse ™ Needle) and centrifuged for 12 min to obtain the serum. The estradiol level was verified by chemiluminescence (Estradiol IMMULITE, Diagnostic Products Corp., Los Angeles, California). All samples were assayed by a technician blinded to the comparison groups, in duplicate and in the same assay to avoid interassay error. The minimum detectable dose of estradiol was 11.0 pg/mL.

Bone Mineral Content Analysis by Multislice Computed Tomography

The femur and left hemimandible were submitted for multislice computed tomography (CT) imaging (BrightSpeed, GE Healthcare, Little Chalfont, United Kingdom) to determine bone mineral content (BMC). The images were analyzed by a trained radiologist, blinded to the comparison groups, with the auxiliary of the OsiriX software (Prixmeo SARL, Geneva, Switzerland). The measurement of BMC in Hounsfield units was based on the study of Kuroda et al (20), with minor adaptations. The BMC of the mandible was measured in the

condyle and in the periapical portion next to the mesial root of the first molar. In the femur, the measurement was carried out in the condyle 2 mm from the growth plate. All measurements were calculated for the cortical and cancellous bone, except for the mandibular condyle, in which it was only possible to identify the cortical bone.

Hematological Parameters

For the determination of differential white blood cell counts, the samples were collected by cardiac puncture (21). Immediately after, a blood drop was taken for smear evaluation by May-Grunewald-Giemsa staining. Differential counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils and immature cells) were estimated under a ×40 objective (Olympus® CH30 model) by counting 100 cells.

Estimation of Liver Oxidative Damage

The levels of reactive oxygen species (ROS) were quantified using the oxidant-sensing fluorescent probe, 2,7-dichlorofluorescein diacetate (DCHF-DA) (22). The oxidation (DCHF-DA) to fluorescent dichlorofluorescein (DCF) was determined at 488 nm for excitation and 525 nm for emission. Liver samples (20 μL) were added to a medium containing Tris HCl buffer (0.01mM; pH 7.4) and DCFH-DA (10μM). After DCFH-DA addition, the medium was incubated in the dark for 1 h, and the fluorescence was then measured. DCF-RS levels were corrected for the protein content (23) and expressed as a percentage of the control.

Local Inflammatory Infiltrate and Histomorphometric Analyses

The right hemimandibles were decalcified in EDTA 17 % (pH 7.0) for 60 days, and the EDTA solution was changed every week. The samples were embedded in paraffin blocks and were sectioned at a thickness of 4 μm in the mesiodistal plane. Two slices of each tooth that showed both the apical foramen and the periapical tissues were selected and stained with hematoxylin and eosin (HE). The analyses were performed by light microscope (Binocular Optical Microscope ZEISS, Axio Lab.A1, Jena, Germany), and the images were transferred to a monitor with a camera apparatus (AxioCam, ERc 5S, Jena, Germany). The intensity of the inflammatory infiltrate in the periapical region of the mesial root of the first molar was evaluated using ×200 magnification and classified as: 1) absent; 2) mild; 3) moderate; or 4) intense infiltrate (18). The histomorphometric analysis of the periapical lesion associated with the mesial root of the first molar was measured in square millimeters (mm^2) using ×50

magnification by ZEN 2 software (ZEISS, Jena, Germany). The area of periapical lesion was calculated by the lesion boundary, considering the outer external cementum surface, the periodontal ligament, and the external alveolar bone surface. The analyses were assessed by a pathologist calibrated and blinded to the experimental groups. Intra-examiner agreement for inflammatory infiltrate analysis was assessed by a weighted kappa (K_w =0.92) and for histomorphometric analysis by intraclass correlation coefficient (ICC=0.83).

Statistical Analysis

The parametric data (uterus weight, estradiol level, BMC, histomorphometric size of the periapical lesion and hematologic values) were evaluated by two-way ANOVA followed by Bonferroni post-hoc test and are expressed as mean ± standard error of the mean. The non-parametric results (local inflammatory infiltrate data) were evaluated by Kruskal-Wallis test followed by Bonferroni post-hoc test. *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed using software package Statistica 11.0 for Windows.

RESULTS

Serum Estradiol Levels and Uterus Weight

For estradiol levels, two-way ANOVA revealed a significant main effect of ovariectomy, treatment, and ovariectomy × treatment interaction [F(1, 27) = 136.71, *P* = .000; 77.45, *P* = .000; and 55.46, *P* = .000, respectively]. The post-hoc test revealed that the OVX and OVX + ZOL groups had significantly lower levels of estradiol than the SHAM and SHAM + ZOL groups (*P* < .05) (Figure 1A).

With regard to uterus weight, two-way ANOVA also revealed a significant main effect of ovariectomy, treatment, and ovariectomy × treatment interaction [F(1, 27) = 195.47, *P* = .000; 10.02, *P* = .003; and 9.88, *P* = .004, respectively]. Bonferroni's test showed that the uteri of the OVX and OVX + ZOL groups weighed less than those of the SHAM and SHAM + ZOL groups (*P* < .05) (Figure 1B).

In addition, post-hoc comparisons revealed that uterus weight (*P* < .05) and estradiol levels (*P* < .05) were affected by ZOL treatment in the SHAM groups (Figure 1A and 1B).

Bone Mineral Content Analysis

The results of BMC analysis of the femur and mandible are described in Table 1.

For BMC of the cortical bone of the femur, two-way ANOVA revealed a significant main effect of treatment and ovariectomy × treatment interaction [F(1, 27) = 13.17, *P* = .001 and 8.74, *P* = .006, respectively]. The post-hoc test revealed that the OVX group showed a significantly lower BMC than the other groups in the condyle of the femur (2 mm) (*P* < .05).

For BMC of cancellous bone of the femur, two-way ANOVA revealed a significant main effect of ovariectomy, treatment, and ovariectomy × treatment interaction [F(1, 27) = 7.4, *P* = .011; 53.27, *P* = .000; and 9.31, *P* = .005, respectively]. The post-hoc test revealed that the OVX group showed a significantly lower BMC than the other groups in the condyle of the femur (2 mm) (*P* < .05). The treatment with ZOL increased the BMC of the cancellous bone of the femur in the groups SHAM + ZOL (*P* = .05) and OVX + ZOL (*P* < .05) groups.

For BMC of the mandible, two-way ANOVA revealed no significant main effect of ovariectomy, treatment, and ovariectomy × treatment interaction [F(1, 22) = 1.14, *P* = .295; 0.2, *P* = .655 and 1.73, *P* = .200, respectively]. The post-hoc test revealed no statistical difference among the groups for BMC of the mandible (*P* > .05).

Local Inflammatory Infiltrate and Histomorphometric Analysis

For local inflammatory infiltrate, there were no statistically significant differences among the experimental groups (*P* > .05). In all groups, there was a predominance of moderate local inflammatory infiltrate.

For periapical lesion size, two-way ANOVA revealed no significant main effect of ovariectomy, treatment, and ovariectomy × treatment interaction [F(1, 25) = 0.15, *P* = .69; 1.38, *P* = .25 and 0.11, *P* = .73, respectively]. The post-hoc test revealed no statistical difference among the groups for periapical lesion size (*P* > .05). The numerical values (mean ± SE) of the apical periodontitis areas (mm^2) were: SHAM: .061 ± .008; SHAM + ZOL: .052 ± .007; OVX: .057 ± .004; OVX + ZOL: .051 ± .004. Figure 2 shows representative images of local inflammatory infiltrate and areas of the apical periodontitis lesions associated with the apices of the mesial roots of the first molars.

Hematological Analysis

After 56 days of estrogen deficiency, there was no systemic inflammatory response in the rats, that is osteoporosis did not affect hematological parameters and ZOL administration did not alter these parameters (Figure 3).

Reactive Oxygen Species

Two-way ANOVA revealed a significant main effect of ovariectomy and treatment [F(1, 27) = 68.05, *P* = .000; 11.42, *P* = .002].The post-hoc test revealed that the OVX group showed higher levels of ROS than the SHAM groups (*P* < .05) and the OVX + ZOL group showed statistically lower ROS levels than the OVX group (*P* < .05) (Figure 4).

DISCUSSION

Recent evidence has emphasized the probable relationship between systemic pathological conditions and their treatments, with inflammatory disorders in the oral environment (2, 24). The anti-resorptive agent ZOL is approved worldwide for the treatment of osteoporosis (12). This drug has high affinity to the hydroxyapatite of mineralized tissues, accumulating rapidly at sites with high bone turnover and thereby reducing bone resorption (25). Because of this characteristic, the aim of this study was to evaluate the influence of a single dose of ZOL in the size of periapical lesions, in the formation of ROS and in the local and systemic inflammatory parameters in osteoporotic and healthy rats.

The histomorphometric analysis showed that there was no statistically significant difference for the size of the periapical lesion among groups. To our knowledge, these results disagree with the single study in the literature that evaluated the effect of ZOL on the development of periapical lesions of ovariectomized rats. A possible explanation is due to methodological differences in drug concentration and application. Wayama et al (14) used high doses of ZOL (0.04 mg/kg body weight, once a week for 4 weeks) and observed that the size of the periapical lesion in OVX rats treated with bisphosphonate was lower than those treated with the saline. In our study, a single dose of ZOL was used, which corresponds to the use protocol for the treatment of osteoporosis in humans, and we observed that the size of the periapical lesions was not affected.

In higher concentrations, ZOL is prescribed to prevent or delay bone metastasis in some types of cancer (26). However, because this drug blocks remodeling, thereby influencing bone formation, there is a great concern regarding the development of osteonecrosis of the mandibular bone when ZOL is administered mainly in oncological doses (15). Despite the fact that this drug, in high and frequent doses, exacerbates the inflammatory response and induces bone lesions which resemble osteonecrosis (27, 15), the present study observed that when administered for the treatment of osteoporosis, in a single and low dose, ZOL does not appear to have a deleterious effect on the mandibular bone. This is in accordance with a systematic review that showed an extremely low incidence of osteonecrosis

in patients with osteoporosis who were treated with ZOL once a year (15). Of the 5 studies included in the review, only 1 reported the occurrence of a single case of osteonecrosis in osteoporotic patients who were given ZOL; moreover, the patient had additional risk factors for the development of osteonecrosis (28).

Interestingly, the results of our study also showed that ZOL administration in SHAM animals produced a significant increase in estrogen levels and in the weight of the uterus compared with the other groups. These results suggest a hypothesis that estrogen has an important protective role in the bone resorption induced by endodontic pathogens. Indeed, studies have demonstrated that this hormone regulates skeletal growth and maintains the adult skeleton (1). However, further research is needed to understand if ZOL really influences estradiol levels and uterine weight and how this mechanism occurs.

Histological analysis of the periapical lesion showed that the local inflammatory process was moderate in all groups, with the presence of irregular periodontal fibers and inflammatory cells distributed sparingly and not restricted to the apex. According to these results, we suggest that periapical inflammation is triggered by infection of the root canal system, and that neither osteoporosis nor its systemic treatment alter the chronic inflammatory parameters of apical periodontitis. The results of the present study are in disagreement with the study by Wayama et al (14), which showed that ZOL administered in OVX rats decreased the inflammatory infiltrate when compared to the untreated OVX rats. Nevertheless, only a small amount of information, some of which is controversial, has been reported in the literature concerning the effect of ZOL on the local inflammatory infiltrate of periapical lesions.

To understand the leukocyte profile of osteoporotic rats treated with ZOL, hematological examinations were performed. After 35 days of periapical lesion induction, no systemic inflammatory profile was observed among the experimental groups. Our results showed a hematological profile within the normal parameters, that is, the presence of a large number of lymphocytes and a low neutrophil count, which characterizes the hematological pattern of healthy rats (21, 29). These results are in agreement with the study of Kuhn and Hardegg (30) that quantified the blood cells of SHAM and OVX rats and observed that in both groups the number of lymphocytes was greater than the number of neutrophils, with no significant difference between them. In a recent clinical study, it was observed that patients with osteoporosis present high levels of the neutrophil to lymphocyte ratio compared to patients in the control group; however, these results should be interpreted with caution

because the sample size was very large and the effect size was very small (N=1635, $P = .02$) (31).

Previous literature (32, 33, 34) has shown that estrogen deficiency does not lead to changes in the alveolar bone architecture of the mandible. It is known that the time required for some manifestation of change in the alveolar bone due to estrogen deficiency is much longer than in the femur (19). The consequences of osteoporosis are manifested more rapidly in the long bones and vertebrae (35). Therefore, the time between ovariectomy and euthanasia may not be sufficient to observe changes in bone mineral density in the mandible (10), and it is likely that mechanical stress derived from functional occlusion prevents bone loss from osteoporosis (36). In accordance with these statements, in the present study, we observed that the femur and the mandible presented different responses under conditions of estrogen deficiency. In the mandible, the BMC was similar among the groups, while in the femur, OVX rats had significantly less BMC than the SHAM animals. Furthermore, it is known that ZOL has the potential to improve osteoporosis by reducing fracture risk and by producing significant gains in bone mineral density (13), and this is in agreement with our results which showed that ZOL administration improved the BMC in the cortical and cancellous femoral bone of the ovariectomized rats.

The presence of local inflammation and resorption in the periapical area may lead to increased free radical production, which causes oxidative damage to cellular molecules (37). In addition, ROS play an important role in osteoclastogenesis, contributing to bone loss associated with postmenopausal osteoporosis (38). On the other hand, osteoporosis treatment with bisphosphonates has been associated with the reduction of oxidative stress markers (39). In the present study, the use of ZOL decreased ROS levels, which may have contributed to the increase in BMC in the femur, involving both the cancellous and cortical bone. However, examples in the literature are scarce and the results of research are limited for the action of ZOL on oxidative stress and its relationship with BMC.

Ovariectomized rats are used as an experimental model to simulate the systemic condition of osteoporosis (17), which is present in most postmenopausal women (35). The efficacy of ovariectomy was confirmed in the present study by the significant difference among the OVX and SHAM groups for serum estradiol levels, uterine atrophy, and BMC in the femur. Removal of the ovaries leads to atrophy of the uterus, estrogen deficiency, and promotes altered bone remodeling patterns (17).

CONCLUSION

ZOL therapy in rats with osteoporosis did not influence the size of periapical lesions and did not affect local and systemic inflammatory conditions. However, treatment with ZOL reduced a marker of oxidative damage.

SIGNIFICANCE

Postmenopausal osteoporosis is a systemic disease that may affect the alveolar bone. Zoledronic acid is an effective drug used for the treatment of osteoporosis which has demonstrated inhibitory effects on osteoclasts. Thus, it is important to carefully consider the influence of this systemic alteration and its treatment on the development of apical periodontitis.

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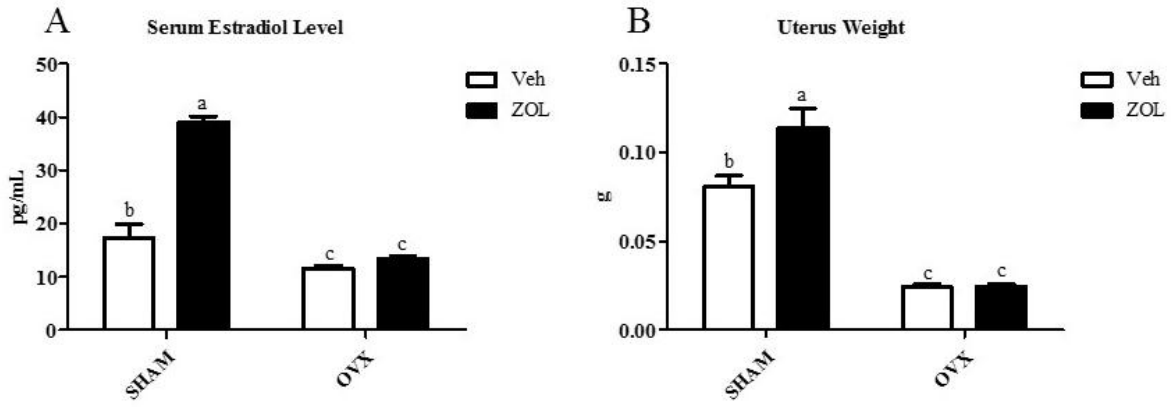

Figure 1. Serum estradiol levels (A) and uterus weight (B) according to the different groups. Each column represents the mean ± SE of 6–9 animals per group. Different letters represent significant statistical differences. $P < .05$ (two-way ANOVA plus Bonferroni's test).

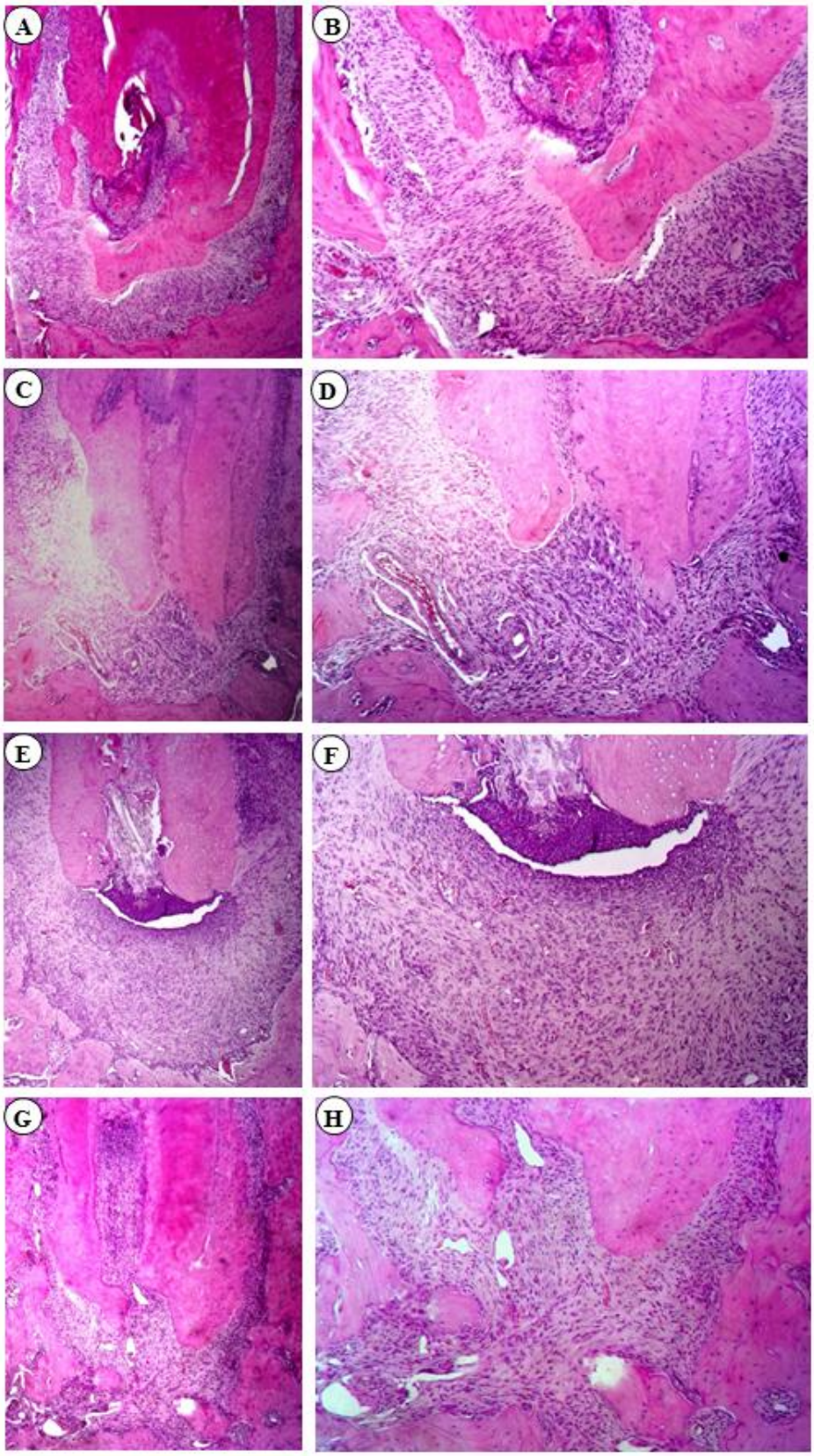

Figure 2. Histological aspects of periapical lesions associated with the apices of mesial roots of right first mandibular molar: (A and B) SHAM; (C and D) SHAM+ZOL; (E and F) OVX; (G and H) OVX+ZOL. Original magnification: periapical lesion measurement, A, C, E and G, ×50; magnitude of inflammation, B, D, F and H, ×200.

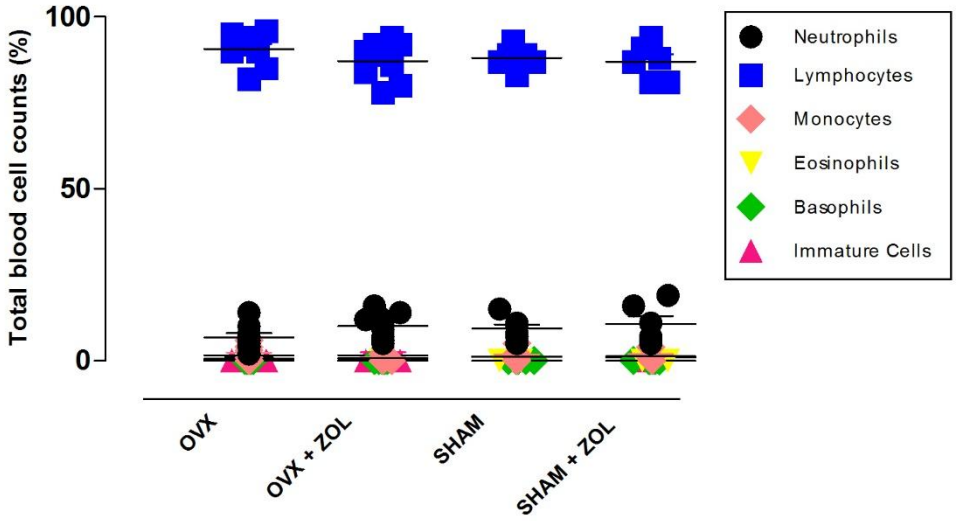

Figure 3. Effects of osteoporosis and ZOL on the white blood cell count. Each column represents the mean ± SE of 6–9 animals per group for blood cell counts, One-way ANOVA plus Bonferroni's test.

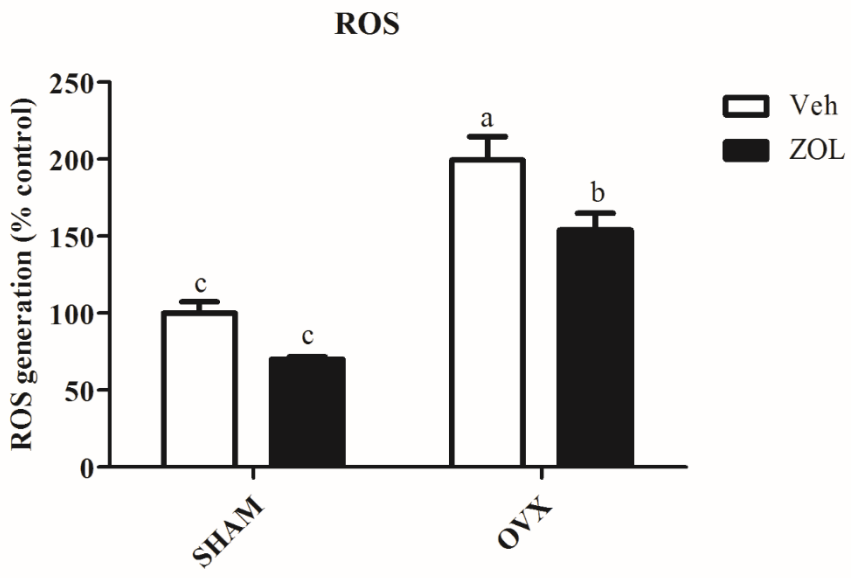

Figure 4. ROS levels in the liver. Each column represents the mean ± SE of 6–9 animals per group. Different letters represent significant statistical differences. $P < .05$ (two-way ANOVA plus Bonferroni's test).

LIST OF TABLE

Table 1. Mean ± standard error (SE) of the BMC in Hounsfield units (HU).

	BMC– Femur		BMC – Mandible		
Groups	**Cortical Bone**	**Cancellous Bone**	**Cortical Bone Periapical**	**Cancellous Bone Periapical**	**Condyle**
SHAM	1476.39 ± 63.10[a]	875.68 ± 95.02[a]	2022.00 ± 87.20	1701.41 ± 69.14	1526.40 ± 87.83
SHAM + ZOL	1520.25 ± 48.35[a]	1153.12 ± 62.55[ac]	1941.20 ± 136.76	1561.70 ± 121.95	1334.39 ± 61.10
OVX	1211.80 ± 70.61[b]	498.50 ± 52.40[b]	1999.00 ± 83.34	1535.68 ± 66.95	1404.23 ± 87.30
OVX + ZOL	1640.64 ± 60.62[a]	1174.69 ± 48.94[c]	2164.18 ± 74.76	1764.89 ± 70.08	1584.44 ± 43.50

In a single column, different superscript letters indicate a significant statistical difference. $P < .05$ (two-way ANOVA plus Bonferroni's test).

4 DISCUSSÃO

A presente Tese apresentou os resultados de dois estudos. O primeiro artigo avaliou a associação entre o BMC e os marcadores de estresse oxidativo, bem como quantificou os marcadores de dano oxidativo e as defesas antioxidantes em ratas com osteoporose e tratadas com ZOL. O principal resultado deste estudo mostrou que há uma correlação entre o BMC e os marcadores de estresse oxidativo, sugerindo que a presença de radicais livres pode contribuir para a perda óssea, influenciando a manifestação da osteoporose; e, que os antioxidantes podem prevenir esta perda.

A presença de ROS, Óxido Nítrico (NOx – do inglês, *nitric oxide*) e Peroxidação Lipídica (LP – do inglês, *lipid peroxidation*) influenciaram na redução do BMC do fêmur, tanto no osso cortical quanto no esponjoso. Por outro lado, observou-se que a Vitamina C (Vit C) influenciou no aumento do BMC do osso esponjoso, o qual é mais susceptível as manifestações do metabolismo ósseo (MORSE et al., 1994; KALU, 1991; PENG et al., 1997). A Vit C tem um papel importante como defesa antioxidante, protegendo o organismo dos efeitos negativos dos radicais livres (WU et al., 2004). Além disso, a Vit C é um cofator essencial na formação de colágeno estável e participa da formação óssea pela estimulação e diferenciação dos osteoblastos (DIXON; WILSON, 1992). Entretanto, observou-se que o ROS foi o único marcador de estresse oxidativo que manteve a associação com o BMC quando a análise de regressão foi ajustada para outras variáveis. De fato, o ROS é um dos principais marcadores de dano oxidativo; esta molécula influencia a diferenciação e função dos osteoclastos (BAX et al., 1992; STEINBECK et al., 1994), pela estimulação da expressão do RANKL (MANOLAGAS, 2010), aumentando a perda óssea.

Os resultados deste estudo também mostraram que as ratas ovariectomizadas apresentaram níveis reduzidos de Vit C e níveis aumentados de ROS, LP e NOx, o que pode justificar o baixo BMC nesses animais. Estes achados estão de acordo com a literatura, que vem observando uma correlação positiva entre os marcadores de estresse oxidativo e a perda óssea pós-menopausa, pelo aumento da reabsorção óssea (CERVELLATI et al., 2013). Foi reportado a presença de altos níveis de moléculas representativas de dano oxidativo e baixos níveis de antioxidantes em mulheres na pós-menopausa (BADR et al., 2008; GOY et al., 2015). Portanto, o estresse oxidativo parece ter um importante papel no desenvolvimento da osteoporose pós-menopausa e, por este motivo a sua relação com a patogênese da osteoporose vem sendo considerada e investigada (SENDUR et al., 2009).

O segundo artigo avaliou a influência da administração sistêmica do ZOL em ratas com osteoporose no desenvolvimento de lesões periapicais decorrentes de infecção microbiana do sistema de canais radiculares. Além disso, foram avaliados: parâmetros inflamatórios sistêmicos a partir da contagem das células brancas do sangue; intensidade do infiltrado inflamatório nos tecidos periapicais e formação de ROS. A partir dos resultados deste estudo pode-se concluir que a osteoporose e o tratamento com ZOL influenciaram a ocorrência de dano oxidativo, uma vez que o grupo com ratas ovariectomizadas apresentou maior quantidade de ROS e a administração do ZOL nesses animais reduziu a produção deste radical livre. Além disso, observou-se nos animais ovariectomizados, que o BMC do fêmur diminuiu, tanto no osso cortical quanto no esponjoso e, que o tratamento com ZOL aumentou o conteúdo mineral no osso esponjoso. Entretanto, estas alterações não foram suficientes para gerar mudanças no BMC da mandíbula e, consequentemente, no tamanho da lesão periapical, bem como no infiltrado inflamatório sistêmico e local.

A relação entre doenças sistêmicas e progressão de patologias periapicais tem sido assunto de interesse na comunidade científica (WOLLE et al., 2012; WOLLE et al., 2013; CASTELLANOS-COSANO et al., 2013; MARTINS et al., 2016; SEGURA-EGEA et al., 2016). Neste sentido, a influência da osteoporose e do seu tratamento com bisfosfonatos no desenvolvimento da periodontite apical tem sido investigada (XIONG et al., 2007; LIU et al., 2010; ZHANG et al., 2011; WAYAMA et al., 2015; BRASIL et al., 2017; QIAN et al., 2016).

Alguns autores sugerem que a redução nos níveis de estrogênio, causa principal da osteoporose, induz ao desenvolvimento de lesões periapicais maiores (XIONG et al., 2007; BRASIL et al., 2016; WAYAMA et al., 2015). Estes resultados divergem dos resultados do presente estudo. Entretanto, a linhagem dos animais utilizados, o tempo entre a ovariectomia e a eutanásia e a dose da medicação devem ser levadas em consideração. Animais *Sprague-Dawley* apresentam um metabolismo mais acelerado, e consequentemente as manifestações ósseas ocorrem mais rápido nesta linhagem de ratas (FANG et al., 2015). Além disso, sabe-se que o tempo necessário para as manifestações da osteoporose serem evidentes no osso alveolar é maior do que no fêmur e nas vértebras (KURODA et al., 2003; Management of osteoporosis in postmenopausal women, 2010), provavelmente porque os ossos maxilares estão em constante função mastigatória, o que retarda as perdas ósseas (INMAN et al., 1999). Assim, pode-se sugerir que a mandíbula precisa de um tempo maior de deficiência de estrogênio quando comparada ao fêmur para desenvolver alterações no padrão ósseo. Essa suposição vai ao encontro com do estudo de BRASIL et al. (2017) que observou que longos períodos de deficiência de estrogênio podem influenciar na progressão da periodontite apical.

Com relação a dose de administração, os resultados deste estudo podem ser comparados apenas com um estudo que também utilizou ZOL. Wayama et al. (2015) observaram lesões periapicais menores após a utilização deste bisfosfonato; entretanto, os diferentes protocolos de administração do ZOL entre os estudos podem explicar a divergência nos resultados.

O estresse oxidativo vem sendo relacionado com a perda óssea da osteoporose (BAEK, 2010; MANOLAGAS, 2010). A geração de radicais livres, especialmente de ROS, influenciam na patogênese da perda óssea, pois ativam o RANKL que participa da transformação dos pré osteoclastos em osteoclastos maduros, promovendo a reabsorção do tecido ósseo (MANOLAGAS, 2010). Assim como o hormônio estrogênio (LEAN et al., 2003), acredita-se que o ZOL também apresenta propriedades antioxidantes, reduzindo, assim, a produção de radicais livres e consequentemente a osteoclastogênese (DOMBRECHT et al., 2006). O ZOL é um bisfosfonato com ação comprovada na remodelação óssea, pois aumenta a densidade mineral óssea, reduzindo a suceptibilidade a fraturas (MARICIC, 2010; SHIKARI et al., 2016). Este medicamento apresenta ação local e prolongada (EUROPEAN MEDICINES AGENCY ACLASTA, 2015), uma vez que fixa-se à hidroxiapatita (NANCOLLAS, 2006). Deste modo, para tratamento da osteoporose, é administrado uma vez ao ano, proporcionando boa adesão dos pacientes ao tratamento (SHIKARI, 2012).

Os bisfosfonatos são medicamentos utilizados para o tratamento de desordens associadas com reabsorção do tecido ósseo, tais como, osteoporose (SHIRAKI et al., 2016), doença de Paget (BAYKAN et al., 2014), neoplasias e metástases ósseas (AHN et al., 2014). O tipo de patologia determina a dose de administração, sendo que quanto maior a dose, maiores são os efeitos adversos (KHAN et al., 2015). O principal efeito adverso relacionado ao uso dos bisfosfonatos é a osteonecrose dos maxilares (GRBIC et al., 2008; KHAN et al., 2015). Esta droga influencia a remodelação óssea, provocando alterações tanto na função dos osteoclastos quanto dos osteoblastos (HUGHES et al., 1995; HUANG et al., 2015) e por este motivo pode levar a complicações inflamatórias e infecciosas sérias no tecido ósseo. Entretanto, quando utilizado para o tratamento de osteoporose, raramente é observada a ocorrência deste efeito adverso (SÁNCHEZ; BLANCO, 2017). É provável que a menor dose e frequência de administração comparado ao protocolo para o tratamento de neoplasias não provoque as alterações necessárias para a ocorrência da complicação (DHILLON, 2016). A baixa evidência relacionada à osteonecrose mandibular com o uso de ZOL (SÁNCHEZ; BLANCO, 2017) pode ser explicada, em parte, pelos resultados do presente estudo, que observaram semelhança entre os grupos quanto ao infiltrado inflamatório local.

A remoção bilateral dos ovários é um modelo experimental eficaz para indução da osteoporose em ratas (TANAKA et al., 1999; PARK et al., 2010). Esta técnica, causa deficiência na produção de estrogênio e, consequentemente, leva à perda da massa óssea, semelhante ao padrão das alterações que ocorrem nas mulheres na menopausa (BACH; MILLER, 1994). O rato tem sido comumente utilizado para estudar os mecanismos reguladores do metabolismo do cálcio em processos patológicos como a osteoporose, pois constitui um bom modelo de estudo para verificar a incidência de perda de massa óssea (HODGKINSON, 1979). No presente estudo, o sucesso da ovariectomia foi confirmado pelos resultados obtidos para o peso do útero, para o nível de estradiol e para o BMC. Os animais ovariectomizados apresentaram menor peso do útero, devido atrofia deste órgão pela redução da produção de estrogênio, menores níveis de estradiol pela remoção do principal órgão produtor deste hormônio e redução do BMC pelo desequilíbrio na remodelação óssea.

Apesar dos estudos em modelos animais colaborarem para o entendimento da influência de doenças sistêmicas na perda óssea de origem endodôntica, os achados são preliminares, controversos e necessitam ser complementados por evidências clínicas. A literatura científica apresenta até o momento apenas um estudo clínico, o qual avaliou a relação entre radiolucidez periapical e densidade mineral óssea em mulheres pós-menopausa. O estudo observou uma correlação marginal entre a presença de lesão periapical e a baixa densidade mineral óssea ($P = 0,05$) (LOPEZ-LOPEZ et al., 2015). Assim, novas investigações devem ser conduzidas para clarear o real efeito da osteoporose e dos medicamentos usados para tratá-la no desenvolvimento e regressão de patologias periapicais de origem endodôntica.

5 CONCLUSÃO

A partir dos estudos desta Tese, podemos conclui que:

- Marcadores de estresse oxidativo estão associados com osteoporose;
- O tratamento da osteoporose com ZOL auxilia na manutenção da massa óssea;
- Um período curto de tempo de deficiência de estrogênio (56 dias) e a administração de ZOL não influenciaram o tamanho das lesões periapicais de origem endodôntica;
- Cinquenta e seis dias de deficiência de estrogênio e a administração de ZOL não provocaram resposta inflamatória sistêmica, ou seja, a osteoporose e seu tratamento não alteraram os parâmetros hematológicos;
- A infecção microbiana do canal radicular promoveu uma resposta inflamatória local no periápice radicular e a osteoporose e a administração de ZOL não alteram o grau do infiltrado inflamatório;
- A geração de ROS aumentou na presença da osteoporose e o tratamento desta doença sistêmica com ZOL diminui este marcador de dano oxidativo;
- O tratamento da osteoporose com ZOL afeta positivamente o conteúdo mineral do osso esponjoso;
- A ovariectomia provoca alterações no peso do útero, nos níveis de estradiol e no BMC.

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[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13.03.03).

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1. General Points on Composition

a. Authors are strongly encouraged to analyze their final draft with both software (*e.g.*, spelling and grammar programs) and colleagues who have expertise in English grammar. References listed at the end of this section provide a more extensive review of rules of English grammar and guidelines for writing a scientific article. Always remember that clarity is the most important feature of scientific writing. Scientific articles must be clear and precise in their content and concise in their delivery since their purpose is to inform the reader. The Editor reserves the right to edit all manuscripts or to reject those manuscripts that lack clarity or precision, or have unacceptable grammar or syntax. The following list represents common errors in manuscripts submitted to the *JOE*:

b. The paragraph is the ideal unit of organization. Paragraphs typically start with an introductory sentence that is followed by sentences that describe additional detail or examples. The last sentence of the paragraph provides conclusions and forms a transition to the next paragraph. Common problems include one-sentence paragraphs, sentences that do not develop the theme of the paragraph (see also section “c” below), or sentences with little to no transition within a paragraph.

c. Keep to the point. The subject of the sentence should support the subject of the paragraph. For example, the introduction of authors’ names in a sentence changes the subject and lengthens the text. In a paragraph on sodium hypochlorite, the sentence, “In 1983, Langeland et al., reported that sodium hypochlorite acts as a lubricating factor during instrumentation and helps to flush debris from the root canals” can be edited to: “Sodium hypochlorite acts as a lubricant during instrumentation and as a vehicle for flushing the generated debris (Langeland et al., 1983)." In this example, the paragraph’s subject is sodium hypochlorite and sentences should focus on this subject.

d. Sentences are stronger when written in the active voice, *i.e.*, the subject performs the action. Passive sentences are identified by the use of passive verbs such as "was," "were," "could," etc. For example: "Dexamethasone was found in this study to be a factor that was associated with reduced inflammation," can be edited to: "Our results demonstrated that dexamethasone reduced inflammation." Sentences written in a direct and active voice are generally more powerful and shorter than sentences written in the passive voice.

e. Reduce verbiage. Short sentences are easier to understand. The inclusion of unnecessary words is often associated with the use of a passive voice, a lack of focus or run-on sentences. This is not to imply that all sentences need be short or even the same length. Indeed, variation in sentence structure and length often helps to maintain reader interest. However, make all words count. A more formal way of stating this point is that the use of subordinate clauses adds variety and information when constructing a paragraph. (This section was written deliberately with sentences of varying length to illustrate this point.)

f. Use parallel construction to express related ideas. For example, the sentence, "Formerly, endodontics was taught by hand instrumentation, while now rotary instrumentation is the common method," can be edited to "Formerly, endodontics was taught using hand instrumentation; now it is commonly taught using rotary instrumentation." The use of parallel construction in sentences simply means that similar ideas are expressed in similar ways, and this helps the reader recognize that the ideas are related.

g. Keep modifying phrases close to the word that they modify. This is a common problem in complex sentences that may confuse the reader. For example, the statement, "Accordingly, when conclusions are drawn from the results of this study, caution must be used," can be edited to "Caution must be used when conclusions are drawn from the results of this study."

h. To summarize these points, effective sentences are clear and precise, and often are short, simple and focused on one key point that supports the paragraph's theme.

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a. 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.

b. 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.

c. 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.

d. 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.

e. 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).

f. 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.

g. 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
1	5	100
3	5	100

h. 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).

i. 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

j. 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

k. 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.

3. Manuscripts Category Classifications and Requirements

Manuscripts submitted to the *JOE* must fall into one of the following categories. The abstracts for all these categories would have a maximum word count of 250 words:

A. CONSORT Randomized Clinical Trial-Manuscripts in this category must strictly adhere to the Consolidated Standards of Reporting Trials-CONSORT- minimum guidelines for the publication of randomized clinical trials. These guidelines can be found at *www.consort-statement.org/*. These manuscripts have a limit of 3,500 words, [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.

B. Review Article-Manuscripts in this category are either narrative articles, or systematic reviews/meta-analyses. Case report/Clinical Technique articles even when followed by extensive review of the literature will should be categorized as "Case Report/Clinical Technique". These manuscripts have a limit of 3,500 words, [including abstract, introduction, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.

C. Clinical Research (*e.g.*, prospective or retrospective studies on patients or patient records, or research on biopsies, excluding the use of human teeth for technique studies). These manuscripts have a limit of 3,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.

D. Basic Research Biology (animal or culture studies on biological research on physiology, development, stem cell differentiation, inflammation or pathology). Manuscripts that have a primary focus on biology should be submitted in this category while manuscripts that have a primary focus on materials should be submitted in the Basic Research Technology category. For example, a study on cytotoxicity of a material should be submitted in the Basic Research Technology category, even if it was performed in animals with histological analyses. These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or 4 tables*.

E. Basic Research Technology (Manuscripts submitted in this category focus primarily on research related to techniques and materials used, or with potential clinical use, in endodontics). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 3 figures and tables *.

F. Case Report/Clinical Technique (*e.g.*, report of an unusual clinical case or the use of cutting-edge technology in a clinical case). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and

acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or tables*.

* Figures, if submitted as multipanel figures must not exceed 1 page length. Manuscripts submitted with more than the allowed number of figures or tables will require approval of the *JOE* Editor or associate editors. If you are not sure whether your manuscript falls within one of the categories above, or would like to request preapproval for submission of additional figures please contact the Editor by email at *jendodontics@uthscsa.edu*.

Importantly, adhering to the general writing methods described in these guidelines (and in the resources listed below) will help to reduce the size of the manuscript while maintaining its focus and significance. Authors are encouraged to focus on only the essential aspects of the study and to avoid inclusion of extraneous text and figures. The Editor may reject manuscripts that exceed these limitations.

Available Resources:

Strunk W, White EB. The Elements of Style. Allyn & Bacon, 4th ed, 2000, ISBN 020530902X.

Day R. How to Write and Publish a Scientific Paper. Oryx Press, 5th ed. 1998. ISBN 1-57356-164-9.

Woods G. English Grammar for Dummies. Hungry Minds:NY, 2001 (an entertaining review of grammar).

Alley M. The Craft of Scientific Writing. Springer, 3rd edition 1996 SBN 0-387-94766-3.

Alley M. The Craft of Editing. Springer, 2000 SBN 0-387-98964-1.

ANEXO C – CARTA DE APROVAÇÃO DO PROTOCOLO DE PESQUISA NA COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL (CEUA - PUCRS).

PUCRS CEUA Comissão de Ética no Uso de Animais

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 31/2015 - CEUA

Porto Alegre, 28 de maio de 2015.

Prezado Sr(a). Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 15/00436, intitulado **"Efeito da medicação intracanal à base de hidróxido de cálcio e da reposição com estrogênio sobre lesões periapicais em ratas com osteoporose induzida".**

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Nº de Animais	Espécie	Duração do Projeto
80 animais	Rattus novergicus	02/2015 – 12/2016

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilma. Sra.
Profa. Dra. Maria Martha Campos
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