Marcelo Rocha Marques

EFEITO DA ADMINISTRAÇÃO INTERMITENTE DE PTH NA PERIODONTITE INDUZIDA EM RATAS COM DEFICIÊNCIA DE ESTRÓGENO

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do Título de Mestre em Biologia Buco-Dental, Área de Histologia e Embriologia.

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pode ser no coração,
pode ser numa canção,
eu só quero ter você por perto"

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"....pensar é estar doente dos olhos."

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SUMÁRIO

RESUMO

Apesar de fisiologicamente o hormônio paratireóideo (PTH) promover reabsorção óssea, estudos demonstram a sua capacidade em promover anabolismo ósseo quando administrado intermitentemente. O tratamento de doenças que envolvem perda óssea em decorrência da deficiência de estrógeno, como osteopenia e osteoporose, tem se beneficiado desta capacidade do PTH. Em ratos, a administração intermitente de PTH é capaz de inibir perda óssea ocasionada por periodontite induzida. Este estudo objetiva analisar a influência do PTH na perda óssea ocasionada por periodontite induzida em ratas com deficiência de estrógeno. Trinta ratas Wistar, com 4 semanas foram divididas em 3 grupos: A,B e C. As ratas dos grupos B e C foram ovariectomizadas e as do A receberam simulação da cirurgia. Após sete dias todos os animais tiveram o primeiro molar inferior esquerdo ligado por fio de algodão, posicionado intrasulcularmente, para indução de periodontite. O grupo C recebeu doses de 40μg/kg de hPTH (1-34), 3 vezes por semana, durante 1 mês. No mesmo período, os grupos A e B receberam 40μg/Kg da solução veículo do PTH. As mandíbulas das ratas foram radiografadas e preparadas para cortes histológicos, que foram examinados através de um programa analisador de imagens para medida da área de perda óssea alveolar na região de furca. As radiografias foram analisadas por um fotodensitômetro. As tíbias foram removidas e analisadas com auxílio de microscopia eletrônica de varredura. Os resultados de perda óssea ocasionada pela periodontite induzida foram (mm^2): Grupo A: 0,40b; Grupo B: 0,48b; Grupo C: 0,21a (onde as letras em minúsculo - a e b - diferentes indicam diferença estatística significante ($p<0,05$)). Foi evidenciada menor densidade radiográfica nas mandíbulas de animais ovariectomizados não tratados com PTH ($p<0,05$). Em uma análise morfológica, observou-se maior porosidade e menor massa óssea na região medular das epífises das tíbias dos animais ovariectomizados não tratados com PTH, em comparação com os outros dois grupos. O PTH foi capaz de diminuir a perda óssea alveolar em animais ovariectomizados que sofreram indução de periodontite. Além disso, o hormônio paratireóideo diminuiu também a perda óssea, ocasionada pela deficiência de estrógeno, nas mandíbulas e nas tíbias dos animais avaliados.

ABSTRACT

Parathyroid hormone (PTH) intermittent administration, a bone anabolic treatment, has been used to treat bone mass decrease in estrogen deficient or/and osteoporotic individuals. Objectives: The aim of this study was analyze the effects of PTH intermittent administration on the periodontitis progression, in ovariectomized rats. Methods: Thirty female rats were set in 3 groups. The rats of the B and C groups were ovariectomized, and the A group was sham operated. The animals received cotton ligature around the first left lower molar, the right molars were left unligated working as control. The C group received PTH doses (40µg/Kg), and the A and B groups received the same dose of the vehicle, three times week. After 30 days the animals were sacrificed, the mandibles were extracted, X-rayed and samples were prepared to obtain decalcified histological sections, scanning electron microscopy of the tibia was also performed to evaluate possible changes in bone structure caused by estrogen deficiency. Results: The samples analyzed under light microscopy, using an image analyzer software for bone loss measurement (mm2) showed: Group A: 0.40b; Group B: 0.48b; Group C: 0.21a; where different letters indicate statistical significant differences among groups $p<0.05$ (ANOVA and Tukey's tests). The photodensitometer analysis demonstrated a lower mandibular bone density in the ovariectomized group that did not receive PTH treatment ($p<0.01$), results corroborated by SEM images. Conclusions: Despite of the presence of a periodontitis inductor and estrogen deficiency, PTH intermittent doses were able to reduce alveolar bone loss, in ovariectomized rats.

1. INTRODUÇÃO

A periodontite, uma doença inflamatória crônica, é um processo complexo, multifatorial no qual as interações entre os componentes da placa bacteriana subgengival e os mecanismos de defesa do hospedeiro, determinam o início e a progressão da doença (CRPD, 1996; D' aiuto*et al.*, 2004). Clinicamente, a periodontite se caracteriza por inflamação dos tecidos de proteção e suporte dos dentes, ocasionando perda dos elementos de suporte dental incluindo o osso alveolar (Listgarten, 1986; Baker, 2000; Crotti *et al*., 2003).

As condições sistêmicas do hospedeiro, podem determinar um maior ou menor risco de desenvolvimento de periodontite (CRPD, 1996; Holmustrup *et al.*, 2003). Dentre os fatores sistêmicos, a osteoporose e a osteopenia vêm sendo relacionados à doença periodontal (Mohammad *et al.*, 1994; von Wowern *et al*., 1994; Wactawski-Wende *et al*., 1996; Mohammad *et al*., 2003). A osteopenia, por definição, é uma diminuição do tecido ósseo, enquanto que a osteoporose, uma osteopenia mais avançada, é caracterizada por uma progressiva e sistêmica perda de mineral e de matriz ósseas, culminando com um aumento à susceptibilidade a fraturas (Kanis *et al*., 1994; Pacifici, 1996). A osteoporose pode ser diagnosticada através do exame de densidade mineral óssea (DMO) (Kanis *et al*., 1994; Pacifici, 1996), e tem como principais fatores de risco: deficiência de estrógeno (Kanis *et al*., 1994), fumo (Hopper *et al*., 1994), alto consumo de álcool (Kimble, 1997), excesso de ingestão de cafeína (Cooper *et al*., 1992) e o uso de glicocorticóides (Daniel, 1996).

Autores têm afirmado que a deficiência de estrógeno é o principal fator de risco para osteopenia e osteoporose (Cumming, 1996; Ross, 1998). O estrógeno é capaz de regular a remodelação óssea pela modulação da produção de citocinas e fatores de crescimento, especialmente: interleucina (IL) 1, fator de necrose tumoral alfa (TNF-α), fator estimulador de colônias de granulócitos e macrófagos (GM-CSF) e IL-6 (Pacifici, 1996). A falta de estrógeno ocorrida na pós-menopausa, gera uma alteração no circuito de citocinas que controlam a atividade das células ósseas (Pacifici, 1996), ocasionando um desequilíbrio na remodelação óssea e culminando com uma diminuição da massa óssea sistêmica, principalmente pela diminuição da atividade dos osteoblastos (Jilka, 1998). Além disso, o acúmulo de osteócitos, que sofrem apoptose em decorrência da queda estrogênica,

pode aumentar a fragilidade óssea, diminuindo a rede sincicial, levando à detecção imperfeita de pequenas lesões. (Hughes *et al*., 1996).

A retomada da atividade do estrógeno através da terapia de reposição hormonal (TRH), regulariza a atividade das células ósseas e é então capaz de melhorar e aumentar a massa óssea do indivíduo na pós-menopausa (Lindsay *et al*., 1976, PACIFICI, 1996). Apesar da TRH ser o principal tratamento para osteoporose pós-menopausa (Iqbal, 2000), existem também outros tratamentos baseados na utilização de drogas anabólicas ósseas, como o hormônio paratireóideo (PTH), que vem sendo utilizado com bons resultados como alternativa no que diz respeito à recuperação da massa óssea perdida (Morley, 1997; Horwitz *et al*., 2003).

O PTH é um mediador da remodelação óssea, sendo o principal regulador da homeostasia do cálcio (Morley, 1997; Rattanakul *et al*., 2003; Goodman, 2004). A capacidade de regular a remodelação óssea é observada em diferentes caminhos, podendo ora promover reabsorção óssea, ora aposição (Morley, 1997; Neer *et al*., 2001). Contínua infusão de PTH provoca um decréscimo na massa óssea, pelo aumento da atividade osteoclástica; já uma administração intermitente promove um aumento da massa óssea, por estimular diferenciação de osteoblastos (Neer *et al*., 2001; Horwitz *et al*., 2003). Os resultados obtidos em pesquisas clínicas favoreceram a liberação do uso do hPTH (1-34), um fragmento do polipeptídeo que constitui o PTH que é administrado de maneira intermitente, em humanos para tratamento da osteoporose (Neer *et al*., 2001; Horwitz *et al*., 2003; Rubin & Bilezikian,.2003).

Apesar da principal conseqüência da osteoporose ser o aumento de fraturas (Kanis *et al*., 1994; Pacifici, 1996), indivíduos com essa doença têm um alto número de dentes extraídos (Groen *et al*., 1968; Kribbs, 1990; Baxter & Fattore, 1993; Mohammad *et al*., 1994; Von Wowern *et al*., 1994; Taguchi *et al*., 1995; Danielle, 1994; Mohammad *et al*., 2003), reduzida densidade mineral óssea mandibular (Mohammad *et al.*, 1994; Kribbs *et al*., 1989) e condilar (Tanaka *et al*., 2000), além de uma aceleração da taxa de reabsorção de osso alveolar residual (Kribbs *et al*., 1989; Atwood, 1971, Von Wowern & Kollerup, 1992; Hirai *et al*., 1993; Klemetti *et al*., 1993). Em adição a isso, vários estudos sugerem a existência de uma correlação entre a redução da densidade óssea esquelética, a diminuição do volume da crista alveolar óssea e a perda de inserção clínica (Kribbs, *et al.,* 1989; Von

Wowern *et al*., 1994; Kribbs, 1990; Jeffcoat *et al*., 1993; Jacobs *et al*., 1996; Loza *et al*., 1996; Krejci, 1996; Wactawski-Wende *et al*., 1996; Mohammad *et al*., 1996; Hildebolt, 1997; Grossi, 1998; Payne *et al*., 1997; Talbot & Craig., 1998; Payne *et al*., 1999; Zachariasen, 1999; Ronderos *et al*., 2000).

Baseado nos achados da literatura citados acima, em que fica clara a relação entre a qualidade óssea sistêmica e saúde oral, e seguindo uma linha existente em nosso laboratório, onde foi verificado que o PTH quando administrado de maneira a promover anabolismo ósseo, é capaz de inibir perda óssea em ratos com periodontite induzida (Barros *et al*., 2003), este trabalho objetiva avaliar o efeito da administração intermitente de PTH, analisando os seus efeitos no ramo mandibular e na instalação e progressão da periodontite induzida em ratas com deficiência de estrógeno.

2. ARTIGOS

2.1 *ARTIGO 1*

Periodontal Disease and Osteoporosis Associations and Mechanisms: A Review of Literature

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Periodontal Disease and Osteoporosis Associations and Mechanisms: A Review of Literature

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Abstract

Periodontitis and osteoporosis, diseases that affect millions of people in world, present bone loss as common hallmark. Prevalence of both osteoporosis and tooth loss increase with advancing age in both women and men. Systemic bone loss has been proposed as a risk factor for periodontal disease with increasing evidences that osteoporosis, and the underlying loss of bone mass characteristic of this disease, is associated with periodontal disease and tooth loss. Periodontitis has long been defined as an infection-mediated destruction of the alveolar bone and soft tissue attachment to the tooth, responsible for most tooth loss in adult populations. Current evidences including several prospective studies support an association of osteoporosis with the onset and progression of periodontal disease in humans. Systemic loss of bone density in osteoporosis, including that of the jaw, may provide a host system that is increasingly susceptible to infectious destruction of periodontal tissue. Studies have provided evidence that hormones, heredity, and other host factors influence periodontal disease's incidence and severity. This paper reviews the role of estrogen deficiency and osteoporosis in oral bone healthy and the current evidences on the association between periodontal disease and osteoporosis.

Key Words: Age, bone loss, periodontitis, osteoporosis.

Introduction

Periodontal disease is initiated by microbial pathogens that elicit a host immune response with subsequent tissue destruction of the periodontal structures, including breakdown of alveolar bone[1]. Although bacteria are a necessary factor in the equation, the reaction of the host's imuno-inflammatory system is responsible for most of the destruction found in periodontal disease. Thus, it makes sense that a number of environmental and acquired factors may modify a patient's risk of developing periodontal disease. Recently, it has been suggested that estrogen can influence tooth retention by preventing the resorption of alveolar bone[1,2,3]. Estrogen deficiency, which affects systemically the sequence of bone resorption and formation, has received increasing attention in relation to the stability of alveolar bone structure in postmenopausal women[2,4]. This paper reviews the scientific evidence for some of the peridontitis risk factors including age and osteoporosis.

Osteoporosis - Biological Aspects

1.1 Bone Remodeling

A balance process of bone resorption continuously remodels normal bone, including alveolar bone, by osteoclasts, followed by bone deposition by osteoblasts[5,10]. Osteoblasts secrete bone matrix proteins, including type-I collagen, proteoglycans, osteocalcin, osteopontin and the growth factors and, later stimulate the bone mineralization[11]. Osteoclastogenesis is also under the control of osteoblasts, since osteoblasts are affected by factors capable of promoting bone resorption, such as parathyroid hormone (PTH), 1,25 dihydroxyvitamin D3, calcitonin and prostaglandin E2 (PGE2)[10,12]. Unlike osteoclasts, osteoblasts do not have a hematopoietic lineage, but are derived from mesenchymal precursors[13]. Precursor cells are attracted chemo tactically, then bone cell mitogens, including transforming growth factor beta (TGFb), platelet-derived growth factor (PDGF), bone morph genetic protein, fibroblast growth factor and insulin-like growth factors-I and II, induce their proliferation and differentiation to osteoblasts. Many of these growth factors are released as osteoclasts dissolve the bone. Resorption thus automatically triggers replacement[11].

1.2 Osteopenia/Osteoporosis

Osteopenia is defined as a reduction in bone mass due to bone resorption[4]. The reduction in bone mass and deterioration in bone architecture, that may occur after age 40, is characteristic of osteoporosis resulting in increased fragility of the bone and its susceptibility to fractures[4,14]. In 50-years–old Caucasian American women, the lifetime risk for total osteoporotic fractures and hip fractures is 45% and 17.5% respectively[4]. About 25 to 30% of all hip fractures occur in men, and male osteoporosis is increasing as men live longer, probably due to a decrease in sex steroids and age-related bone loss[4,14]. Osteoporosis can be further characterized as either primary or secondary. Primary osteoporosis can occur in both sexes at all ages, but often follows menopause in women and occurs later in life in men. In contrast, secondary osteoporosis is a result of medications (e.g. glucocorticoids) or other conditions (e.g. hypogonadism)[15].

Currently there is no accurate method to measure the overall bone strength. Bone mineral density (BMD) is frequently used as a proxy measure and accounts for approximately 70% of bone strength[14]. The World Health Organization (WHO) operationally defines osteoporosis as bone density 2.5 SDs below the mean for young white adult women. It is not clear how to apply this diagnostic criterion to men and children, or across ethnic groups. Because of the difficulty of accurate measurement and standardization between instruments and sites, controversy exists among experts regarding the continued use of this diagnostic criterion[5,14,15].

1.3 Risk factors for osteoporosis

The prevalence of osteoporosis and the incidence of fracture vary by sex and race/ethnicity. Both men and women experience an age-related decline in BMD starting in midlife. Women experience more rapid bone loss in the early years following menopause, which places them at earlier risk for fractures[5,18].

Risks associated with low BMD are supported by evidence that includes large prospective studies. Predictors of low bone mass include female sex, increased age, white race, low weight and body mass index (BMI), family history of osteoporosis, smoking, and history of prior fracture. Use of alcohol and caffeine-containing beverages is inconsistently associated with decreased bone mass[15]. The most common cause of

osteoporosis in women is the decrease in estrogen that accompanies menopause. Estrogen loss is associated with elevated bone resorption caused by a rise crease in the cytokines that regulate osteoclast generation, as follows: RANK–ligand; TNF-a (tumor necrosis factor-a); interleukin-1 (IL-1), IL-2, IL-6; M-CSF (macrophage-colony stimulating factor), and prostaglandin E[11]. Production of all of these cytokines is either directly or indirectly suppressed or regulated by estrogen[4].

Glucocorticoid use causes the most common form of drugrelated osteoporosis, and the long-term administration of glucocorticoids for disorders such as rheumatoid arthritis and chronic obstructive pulmonary disease is associated with a high rate of bone fracture[17,5]. People who have undergone organ transplantation are at high risk for osteoporosis due to a variety of factors[18,5]. Hyperthyroidism is also a well-described risk factor for osteoporosis[5].

Osteoporosis x Periodontal Disease

A number of studies have investigated a possible relationship between periodontitis and osteoporosis, and although the literature supports such relationship, its extent remains unclear, due to small sample sizes, noncomparable study populations and different study methods used to assess periodontitis and osteoporosis[6,7,8,9]. In spite of these limitations, recent investigations have been designed to provide more specific information.

Periodontal disease is a chronic inflammatory disease that leads eventually to loss of the supporting structures of the teeth, including resorption of alveolar bone of the jaw. Periodontitis are the most prevalent of the diseases of the bone in humans, being severe enough to lead to tooth loss in 10 to 15% of adults[19,20] and can be exacerbated by certain systemic factors, such as estrogen-deficiency[21].

Estrogen-deficiency enhances the rate of breakdown of connective tissue components of the gingiva by stimulating synthesis of matrix metalloproteinases (MMP-8, and MMP-13)[22], nitric oxide[23] and several cytokines implicated in bone resorption[24]. Estrogen deficiency increases IL-6 concentrations in bone marrow[8,25,26], serum[8,27], and gingiva[21,8], cooperatively stimulating osteoclast bone resorption. A cross-sectional study of

pre and postmenopausal women report significant correlation between alveolar and metacarpal BMD and elevated salivary IL-6 concentrations in postmenopausal women[9].

Preliminary data from the oral ancillary study of the Women's Health Initiative[8,28], which was designed to determine a possible association between systemic osteoporosis and oral bone loss, suggested a significant correlation between the mandibular basal bone mineral density and hip bone mineral density[6,8]. Krall et al 2001[1] have correlated calcium and vitamin D supplements with a lower risk of tooth loss in elderly men and women.

Others have reported diminished tooth loss in estrogen users[29], gingival plaque and body mass index; the authors demonstrated that loss of skeletal bone mineral density was related substantially to alveolar bone loss. To a lesser extent, skeletal bone mass was also related to CAL (clinical attachment loss). These data implicate postmenopausal osteoporosis as risk indicator for periodontal disease in postmenopausal white women[8,7].

The relationship between skeletal loss of mineral density and increased periodontal bone loss may be due to several factors. It may be that more periodontal bone loss occurs simply because the bone surrounding the teeth is less dense and therefore less resistant to resorption. Genetic predisposition to systemic and periodontal bone loss also may be factor, as well as environmental or lifestyle factors that predispose some people to both diseases. Many possible factors contribute to the development of osteoporosis and periodontal diseases being difficult establish the direct correlation between tooth loss, bone loss, and loss of attachment resulting from periodontitis and decreased BMD associated with osteoporosis, but studies are ongoing[8]. Understanding the association between these common diseases and the mechanisms underlying those associations will aid health professionals to provide improved means to prevent, diagnose, and treat these very common diseases.

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2.2 *ARTIGO 2*

Effect Of Intermittent PTH Administration In The Periodontitis - Associated Bone Loss In Ovariectomized Rats

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Effect Of Intermittent PTH Administration In The Periodontitis-Associated Bone Loss In Ovariectomized Rats.

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Short title: Estrogen deficiency and PTH in periodontal disease.

ABSTRACT

Background: PTH intermittent administration has been considered to treat bone mass decrease in estrogen deficient or/and osteoporotic individuals. Changes in hormone levels, due to puberty, pregnancy and menopause, have been proposed as a risk factor for periodontal disease.

Objectives: The aim of this study was analyze the effects of PTH intermittent administration on the periodontitis progression, in estrogen deficiency rats.

Methods: Thirty female rats were set in 3 groups. Groups C and B were ovariectomized and A group was Sham operated. After one week the animals received cotton ligature around the first lower molar. During 30 days animals from C group received PTH doses (40µg/Kg), and the A and B groups received the vehicle, three times a week. After that the animals were sacrificed, the mandibles were extracted, X-rayed and the samples were prepared to histological sections. Samples were histomorphometrically analyzed under light microscopy, using image analyzer software. Scanning electron microscopy (SEM) of the tibia was also performed to evaluate the possible changes in bone structure caused by estrogen deficiency.

Results: Histomorphometric results indicated the anabolic PTH effect on ovariectomized rats with inhibition of periodontitis manifestation, thus neutralizing the periodontitis inductor effects. The photo densitometry demonstrated a lower mandibular optical density in the ovariectomized group that did not receive PTH ($p<0.05$). SEM images confirmed the early effect of estrogen deficiency in osseous tissue and the PTH anabolic effect. .

Conclusion: PTH was able to reduce alveolar bone loss in ovariectomized rats, despite the presence of a periodontitis inductor and estrogen deficiency.

Key words: Parathyroid Hormone (PTH 1-34), periodontitis, estrogen deficiency.

INTRODUCTION

Estrogen deficiency is the dominant pathogenic factor for osteoporosis in women. The impact of estrogen deficiency and osteopenia/osteoporosis on periodontitis is unclear. [1-3]. Estrogen modulates some cytokines that are important regulators of the bone metabolism and also regulators of the host response to infections. Among these cytokines are interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), macrophage colony stimulating factor (M-CSF)[4]. Estrogen deficiency promotes an unbalance on bone metabolism interfering in the bone mass density (BMD) in postmenopausal women[4,5].

Due to the effect in the process of bone formation/resorption, the estrogen deficiency has been investigated in relation to the stability of alveolar bone structure and, several studies have reported a positive correlation between estrogen deficiency and periodontal disease[2,6-8]. Skeletal BMD has been related to interproximal alveolar bone loss and, to a lesser extent, to clinical attachment loss, implicating postmenopausal osteopenia as a risk indicator for periodontal disease in postmenopausal women[9]. The systemic increase of the BMD observed with the hormone/estrogen replacement therapy (H/ERT) in postmenopausal women, has been demonstrated to be accompanied by the increase of alveolar BMD[2].

Although H/ERT in postmenopausal women is the main treatment for osteopenia and osteoporosis, recent studies indicate parathyroid hormone (PTH) treatment in intermittent doses as an efficient anabolic treatment, avoiding the bone loss due to estrogen deficiency[10-12].

PTH functions as a major mediator of bone remodeling and as an essential regulator of calcium homeostasis, producing several distinct and independent effects on the bone remodeling process, resulting in both, bone formation (anabolic activity) and bone resorption (catabolic activity), e.g. continuous infusion of PTH decreases bone mass by stimulating osteoclast activity, while intermittent administration increases bone mass by stimulating osteoblast differentiation[12]. PTH capacity to promote an increase in the skeletal bone mass is also observed in mandibular bone of the ovariectomized rats[13]. Recently, Barros *et al.* 2003[14] reported that intermittent PTH administration in a rodent model was able to protect against experimental periodontitis-associated bone loss.

Therefore, the purpose of the present study was to evaluate the effects of intermittent PTH administration in rats under estrogen deficiency (ovariectomy), analyzing the progression of experimental periodontitis.

MATERIALS AND METHODS

Animals

A total of 30 female Wistar rats, aged four weeks old in the beginning of the experiment, were maintained in a room with 12-hour day/night cycles and an ambient temperature of 21°C, with food and water *ad libitum*. Experimental procedures were approved by the Institutional Animal Research Committee at the University of Campinas (São Paulo-Brazil).

Surgical Protocols

General anesthesia was obtained by intramuscular injection of ketamine of 1.0ml/kg body weight and xylazine at 10mg/Kg body weight. Bilateral ovariectomies were performed in 20 rats. Sham surgeries were performed in other 10 rats when, the ovaries were exposed but not removed [13].

After one week of the ovariectomies and sham surgeries, all animals were treated under general anesthesia obtained by intramuscular administration of ketamine (1.0ml/kg), in order to induce periodontitis, for that one of the mandibular first molars of each animal was randomly assigned to receive cotton ligature (Corrente™ #10, São Paulo, SP, Brazil), placed sub marginally.

Treatments

The ovariectomized rats (Ovx) were then divided into two groups. In ten Ovx animals were given 40µg/kg of PTH (1-34) prepared in 1% acetic acid, injected subcutaneously, 3 times a week for 4 weeks. This group is designated the "Ovx + PTH" group. The remaining 10 ovariectomized animals received the same volume of vehicle (1% acetic acid in water) and were designed as "Ovx" group. The 10 Sham-operated animals ("Sham" group) also received the same volume of vehicle. All the injections followed the 3 times a week protocol. The intermittent PTH schedule and dose used in the present study were based on previous studies by Hagino *et al.* (2001)[14], Iida-Klein *et al.* (2002)[15] and Barros *et al*. (2003)[16].

Radiographic Procedures

After 4 weeks of treatment, the animals were sacrificed (twenty-four hours after the last injection). The jaws were removed and fixed in 4% neutral formalin for 48 hours. Radiographs were taken using a dental X-ray unit (GE 1000) with an exposure time of 0.1 seconds (70kVp, 10mA) and 31x41mm dental radiographic film (Insight Film, Eastman Kodak, Rochester, NY) and the radiographs were densitometrically evaluated. The optical density values were obtained from 5 measurements in the mandible, as indicated in Figure1.

Histological Procedures

After radiodensitometric analysis, all specimens were demineralized in a 5% EDTA/ phosphate buffered saline solution for around 60 days. Paraffin serial sections (7 µm), prepared in a mesio-distal direction, were obtained and stained with hematoxylin and eosin. Histomorphometric analysis of the specimens were done using an image analysis system (Image-Pro®; Media Cybernetics, Silver Spring, MD, USA) where the area between the bone crest and cementum surface in the furcation region were histometrically determined using 5 sections per specimen.

Scanning Electron Microscopy

For scanning electron microscopy (SEM) evaluation, tibias from all animals were cut in the proximal metaphysis area[17]. The specimens were immersed in 1% vol/vol Triton-X-100 (Sigma) for 20 min at room temperature in an ultrasonic cleaner in order to partially remove the organic material. The specimens were dehydrated by acetone (Merck) and were then mounted on metal stubs, coated with gold using a Balzers MED 010 sputter coater, and examined using a scanning electron microscope (JEOL 6100) for observation and description of the cancellous bone pattern.

Statistical Analysis

Histomorphometric area recordings were expressed in mm^2, statistical differences in bone loss area and relative radio densitometry were subjected to one-way ANOVA and Tukey's Multiple Comparison Test at 5% level of significance.

RESULTS

Histological observations

Figure 2A, illustrate area between the bone crest and cementum surface in the furcation region of unligated teeth. Figures 2 B, 2C and 2D illustrate bone loss in the furcation area of ligated teeth in the different experimental conditions. The results from histomorphometry (Fig 3) analyzing bone loss obtained from ligated teeth demonstrated that Ovx group (0.488 mm^2) and Sham group (0.405mm^2) presented significant bone loss in the molar teeth furcation area ($P<0.05$) when compared with the Ovx + PTH group (0,210 mm^2). When Ovx group and Sham group were compared, the analysis of bone loss in the furca area did not reveal a statistically significant difference ($P>0.6$).

Radiographic observations

Statistical analysis of the optical density revealed significant differences ($P<0.05$) when the Ovx group was compared to the others, with average densities measurements of 1.770, for Ovx + PTH group, 1.761 for the Sham group and 1.801 for Ovx group, confirming the early effect of estrogen deficiency and the anabolism promoted by PTH (Fig. 4).

Scanning electron microscopy observations

Ultrastructurally, trabecular bone aspect of the tibia in the Sham group (Fig. 5A) was not different from the obtained in the Ovx + PTH group (Fig.5C), however the Ovx group (Fig.5B) exhibited bone with a higher resorptive aspect, whereas in Sham operated and PTH treated animals a reduced area appeared pitted. SEM of the spongy bone showed marked trabecular disconnection in Ovx group contrasting with Sham and Ovx + PTH groups, which presented similar pattern consisting of rod and plate-like structures with numerous connections.

DISCUSSION

Estrogen deficiency, an important systemic factor related with periodontits[1-3], can be experimentally provoked in animals through ovariectomy[13,8]. In this study, our primary goal was to determine whether PTH intermittent treatment was able to interfere in induced periodontitis-associate bone loss in ovariectomized rats, since we had data

presented by Barros *et al.* 2003[14] showing that PTH intermittent administration presented a direct effect in experimental periodontal disease progression.

The mechanisms underlying periodontitis process involve both, direct tissue damage resulting from plaque bacterial products and indirect damage through bacterial induction of the host inflammatory and immune responses[18]. Such host responses reflect complex interactions between cells, extracellular matrix and circulating cytokines. Some cytokines as IL-1β, TNF-α, IL-6, IL-8 e IGF, have been found at increased levels in inflamed gingival tissue in concentrations capable of inducing bone resorption[19-23].

Kawase *et al*. 2000[8] have demonstrated that estrogen deficiency increased significantly bone loss in experimental periodontitis. Estrogen deficiency has been associated to elevated bone resorption caused by an increase in the number of osteoclasts, driven by cytokines that regulate osteoblast generation[4]. Expression of RANKL (receptor for activator of nuclear factor-κB) ligand; TNF-α (tumor necrosis factor-α), interleukins (IL) IL-1, IL-6; IL-11; M-CSF (macrophage-colony stimulating factor) and prostaglandin E[24-27] have been demonstrated to affect bone metabolism. RANK ligand (RANKL) has been shown to fully control osteoclast formation in mice[27]. The production of all of these cytokines is either directly or indirectly regulated by estrogen[29].

Our results showed that the bone loss area detected in the Sham operated and ovariectomized groups were similar, such finding is probably due to the age of the animals; other studies with estrogen deficiency tend to work with aged females rats[11, 13]; we worked with younger animals, aged 4 weeks at the beginning of the experiment, with the objective to compare the PTH anabolic treatment results with the ones presented by Barros et al [16].

We observed an unbalance in bone metabolism caused by the estrogen deficiency, exhibiting morphologic aspect consisted of thin rod-like spicules with few connections (Fig.5B), such aspect, compatible with osteopenia, could also be detected by radiodensitometric analysis where the Ovx group showed significant bone loss in the mandible in just 4 weeks after ovariectomy (Fig. 4). Also Miller *et al*. 1991[30] have noted an increase in bone turnover in the rat mandible in a short period after ovariectomy, while Elovic *et al*. 1995[31] reported some reductions in bone area and stiffness at later periods in ovariectomized rats.

In our results the ovariectomized - PTH treated group showed a reduced bone loss in the furca area in comparison to the ligated teeth from the other experimental groups.

Forteo (Teriparatide) is the recombinant human 1-34 amino acid sequence of parathyroid hormone recently approved in the US for the treatment of men and postmenopausal women at high risk for osteoporotic fracture and in Europe for the treatment of postmenopausal women with osteoporosis [32], however PTH anabolic effects have not yet been presented in alveolar bone under estrogen deficiency condition. We could observe the PTH influence in alveolar bone preventing bone loss in furca area even in the presence of a local irritant agent acting synergistically with the osteopenia condition.

There are evidences that PTH can increase osteoblasts in number and activity by inducing bone lining cells to become osteoblasts without stimulating proliferation of precursor cells[8, 41, 42], which could be explained by anabolic PTH activity increasing the life-span of mature osteoblasts by preventing apoptosis[42].

In spite of intermittent PTH administration has proved to be an efficient treatment minimizing bone loss in experimental periodontitis in a rodent model, the precise mechanisms, which lead to this favorable condition are still subject of investigations.

The results suggest that PTH might exert a potential benefit in treatments of periodontal disease in osteoporotic individuals, working synergistically to the conventional periodontitis therapies.

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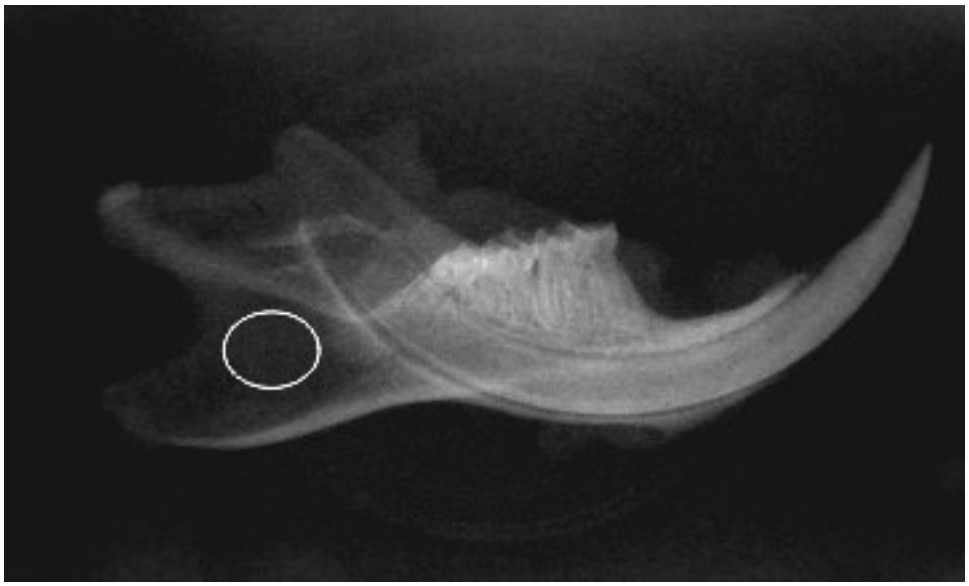

Figure1 - Radiographic image illustrating the mandibular area from where photodensitometric measurements were done to obtain the optical densities.

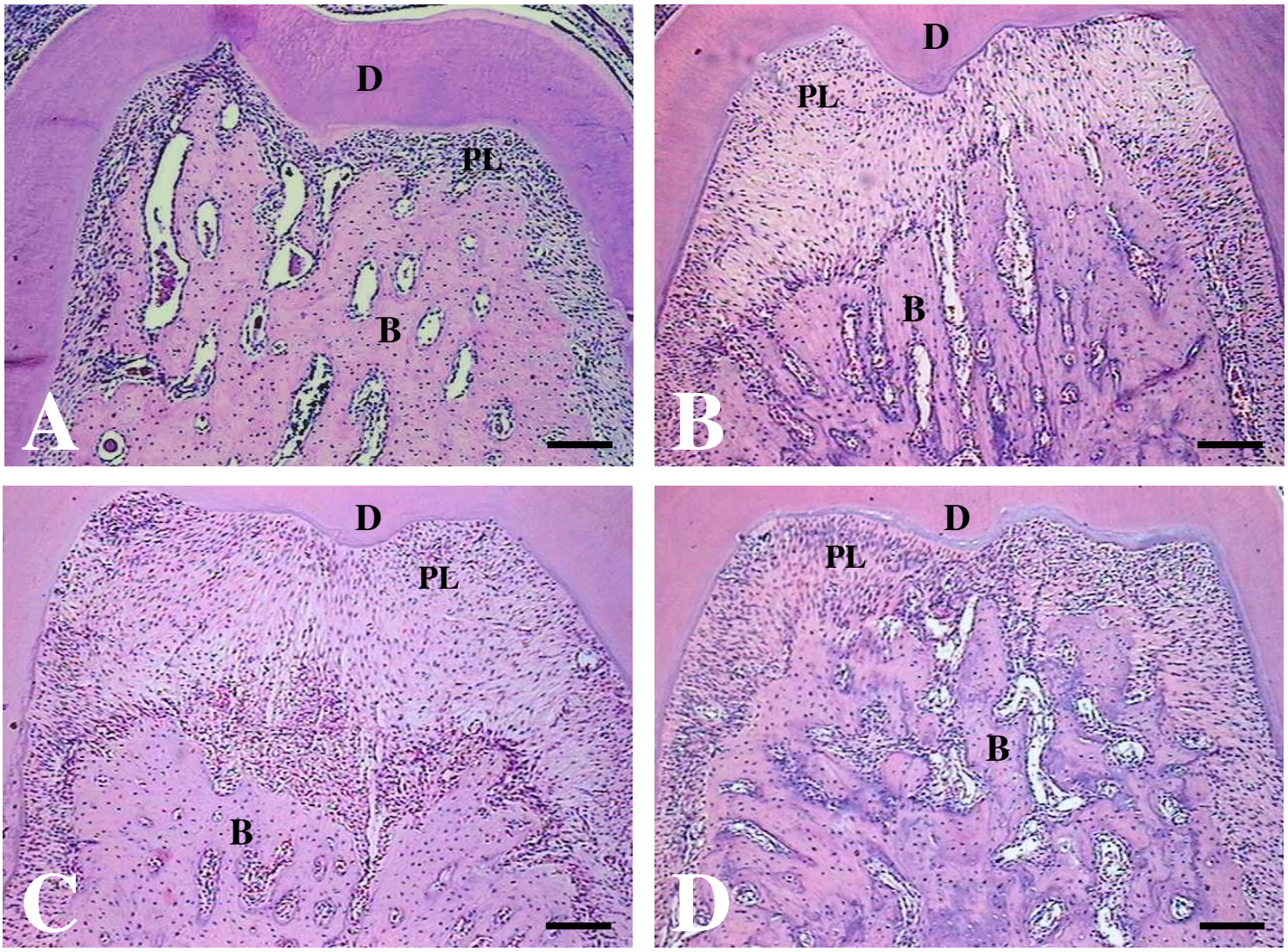

Figure2 - Histological aspect of molar furcation area for the different experimental groups. (**A**) Represents unligated tooth from the control group. (**B**) Ligated tooth from the Sham group. (**C**) Ligated tooth from Ovx group. (**D**) Ligated tooth from Ovx + PTH group. Dentine (D), periodontal ligament area (PL), alveolar bone (B). H&E. The extension of the PL area indicates bone loss due to induction of periodontitis Scale bar represents 0.30 mm.

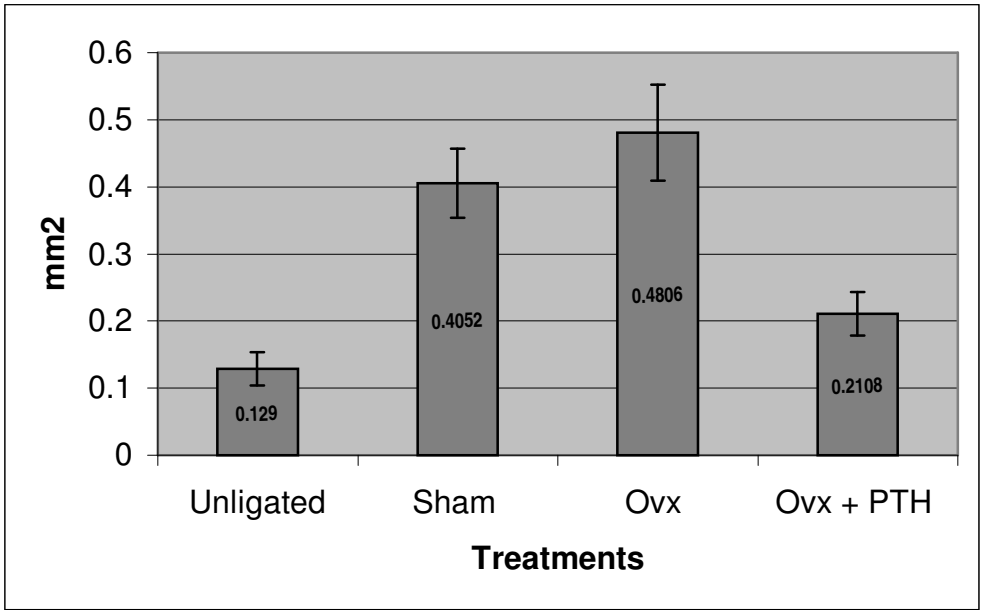

Figure 3 - Mean ± standard deviation of the area between bone crest and cementum surface (mm^2) in the furcation area of unligated teeth and ligated teeth from Sham operated animals, ovariectomized and ovariectomized associated with PTH treated groups of animals. N = 10/group.

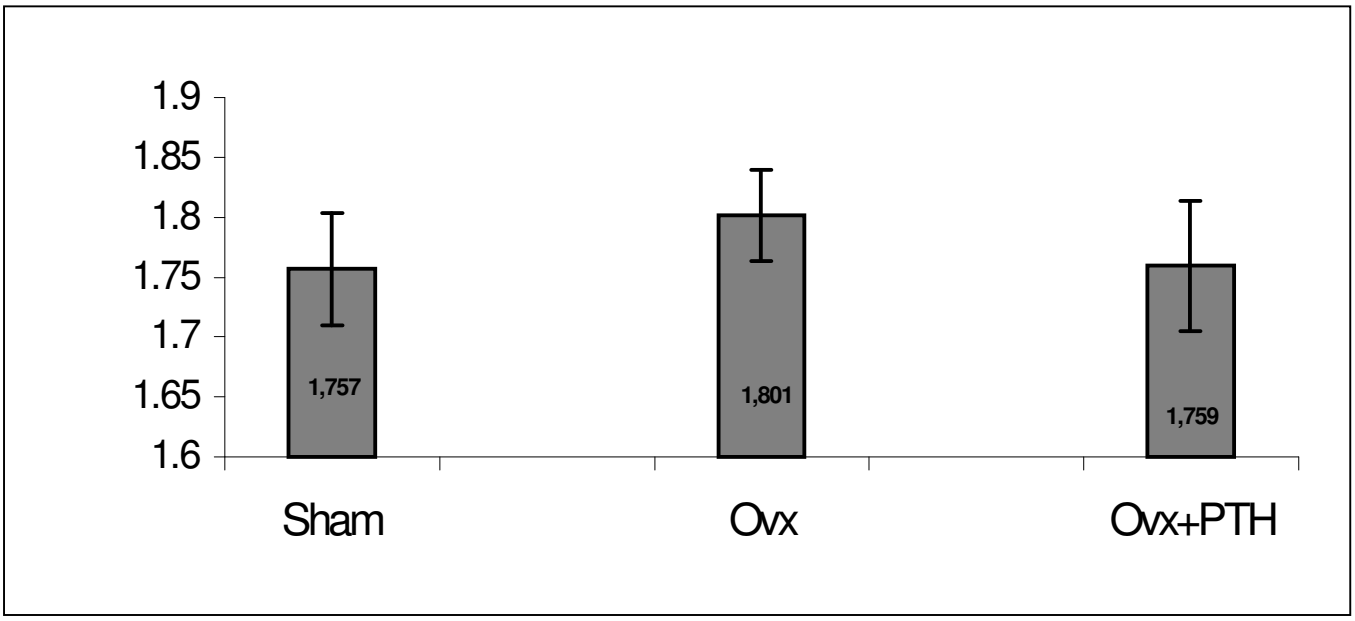

Figure 4 - Mean ± standard deviation of the radiographic optical density measurements, in the groups: Sham operated, ovariectomized and ovariectomized + PTH treated.

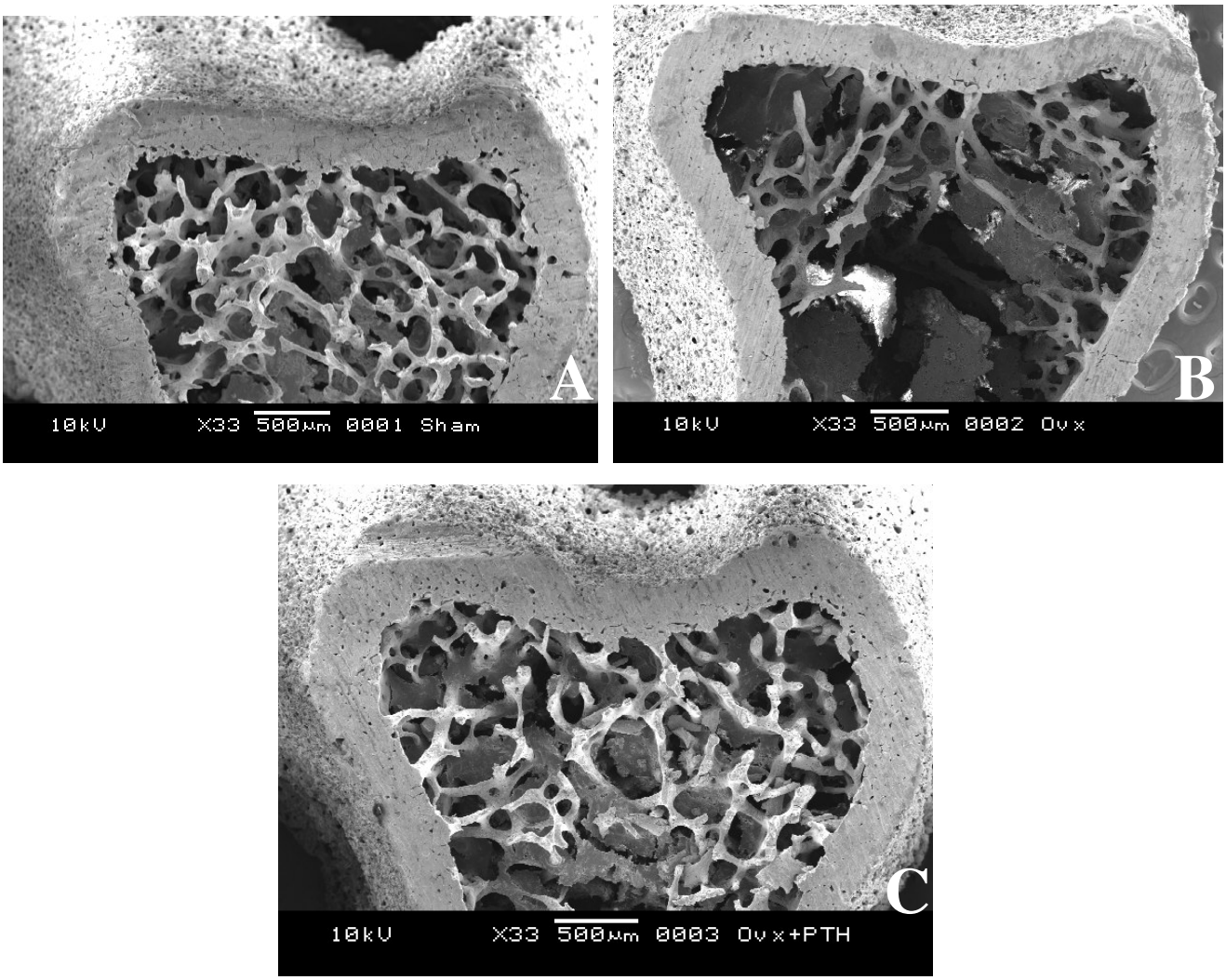

Figure 5 - Scanning Electron Microscopy showing trabecular bone aspect of the tibia. - Sham group (A) and Ovx + PTH group (B) presented similar cancellous bone pattern consisting of rod and plate-like structures with numerous connections. C - Spongy bone showing marked trabecular disconnection in Ovx group.

3. DISCUSSÃO

Apesar de existir uma clara relação entre osteoporose e perda dentaria (Daniel, 1983; Krall *et al*., 1994; Taguchi *et al*., 1995; Krall *et al*., 1996; Gur *et al*., 2003), as causas existentes em relação à associação entre osteopenia/osteoporose e a doença periodontal continuam pouco definidas. Amostras pequenas e diferenças fundamentais no planejamento dos estudos, o tipo de população examinada (isto é, só mulher *versus* homem e mulher), a idade da população estudada, a metodologia e a verificação de doença periodontal e osteopenia esquelética, impedem a interpretação e comparação dos estudos.

Wactaski-Wende *et al*. (1996) observaram em um estudo com 70 mulheres, na pós-menopausa, uma relação significativa entre a altura da crista óssea alveolar como medida da periodontite e a osteopenia esquelética (fêmur e coluna lombar) medida por meio de absorção dual de fótons (ADF). Esta relação foi vista após controle de possíveis variáveis, como a placa dentária, anos de menopausa e tabagismo. Além disso, houve uma relação entre osteopenia da bacia e verificação da perda de inserção no mesmo grupo. De maneira semelhante, Von Wovern *et al*. (1994), em um estudo de controle de casos, comparando 12 pacientes do sexo feminino com fraturas osteoporóticas e 14 mulheres normais, relataram significativamente maior perda de inserção periodontal na mulher com osteoporose, quando comparada com a normal. Eles verificaram que as mulheres com osteoporose tinham menos conteúdo mineral mandibular ósseo, medido pelo ADF, do que as 14 mulheres normais.

A relação entre osteopenia e a gravidade da doença periodontal foi também examinada em uma amostra da Third National Health and Nutrition Examination Survey (NHANES III) (Grossi *et al*. 1998) de 11.247 indivíduos com 20 a 90 anos, onde a osteopenia da bacia foi associada à gravidade da doença periodontal (perda de inserção mínima maior ou igual a 1,5 mm) nas mulheres e homens com quadros semelhantes de osteopenia, independentemente dos efeitos da idade, sexo, tabagismo ou quantidade diária de cálcio ingerida. Esta associação foi aumentada ainda mais nas mulheres na pós-menopausa. Em um estudo mais recente, Mohammad *et al*. (2003) demonstraram existir uma correlação entre diminuição da densidade mineral óssea em mulheres pós-menopausa e um aumento da perda de inserção clínica, independente da quantidade de placa bacteriana

existentes nestas mulheres. Conseqüentemente, embora limitadas, as evidências sugeriram uma associação entre osteopenia, osteoporose e doença periodontal. Demonstrando que a deficiência de estrógeno possa explicar, em parte, a natureza desta associação.

A deficiência de estrógeno é o fator de risco mais associado à osteoporose pós-menopausa (Cumming *et al*., 1996; Ross *et al*., 1998; Riggs *et al*., 2003). A deficiência de estrógeno em mulheres pós-menopausa e sem ovário está associada à diminuição da densidade mineral da coluna lombar e aumento de incidência de fraturas nas vértebras e bacias (Lindsay *et al*., 1976; Hornsman *et al*., 1983; Pacifici *et al*., 1991; Stone *et al*., 2003). O estrógeno regula a remodelação óssea pela modulação da produção de citocinas e fatores de crescimento, como: interleucina 1 beta (IL-1β), fator de necrose tumoral alfa (TNF-α), fator estimular de colônia de macrófagos e granulócitos (GM-CSF) a partir de células ósseas (Pacifici, 1996). A IL-1β e o TNF-α estimulam a maturação de osteoclastos, modulam a proliferação de células ósseas e induzem reabsorção *in vivo* (Pacifici, 1992; Tatakis, 1993). Além disso, a IL-1, TNF-α, e GM-CSF contribuem também para a reabsorção óssea, recrutando osteoclastos e promovendo a diferenciação de células precursoras da medula óssea (Pacifici, 1996). Os precursores dos osteoblastos respondem à perda de estrógeno pela secreção de IL-6, que então induz à osteoclastogênese (Girasole *et al*., 1992). A perda de estrógeno que acompanha a menopausa resulta em aumento das citocinas de remodelação óssea (Pacifici, 1996). Os indivíduos com muita renovação óssea na osteoporose secretam quantidades aumentadas de IL-1, que é bloqueada pelo tratamento com estrógeno progesterona (Pacifici *et al*., 1991).

Alguns estudos examinaram a relação direta entre o nível de estrógeno e a doença periodontal. Norderyd *et al*. (1993) relataram baixos níveis de inserção clínica, embora não estatisticamente significante, em mulheres na pós-menopausa recebendo suplementação de estrógeno em comparação com mulheres na pós-menopausa que não receberam suplementação. Nas mulheres que receberam suplementação observou-se também menor sangramento gengival. Tezal *et al.* (2000) relataram que reduzida DMO em mulheres pós-menopausa está relacionada com perda óssea alveolar na região interproximal, e aumento da perda de inserção clínica.

Os resultados obtidos no nosso trabalho (Figure 2), em que animais com deficiência tiveram uma maior perda óssea ocasionada pela periodontite corroboram com

um estudo de Kawase *et al*. (2000), no qual ratas com deficiência de estrógeno, também por meio de ovariectomia, apresentam maior perda óssea alveolar quando submetidas à periodontite induzida, em comparação com as ratas não ovariectomizadas. Neste mesmo estudo, Kawase *et al*. (2000) também observaram que o grupo ovariectomizado apresentou uma maior marcação para fosfatase ácida, o que indica uma maior atividade osteoclástica neste grupo.

Além de sofrer ação do estrógeno e outros hormônios, as células ósseas são reguladas diretamente pelo PTH. Este apesar de ser conhecido classicamente como promotor de reabsorção óssea, quando administrado intermitentemente, é capaz de promover potente anabolismo ósseo (Horwitz *et al*., 2003; Misof *et al*., 2003). Os trabalhos clínicos já realizados demonstraram que a utilização do PTH como promotor de anabolismo ósseo, é capaz de tratar pacientes com osteoporose (Fujita *et al*., 1999; Neer *et al*., 2001, Horwitz *et al*., 2003).

No nosso estudo, a capacidade do PTH promover anabolismo ósseo ficou clara, ao analisar a densidade radiográfica das mandíbulas (Figure 4) e ainda pela análise das tíbias, feita por microscopia eletrônica de varredura, em que o PTH foi capaz de reduzir os danos ocasionados pela deficiência de estrógeno (Figure 5). Nossos resultados corroboram com os estudos de Miller *et al*., (1997), Hunziker *et al*., (2000) e De KAWANE *et al*, (2002), em que observaram que a administração intermitente de PTH foi capaz de estimular formação óssea em mandíbulas de ratas ovariectomizadas.

Há apenas um relato na literatura que relaciona a utilização do PTH para promover anabolismo ósseo frente à doença periodontal. Em um estudo realizado em nosso laboratório, Barros *et al*. (2003) verificaram que o PTH é capaz de inibir a perda óssea ocasionada por periodontite induzida em primeiros molares de ratos, achados estes que se assemelham aos aqui apresentados (Figure 2 e Figure 3), em que PTH apesar de não inibir totalmente a perda óssea, foi capaz de reduzi-la.

Na perda óssea alveolar observada no modelo de indução de periodontite em animais, que foi usado em nosso trabalho, é relatado que o Lipopolissacarídeo (LPS) bacteriano no biofilme é o principal agente que induz reação inflamatória no tecido periodontal, sendo capaz de culminar com reabsorção óssea alveolar (Rovin *et al*. 1966, Shoji *et al*., 1995; Mitsuta *et al*., 2002; Barros *et al*., 2003). A presença do LPS bacteriano

na gengiva promove o acumulo de células inflamatórias, como neutrófilos, linfócitos e monócitos levando a instalação de um forte processo inflamatório (Fujihashi *et al*., 1993; Baker, 2000). Vários fatores de reabsorção óssea, como IL-1β, TNF-α, IL-6, IL-8 e IGF, estão presentes na gengiva inflamada durante a periodontite em concentrações capazes de induzir reabsorção óssea (Fujihashi *et al*., 1993; Wiebe *et al*., 1996; Fujihashi *et al*., 1996; Gemmell *et al*., 1997; Baker, 2000). Dentre estas citocinas, a IL-1β provavelmente apresenta o papel mais importante na patogênese da doença periodontal (Howells, 1995), uma vez que os níveis de mRNA desta citocina estão aumentados em gengivas inflamadas (Tokoro *et al*., 1996) e no fluído gengival crevicular de sítios periodontalmente doentes (Honig *et al*., 1989; Masada *et al*., 1990). A IL-1β encontra-se também dentre os indutores de reabsorção óssea (Heath *et al*., 1985; Gowen & Mundy, 1986) e promove degradação do tecido conjuntivo por meio de indução de metaloproteases da matriz (MMP) (Meikle *et al*., 1989).

Análises imunohistoquímicas de tecidos gengivais demonstraram um aumento na concentração de IL-6 em gengiva inflamada (Takahashi *et al*., 1994). Altos níveis de IL-6 e do seu mRNA são encontrados em gengivas inflamadas de regiões com periodontite (Matsuki *et al*., 1992; Takahashi *et al*., 1994), em contraste com baixos níveis encontrados em tecido gengival sadio. A IL-6 pode ser produzida por osteoblastos em resposta a agentes de reabsorção óssea presente em tecido gengival inflamado como IL-1, TNF-α e LPS (Littlewood *et al*., 1991) e age como um potente indutor de osteoclastogênese *in vitro* (Kurihara *et al*., 1990).

Pudemos observar neste estudo, uma diminuição na taxa de reabsorção óssea na região de furca, decorrente da periodontite induzida nos animais tratados com doses intermitentes de PTH. Estes resultados explicam-se em parte pelo fato de que ao se ligar a um receptor (G protein-coupled receptors- GPCR), que está presente nas células osteoblásticas (SWARTHOUT *et al*., 2002), o PTH controla diretamente a estimulação da atividade osteoblástica (proliferação síntese protéica e expressão gênica) e indiretamente, via osteoblasto, a atividade osteoclástica (Canalis *et al.,* 1983; Martin E Ng, 1994**).** Além disso, o PTH apresenta-se também interferindo na atuação de outros fatores, como a osteoprotegerina (OPG) e o RANKL (receptor activator of NK-KappaB ligand) secretados

pelos osteoblastos e linfócitos T, que são ligados à aposição e reabsorção óssea respectivamente (Simonet *et al*., 1997; Swarthout *et al*., 2002)

Podemos então inferir que o estímulo da proliferação de células osteoblásticas e o aumento da síntese de matriz, ocasionados pela administração intermitente de PTH, sejam responsáveis pela diminuição dos efeitos da deficiência de estrógeno e da reabsorção óssea decorrente da doença periodontal observada neste estudo. Entretanto após a realização deste trabalho, que se apresenta como o início do estudo da relação entre periodontite, deficiência de estrógeno e PTH, podemos sugerir a investigação de fatores presentes neste processo, visto que o mesmo continua pouco explicado. Apontamos identificar a presença e concentração dos principais indutores de reabsorção óssea no periodonto doente de animais tratados com PTH, como um possível meio de elucidar o verdadeiro papel do PTH anabólico durante a progressão da doença. Além disso, seria interessante também realizar um estudo onde observássemos as diferenças existentes entre o curso do tratamento e as condições periodontais de pacientes osteoporóticos que recebam tratamento com doses intermitentes de PTH, no intuito de verificar os possíveis efeitos clínicos do hormônio paratireóideo na saúde periodontal humana.

4. CONCLUSÕES

A administração intermitente de PTH foi capaz de diminuir a perda óssea ocasionada por periodontite induzida em primeiros molares de ratas com deficiência de estrógeno.

A deficiência de estrógeno aumentou a perda óssea na periodontite induzida no período estudado.

Trinta dias após a ovariectomia, foi possível detectar perda óssea significativa no ramo mandibular das ratas através da análise da densidade radiográfica.

A perda óssea no ramo mandibular de ratas, ocasionada por ovariectomia, pôde ser inibida por PTH no período estudado.

A análise das tíbias dos animais, por meio de microscopia eletrônica de varredura, permitiu verificar a capacidade anabólica do PTH, que impediu a perda óssea que seria normalmente encontrada nas ratas ovariectomizadas.

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6. ANEXOS