POLIANA MENDES DUARTE

IMPACTO DA DEFICIÊNCIA DE ESTRÓGENO E SUAS TERAPIAS SOBRE O TECIDO ÓSSEO AO REDOR DE IMPLANTES DE TITÂNIO E NA PERIODONTITE INDUZIDA EM RATAS OVARIECTOMIZADAS.

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de doutor em Clínica Odontológica, Área de Periodontia.

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SUMÁRIO

PREFÁCIO

Esta tese está baseada nos seguintes artigos científicos:

1. Estrogen deficiency affects bone healing around titanium implants. A histometric study in rats. ***Implant Dentistry, v.12, p.340-345, 2003***
2. *Effect of estrogen replacement and calcit*onin therapies on bone around titanium implants placed in ovariectomized rats: a histometric study. ***JOMI, v.17, n.6, p.786-792, 2002***
3. Effect of estrogen and calcitonin therapies on the bone density in a lateral area adjacent to implants placed in the tibiae of ovariectomized rats. ***Journal of Periodontology, v.74, n.11, p.1618-1624, 2003***
4. Alendronate therapy may be effective to prevent bone loss around titanium implants inserted in estrogen deficient rats. ***Journal of Periodontology (aceito)***
5. Age-related and surgically induced estrogen deficiencies may differently affect bone around titanium implants inserted in rats. ***Journal of Periodontology (submetido)***
6. Effect of an estrogen-deficient state and its therapy on bone loss resulting from an experimental periodontitis in rats. ***Journal Periodontol Research, v.39, n.2, p.107-110, 2004***
7. Alendronate protects against increased periodontitis-related bone loss in estrogen-deficient rats. ***Journal of Periodontology, v.75, n.9, p.1196-1202, 2004***

RESUMO

Osteoporose e osteopenia são doenças osteometabólicas caracterizadas por uma diminuição progressiva de massa óssea gerada pela deficiência de estrógeno na pós-menopausa ou pela ovariectomia. Diversas terapias têm sido propostas para o tratamento destas patologias como, por exemplo, a terapia de reposição estrogênica, os bisfosfonatos e a calcitonina. Assim como os ossos longos, a doença parece atingir os ossos orais, o que poderia afetar o sucesso de implantes dentais e a progressão da doença periodontal. Os objetivos deste trabalho foram avaliar, através de análise histométrica: 1 - a influência da deficiência de estrógeno induzida (DEI) sobre o tecido ósseo ao redor de implantes de titânio e na perda óssea proveniente da periodontite induzida (PI) em ratas ovariectomizadas (OVX); 2 - a influência da terapia de reposição estrogênica (TRE), da calcitonina (CT) e do alendronato (ALD) sobre o tecido ósseo ao redor de implantes de titânio e na perda óssea proveniente da PI em OVX; 3 - o efeito residual da TRE e do ALD; 4 - o efeito da deficiência de estrógeno relacionada à idade (DERI) , comparativamente a DEI, sobre o tecido ósseo ao redor de implantes de titânio. Os resultados demonstraram um efeito negativo da DEI no tecido ósseo preexistente e neoformado ao redor dos implantes de titânio e na perda óssea decorrente da PI. A TRE contínua e o ALD (contínuo-C e interrompido-I) demonstraram-se capazes de prevenir a influência negativa da deficiência de estrógeno endógeno ao redor dos implantes. Somente o ALD (C/I) apresentou efeito positivo na prevenção da progressão da perda óssea decorrente da PI. A DERI apresentou impacto negativo apenas no osso preexistente ao redor dos implantes de titânio.

PALAVRAS - CHAVE: implantes dentais, periodontite, deficiência de estrógeno, terapia de reposição hormonal, calcitonina, alendronato, osteoporose.

ABSTRACT

Osteoporosis and osteopenia has been defined as systemic skeletal diseases characterized by gradual loss of bone tissue. The most common cause of osteoporosis is the decrease of estrogen level, in menopause or ovariectomy. Estrogen replacement, calcitonin and bisphosphonates therapies have been considered for preventing and treating osteoporosis. Significant relationships have been reported between oral bone and skeletal bone mass in postmenopausal women with osteoporosis. Osteoporosis could, therefore, be considered as a risk factor for dental implants and periodontitis progression. The purpose of this study was, by histometric analysis, to evaluate: 1 - the influence of an induced estrogen-deficient (IED) state on bone around titanium implants placed in ovariectomized rats (OVX) and on bone loss resulting from an experimental periodontitis (EP); 2 - the impact of estrogen (E), calcitonin (CT) and alendronate (ALD) administrations on bone density and healing around titanium implants and on bone loss resulting from an EP; 3 - whether ALD and E therapies would provide a residual effect; 4 - the influence of age-related (ARED) and surgically IED, comparatively, on bone around titanium implants inserted in rats. The results demonstrated that IED affects bone healing and bone density around titanium implants and bone loss resulting from EP. Continuous (C) E and interrupted (I) and C-ALD may prevent the negative influence of IED on bone around titanium implants. Only ALD (C/I) may protect against the impact of IED on alveolar bone loss resulting from EP. ARED mainly affects pre-existing bone while IED more significantly affects both newly formed and pre-existing bone.

KEY WORDS: Dental implants, periodontitis, estrogen deficiency, hormone replacement therapy, calcitonin, alendronate, osteoporosis.

INTRODUÇÃO

Fatores de riscos para os implantes dentais

Os implantes dentais de titânio são considerados atualmente dispositivos seguros para substituição de dentes perdidos, cada vez mais empregados na rotina odontológica. Estudos longitudinais têm demonstrado que a reabilitação de regiões edêntulas por implantes osseointegráveis é um procedimento com taxa de sucesso acima de 90% (BAIN & MOY, 1993; JONES *et al*., 1999). Entretanto, alguns fatores exógenos, relativos ao operador e ao biomaterial; endógenos locais, relativos à quantidade e qualidade óssea e endógenos sistêmicos inerentes aos hábitos e estado de saúde do paciente podem contribuir para o insucesso desses implantes (ESPOSITO *et al.,* 1998).

Os principais fatores locais relacionados às falhas de implantes consistem basicamente na característica anatômica e na qualidade óssea do sítio receptor. JAFFIN & BERMAN (1991) encontraram 37% de falhas em implantes colocados em osso tipo IV e apenas 3% de falhas em implantes colocados em ossos dos tipos I, II e III. FRIBERG (1994), através de estudo retrospectivo, avaliou mais de 4000 implantes e concluiu que regiões com qualidade óssea pobre, principalmente posterior da maxila e mandíbula, apresentam maior risco para a estabilidade inicial do implante.

Em relação aos fatores de ordem sistêmica, além do consumo de cigarros, as doenças ósseas, imunológicas e hormonais parecem influenciar o resultado da osseointegração, demonstrando que o estado de saúde geral do paciente apresenta um importante papel na sobrevida dos implantes dentais (ADELL, 1992).

Osteoporose e os implantes dentais

Osteoporose e é uma doença osteometabólica, conseqüente de uma perda gradativa do conteúdo mineral e orgânico do tecido ósseo, caracterizada pela presença de uma massa óssea inferior a valores pré-estabelecidos para determinada idade e sexo (ALBRIGHT, 1941). A doença pode ser classificada, de acordo com sua etiologia, em osteoporose primária e secundária. A secundária é uma conseqüência de distúrbios endócrinos, como por exemplo, hiperparatireodismo, hipertireoidismo e ingestão de glicocorticóides. A osteoporose primária, por sua vez, decorre do envelhecimento ou da diminuição dos níveis plasmáticos de estrógeno na menopausa, sendo esta

última a causa mais comum de osteoporose na mulher. Embora o nível estimado dessa perda diferencie de uma população para outra e de acordo com as formas de diagnóstico empregadas, tem sido reportado uma ocorrência de 0,5% a 1% de perda óssea por ano (VICO *et al.,* 1992).

Assim como os ossos longos, a osteoporose também atinge os ossos da face. Dessa forma, alguns estudos têm demonstrado que a presença desta doença pode implicar em alterações no tratamento odontológico no que diz respeito à colocação de implantes, próteses, progressão de doença periodontal e risco de perdas dentárias (KRIBBS *et al*., 1989; KRIBBS *et al*., 1990a; KRIBBS, 1990b).

Um dos principais modelos de indução de osteoporose em animais consiste na excisão cirúrgica bilateral dos ovários (ovariectomia), onde a diminuição do nível de estrógeno resulta num quadro de osteopenia qualitativamente similar ao desenvolvida na osteoporose pós-menopausa humana (WRONSKI *et al.,*1988). Embora estudos clínicos não tenham sugerido que a osteoporose é um fator de risco para o insucesso dos implantes, estudos em ratas ovariectomizadas são praticamente unânimes em demonstrar que a deficiência de estrógeno ocasiona menor área de contato, quantidade e qualidade óssea ao redor de implantes, bem como em uma menor resistência ao torque. (YAMAZAKI *et al,* 1999).

Mecanismo de ação do estrógeno

Estudos laboratoriais têm demonstrado que o estrógeno age diretamente sobre as células ósseas, inibindo a reabsorção e promovendo osteogênese, uma vez que receptores para esse hormônio têm sido encontrados em todos os principais tipos de células do tecido ósseo (TURNER *et al.,* 1994). A deficiência de estrógeno, por sua vez, está relacionada a um aumento do número, do tempo de vida e da quimiotaxia de osteoclastos e seus precursores. É possível que a ação deste hormônio esteja associada à inibição da maturação e da atividade de reabsorção dos osteoclastos (JILKA *et al.* 1992). Em cultura de osteoblastos, o estrógeno parece estimular a proliferação e diferenciação dessas células (TURNER *et al.,* 1994).

O estrógeno pode ainda modular a produção de reguladores ósseos liberados local e sistemicamente por monócitos, macrófagos e linfócitos T (RAISZ, 1988). GIRASOLE *et al.* (1995) demonstraram o efeito do estrógeno na secreção de interleucina-6 (IL-6) e sua relação com

diferenciação osteoclástica. A IL-6 exerce importante papel no metabolismo ósseo por estimular sua reabsorção pela quimiotaxia e diferenciação de osteoclastos. (GIRASOLE *et al.,*1992). A interleucina-1 (IL-1) é o mais potente estimulador da atividade osteoclástica promovendo reabsorção óssea em estudos desenvolvidos *in vitro* (GOWEN *et al.,* 1983) e *in vivo* (LORENZO *et al.,*1998). Exerce efeito na síntese de proteínas colágenas e não-colágenas e induz as células ósseas a secretarem várias citocinas como IL-6, fator estimulador de colônias de macrófagos (M-CSF) e fator estimulador de colônias de macrófagos granulócitos (GM-CSF). Estas, por sua vez, regulam a proliferação de precursores osteoclásticos e sua diferenciação em osteoclastos (PACIFICI, 1992). PACIFICI *et al.,* em 1989, demonstraram que monócitos de pacientes osteoporóticos apresentaram maior atividade de IL-1 quando comparados a pacientes saudáveis. Os autores demonstraram que a atividade desta citocina aumentava após a menopausa, permanecia elevada por um período maior em mulheres portadoras de osteoporose e retornava a níveis pré-menopausa quando empregado tratamento com estrógeno. Efeito similar foi observado para o fator de necrose tumoral α (TNF-α) e o GM-CSF. Os monócitos humanos secretam simultaneamente IL-1 e um inibidor de IL-1. A deficiência de estrógeno possivelmente desequilibra o nível destas substâncias, diminuindo a secreção do inibidor e elevando o nível de IL-1 (PACIFICI, 1992).

Em nível molecular, tem sido demonstrado que a reabsorção óssea ocorre por intermédio de duas moléculas-chave, o ligante ativador do receptor NF-Kappa (RANKL) e a osteoprotegerina (OPG). O mecanismo de reabsorção óssea é significantemente reduzido pela inibição da função de RANKL nas células osteoclásticas, através da ação de OPG no receptor específico. O estrógeno, por sua vez, exerce um importante papel no controle da reabsorção óssea através de sua ação estimulatória em OPG. Dessa forma, tem sido sugerido que a deficiência de estrógeno induz um desequilíbrio no sistema RANKL/OPG, favorecendo assim a reabsorção óssea (LINDBERG *et al.,* 2001).

Tratamentos para a osteoporose

Diversos tratamentos têm sido propostos para a osteoporose com o objetivo de prevenir ou evitar a progressão da reabsorção óssea. Entre os tratamentos farmacológicos estão os agentes anti-reabsorção como a terapia de reposição estrogênica, os moduladores seletivos de receptores

de estrógenos (SERMs), os bisfosfonatos e a calcitonina (CT); e os agentes estimuladores da formação óssea como o paratormônio (PTH) (National Institute of Health- NIH, 2001).

Baseados nos mecanismos de ação do estrógeno no metabolismo ósseo, muitos estudos têm sugerido que a terapia de reposição estrogênica é capaz de prevenir a osteoporose e reduzir riscos de fraturas ósseas em mulheres no período pós-menopausa (MICHAELSSON *et al.,* 1998). Dessa forma, a terapia de reposição hormonal com estrógenos, administrados oralmente, de forma injetável ou através de adesivos cutâneos, tem sido assim considerada a principal forma de tratamento e prevenção de osteoporose pós-menopausa. Trabalhos clínicos e laboratoriais têm comprovado que a presença do hormônio reduz significantemente o ritmo de reabsorção óssea (CHRISTIANSEN *et al.,*1993). MICHAELSSON *et al.,* em 1998, em um estudo epidemiológico retrospectivo sugeriram que a terapia de reposição hormonal pode reduzir o risco de fratura em mais de 50% dos casos quando os sujeitos estão sob tratamento ou receberam o hormônio nos últimos cinco anos. WRONSKI *et al.,* em 1988, concluíram que o tratamento de ratas ovariectomizadas com estrógeno promoveu completa proteção contra o desenvolvimento de osteopenia na tíbia.

Embora essa terapia ofereça benefícios indiscutíveis para o tecido ósseo, a mesma tem demonstrado contra-indicações e risco de efeitos colaterais como aumento da incidência de câncer e de doenças cardiovasculares (Woman's Health Initiative, 2002). Dessa forma, muitos pacientes interrompem o tratamento ou procuram terapias alternativas para a osteoporose pós-menopausa (RODAN & MARTIN, 2000). As conseqüências do abandono do tratamento para o tecido ósseo têm sido investigadas por estudos em humanos e em animais. CHRISTIANSEN *et al.,* (1981) demonstraram uma acelerada taxa de perda óssea após a interrupção da terapia de reposição hormonal em mulheres no período pós-menopausa. WRONSKI *et al*., (1993) estudaram o efeito da interrupção da administração de estrógeno em ratas ovariectomizadas e concluíram que o efeito de proteção contra perda óssea exercido pelo hormônio foi completamente perdido após a retirada do mesmo.

Bisfosfonatos são agentes anti-reabsortivos derivados do ácido pirofosfônico, que constituem uma terapia alternativa para a prevenção da perda óssea gerada pela deficiência de estrógeno (TIRAS *et al.,* 2000). Alendronato é um dos mais potentes bisfosfonatos que apresenta

ação óssea específica além de grande afinidade e capacidade de adsorção ao tecido ósseo. Sua meia-vida óssea terminal é estimada em mais de dez anos, o que faz com que o medicamento apresente um efeito prolongado mesmo após a interrupção do uso (WRONSKI *et al*., 1993), na maioria das vezes devido a distúrbio gástrico (DONAHUE *et al.,* 2002). Seu mecanismo de ação parece estar relacionado à inibição seletiva da atividade osteoclástica (BUFFO *et al,* 1996) e, segundo estudos *in vitro,* a um efeito positivo na diferenciação e atividade osteoblástica. (GANDOLFI *et al,* 1999). Baseados nessas propriedades, estudos randomizados e placebos controlados têm demonstrado que o alendronato aumenta a massa óssea e reduz o risco de fraturas em 30% a 50% em mulheres osteoporóticas (GREENSPAN *et al.,* 2002). Tais dados clínicos estão de acordo com estudos desenvolvidos em animais ovariectomizados, que têm demonstrado que o alendronato é capaz de prevenir as alterações ósseas decorrentes da deficiência de estrógeno (THOMPSON *et al.,* 1992).

A calcitonina, isolada principalmente do salmão, também tem sido prescrita como terapia alternativa para o tratamento ou prevenção de desordens ósseas por inibir a reabsorção do tecido ósseo (GONZÁLEZ *et al.,* 1987; NODA & KUWAHARA*,* 1993) e por apresentar propriedades analgésicas (LYRITIS *et al.,* 2002). Este hormônio parece reduzir transitoriamente as concentrações plasmáticas de cálcio através de um efeito imediato na diminuição da atividade osteoclástica. Um efeito tardio desse hormônio consiste na diminuição da formação de osteoclastos (GUYTON, 1992). KALLIO *et al.,* em 1972, demonstraram que a calcitonina além de manter os osteoclastos separados da superfície óssea, provoca inibição da formação da "bordadura em escova" da membrana plasmática dessas células, estrutura fundamental em sua atividade de reabsorção. Em 1988, CIVITELLI *et al.,* afirmaram que existe ganho de massa e menor perda óssea em pacientes osteoporóticos tratados com calcitonina. WRONSKI *et al.,* em 1991, sugeriram que o hormônio promove, em curto prazo, proteção contra a osteopenia desenvolvida pela deficiência de estrógeno. Em 1996, SHEN *et al.* avaliaram, por meio de técnicas histométricas, as alterações ósseas ocorridas em ratas ovariectomizadas, tratadas por um longo período com calcitonina, e as conseqüências da interrupção desse tratamento. Os resultados demonstraram que a calcitonina diminuiu o remodelamento ósseo, mas foi capaz de prevenir apenas parcialmente o desenvolvimento de osteopenia no osso trabecular. A retirada do hormônio resultou em uma rápida perda do tecido ósseo.

Tratamentos para a osteoporose e os implantes dentais

Embora esteja bem documentado na literatura o benefício de cada medicamento para a osteoporose no tecido ósseo esquelético, poucos são os relatos sobre o efeito dos mesmos no tecido ósseo preexistente e neoformado ao redor de implantes, bem como o efeito dos mesmos no sucesso da osseointegração.

MINSK & POLSON (1998), não encontraram correlação entre a taxa de sucesso dos implantes dentais em mulheres pós-menopausa recebendo ou não reposição hormonal. AUGUST *et al.*, em 2001, compararam 168 mulheres pós-menopausa sem terapia de reposição hormonal, 75 com reposição hormonal, 114 mulheres pré-menopausa, 59 homens com menos de cinqüenta anos e 110 homens com mais de 50 anos em relação ao sucesso da osseointegração. Mulheres sem reposição hormonal obtiveram nível de fracasso maxilar significantemente maior (13,6%) que mulheres pré-menopausa (6,3%) e homens com mais de 50 anos (7,6%). Mulheres tratadas tiveram menor taxa de insucesso quando comparadas às não tratadas, embora essa diferença não tenha sido estatisticamente diferente (8,1%).

NARAI *et al.*, em 2003, demonstraram que o tratamento de ratas osteoporóticas com alendronato aumentou significantemente o torque de remoção, fazendo com os animais tratados com esse medicamento apresentassem mesma resistência ao torque que os ratos normais.

JANURIO *et al.* (2001) estudaram o efeito da calcitonina em implantes de titânio inseridos em tíbias de coelhos saudáveis para verificar se o hormônio era capaz de acelerar o processo de maturação óssea. Análises histométricas de 6, 8, 12 e 18 semanas após colocação dos implantes demonstram que a calcitonina é capaz de aumentar a massa óssea ao redor de implantes de titânio nos estágios tardios do processo de reparo. Esse estudo, porém, foi desenvolvido em animais saudáveis e objetivava observar o efeito da calcitonina na formação óssea ao invés de seu papel na contenção da reabsorção óssea.

Osteoporose, seus tratamentos e a doença periodontal

Periodontite consiste em uma doença inflamatória dos tecidos de suporte dental resultando em reabsorção do osso alveolar e perda de ligamento periodontal, sendo uma das maiores

responsáveis pela perda de dentes no adulto (PAGE & SCHROEDER, 1976). Embora seu agente etiológico seja o biofilme bacteriano específico, estudos clínicos e laboratoriais têm demonstrado que alguns fatores sistêmicos podem exercer um importante papel no estabelecimento e progressão da periodontite (GENCO, 1996). Desde de que alterações osteoporóticas têm sido observadas no tecido ósseo facial (KRIBBS, 1990), a baixa densidade óssea mineral tem sido considerada um indicador de risco para a doença periodontal (WACTAWSKI-WENDE, 1996).

O verdadeiro papel da osteoporose na etiopatogenia da doença periodontal ainda não está totalmente esclarecido. Ambas as doenças são multifatoriais e apresentam inúmeros fatores de risco em comum, como fumo, deficiências nutricionais, idade e disfunções do sistema imune (WACTAWSKI-WENDE, 1996). Assim, a dificuldade de isolamento dos fatores de confundimento tem sido uma das principais limitações dos estudos em humanos que correlacionam osteoporose e doença periodontal (WACTAWSKI-WENDE, 1996). Estudos clínicos correlacionando densidade óssea mineral (DOM) e doença periodontal (DP) têm apresentado resultados controversos (INAGAKI et al., 2001; MOHAMMAD *et al*., 2003). Enquanto alguns trabalhos sugerem ausência de associação significativa entre a DOM e a DP (MOHAMMAD *et al*., 1996), outros têm demonstrado que uma baixa DOM está positivamente correlacionada com uma reduzida altura de crista alveolar e maior perda de inserção periodontal (INAGAKI *et al*., 2001; MOHAMMAD *et al*., 2003).

Não há informações consistentes sobre o papel dos diferentes tratamentos para a osteoporose na doença periodontal. Estudos realizados em mulheres na pós-menopausa demonstraram que a terapia de reposição hormonal diminuiu a ocorrência de edentulismo e sangramento gengival (KRIBBS *et al.,* 1990*).* A terapia de reposição hormonal tem sido também associada a um aumento da densidade da crista óssea alveolar, embora ainda não esteja bem esclarecido se uma maior densidade alveolar reflete em menor perda de inserção periodontal (RONDEROS et al., 2000). Mulheres na pós-menopausa que se submeteram à terapia de reposição hormonal apresentam menor perda de inserção, demonstrando que o risco é atenuado na presença de terapia de reposição hormonal (ALBANDAR, 2002).

Além disso, dados de periodontite natural ou induzida têm sugerido que os bisfosfonatos podem retardar a perda óssea ao redor de dentes periodontalmente afetados, embora não ocorra redução dos sinais clínicos de inflamação da periodontite (REDDY et al. 1995).

PROPOSIÇÕES GERAIS

Os objetivos do presente trabalho, divido em sete artigos científicos, foram:

1- Avaliar o efeito da deficiência induzida de estrógeno sobre o reparo e densidade óssea ao redor de implantes de titânio inseridos em ratas ovariectomizadas;

2- Avaliar a influência do estrógeno e da calcitonina de salmão no contato e na área óssea dentro das roscas de implantes de titânio inseridos em ratas ovariectomizadas;

3- Avaliar a influência do estrógeno e da calcitonina de salmão no osso preexistente em uma região lateral a superfície de implantes inseridos em tíbias de ratas ovariectomizadas;

4- Avaliar a influência do estrógeno e do alendronato, bem como seus efeitos residuais, no osso neoformado e preexistente ao redor de implantes inseridos em ratas ovariectomizadas;

5- Avaliar o efeito da deficiência de estrógeno relacionada à idade, comparativamente ao efeito da deficiência de estrógeno induzida, sobre o reparo e densidade óssea ao redor de implantes de titânio;

6- Avaliar o efeito da deficiência de estrógeno e das terapias com estrógeno e calcitonina na perda óssea alveolar resultante da periodontite induzida;

7- Avaliar o impacto do alendronato, do estrógeno e da interrupção de ambos os medicamentos na perda óssea resultante da periodontite experimental em ratas ovariectomizadas.

CAPÍTULO 1

Implant Dentistry, v.12, p.340-345, 2003.

ESTROGEN DEFICIENCY AFFECTS BONE HEALING AROUND TITANIUM IMPLANTS. A HISTOMETRIC STUDY IN RATS.

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ABSTRACT

The purpose of this study was to evaluate the influence of an estrogen-deficient state on bone around titanium implants placed in rats. Thirty female Wistar rats were divided into two groups: test (n=15): ovariectomized rats (OVX); and control (n=15): sham operated rats. Screw-type titanium implants were placed bilaterally in rats 21 days after ovariectomy or sham surgery. After sixty days, the animals were sacrificed and undecalcified sections obtained. Blood samples were collected to obtain serum levels of alkaline phosphatase at the time of sacrifice. Bone-to-implant contact (BIC), bone area (BA) around the implants and bone density (BD) in a 500µm-wide zone lateral to the implant were obtained and arranged separately for the cortical (Zone A) and cancellous (Zone B) regions. In zone A, there was no significant difference between test and control groups regarding BIC and BD ($p>0.05$). It was observed a lower BA for the estrogen deficient animals ($p<0.05$). In Zone B, data analysis showed that estrogen deficiency may result in a lower percentage of BIC, BA and BD

($p<0.05$). In addition, a higher concentration of alkaline phosphatase was observed for the test group. Therefore, estrogen-deficient state may affect bone healing and bone density around titanium implants placed in rats, especially in the cancellous bone area.

KEY WORDS: estrogen deficiency, ovariectomy, titanium implants and osteoporosis

INTRODUCTION

The use of titanium endosseous dental implants in the treatment of edentulous or partially edentulous patients has become an alternative to restore function and esthetic[1-3]. The contact between bone tissue and implant surface is known as osseointegration and involves numerous complex factors[4]. These include not only implant-related factors, such as material, shape and surface chemistry, but mechanical loading, surgical technique, and patient variables, such as bone quantity and quality[4]. There are various risk factors that might affect bone quality, quantity and healing around titanium implants[5]. The influence of bone quality and quantity on the outcome of dental implants have been discussed in several studies, in which a high failure rate has been observed in patients presenting a poor bone quality and an inadequate bone volume [5-7].

Osteoporosis has been defined as a systemic skeletal disease characterized by gradual loss and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture [8]. Pathological conditions causing bone loss, such as hyperparathyroidism, hyperthyroidism and Cushing disease, and glucorticoid treatment can induce osteoporosis [9]. However, the most common cause of osteoporosis is the decrease of estrogen in menopause or ovariectomy [8-9].

Estrogen plays an important role in the regulation of bone turnover in adult bone. Thus, estrogen is associated with inhibition of osteoclasts differentiation and reduction of their action, inducing cancellous as well as cortical bone loss[10-11]. Besides, it has been reported that interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and other cytokines have either direct or indirect effects in the pathogenesis of post menopause osteoporosis. The production of all of these cytokines is suppressed or regulated by the estrogen level [12-13].

Significant relationships have been reported between oral bone and skeletal bone mass in postmenopausal women with osteoporosis [14-15]. Osteoporosis could, therefore, be considered as a risk factor in treatment planning and in determining the prognosis for patients considering dental implants [16]. However, clinical reports have been contradictory in correlating postmenopausal

osteoporosis as a risk factor for dental implants[17-18]. On the order hand, few animal studies, using ovariectomized animals model, have demonstrated that estrogen deficiency may induce histological changes around hydroxyapatite-coated and titanium screw dental implants [19-21].

The aim of the present study was to evaluate, by histometric analysis, whether estrogen deficiency, prior to implant placement, would influence bone healing and bone density around titanium implants placed in rats.

MATERIALS AND METHODS

ANIMALS

Thirty female Wistar rats aged 90 days old (190-220g) were included in this study. The animals were kept in plastic cages and provided drinking water *ad libitum*. The food consumption of the ovariectomized rats (OVX) was restricted to that of control rats (pair feeding) to minimize the increase in body weight associated with ovariectomy[22]. The whole protocol was approved by University of Campinas Institutional Animal Care and Use Committee.

OVARIECTOMY AND EXPERIMENTAL DESIGN

Ovariectomy or sham surgeries were performed at the beginning of the study (Fig 1). The rats were anesthetized with intramuscular administration of ketamine (0.5ml/kg) and divided into test and control groups. The test animals (n=15) had their ovaries exposed and completely excised from a dorsal approach. On the other hand, the control animals (n=15) had their ovaries lifted up and returned intact to the original position.

IMPLANTS SURGERY

Twenty-one days after the ovariectomies, general anesthesia was performed using intramuscular administration of ketamine (0.5ml/kg). The tibiae skin was shaved and disinfected with iodine surgical soap. A 10mm incision was made and the bone surface of the tibiae surgically exposed by blunt dissection. Under profuse saline solution irrigation, bicortical implant beds were drilled at a rotary speed not exceeding 1500 rpm. A screw-shaped commercially available pure titanium implant, of 4.0 mm in length and 2.2 mm in diameter, was placed bilaterally until the screw thread had been completely introduced into the bone cortex (Fig. 2). Finally, soft tissues were replaced and sutured. Postoperatively, the animals received antibiotic (Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil) given as a single intramuscular injection.

CLINICAL ANALYSES

In order to confirm the success of the ovariectomy procedure, estrus cycle was monitored two weeks after the sham and ovariectomy surgeries. The changes in the vaginal smear during 4-5 days of the estrus cycles were observed in each group. At autopsy, success of the ovariectomy was also confirmed by absence of ovaries and atrophy of uterine horns.

BIOCHEMICAL SERUM ANALYSES

Blood samples were collected to measure plasma concentration of alkaline phosphatase at the time of sacrifice (60 days after implant insertion). Using automated laboratory techniques, alkaline phosphatase activity was obtained colorimetrically (Gold Analisa Diagnóstica, Belo Horizonte, MG, Brazil).

HISTOMETRIC PROCEDURE

After 60 days, the animals were sacrificed, the tibiae were removed and fixed in 4% neutral formalin for 48 hours. Undecalcified sections were prepared as previously described[23], i.e. the blocks were dehydrated by using an ascending series of ethanol (60-100%) and embedded in glycolmethacrylate (Technovit 7200®; Heraeus Kulzer GmbH, Wehrheim, Germany).Subsequently, the sections (20-30µm) were obtained and stained using 1% toluidine blue. The percentages of bone-to-implant contact (BIC) and bone area (BA) within the threads of the implants were obtained bilaterally. Besides, bone density (BD), i.e., the proportion of mineralized matrix, in a 500 µm-wide zone lateral to the implant surface, was separately recorded for both sides of the implant (Image-Pro®; Media Cybernetics, Silver Spring, MD, USA). Data were arranged separately in cortical (Zone A) and cancellous bone (Zone B).

STATISTICAL ANALYSIS

Data from Zones A and B (cortical and cancellous bone, respectively) were separately averaged. The hypothesis that estrogen deficiency had no influence on the bone density and bone healing was tested using Mann-Whitney's test (alpha = 0.05). In addition, to test the hypothesis that estrogen deficiency did not influence alkaline phosphatase serum level and that there was no difference between the groups with respect to the animals´ body weight during experimental period, the t-Student test was used (alpha = 0.05).

RESULTS

CLINICAL ANALYSES

All animals gained weight during the course of the study, however there was no difference between test and control groups at the beginning and the end of the experiment ($p>0.05$) (Table 1).

In the present study, macro analysis of the uterine horns, the absence of ovaries and assessment of the estrous cycle of the rats confirmed the success of the ovariectomy surgery. All OVX animals presented *diestrus* smear and their reproductive organs atrophied, therefore confirming the reduction of serum estrogen levels in this group. On the order hand, the four stages of the estrous cycle (*estrus, metastrus, diestrus and proestrus*) and a pink and fluid filled uteri were clearly identified in the sham group, assuring that the serum estrogen levels were kept normal in these animals.

BIOCHEMICAL SERUM ANALYSIS

Serum concentrations of alkaline phosphatase (UI) and standard deviation, performed at the time of sacrifice, were 80.47 $\pm$ 20.16 and 29.13 $\pm$ 10.93 for test and control groups, respectively. Intergroup analyses demonstrated a significant difference between the test (OVX) and control (sham) groups ($p<0.05$), regarding the alkaline phosphatase level.

HISTOMETRIC RESULTS

In zone A, the data showed no significant difference between test and control groups regarding BIC ($p=0.237$) and BD ($p=0.074$). On the other hand, estrogen deficiency decreased the percentage of BA within the limits of the threads of the implants ($p=0.0310$) (Table 2). In Zone B, data analysis showed that estrogen deficiency may result in a lower percentage of BIC ($p=0.0152$), BA ($p=0.0181$) and BD ($p<0.01$) (Table 2). Figure 3 illustrates the histological aspects observed for both groups.

DISCUSSION

High long-term success rates have been reported for implant-supported prostheses in fully and partially edentulous patients[1-3]. However, among other factors, the medical status of the patient has been associated with biological failures of dental implants[5]. With the growth of the elderly population, the number of patients with senile or postmenopausal osteoporosis asking for implant treatment has increased[5]. The hypothesis that postmenopausal osteoporosis is a negative factor for dental implants outcomes is based on the fact that besides affecting other parts of the skeleton, it affects oral bone as well, [14-15] resulting in alterated bone metabolism and reduced bone healing capacity [8, 10-11].

Therefore, the present study aimed to investigate in rats the influence of estrogen deficiency, prior to implant placement, on the bone healing process around titanium implants. Ovariectomized rats have long been described as a reliable model to study estrogen deficiency, and therefore, were used in the present study [19-22].

In the current study, implant placement was performed 21 days after ovariectomy and the bone around implants was evaluated 60 days after the implant placement[19]. The analysis of estrus cycle and autopsy confirmed the success of ovariectomy in test group and it could have detected animals in which the ovariectomy could have failed, if there were any. Results indicated that estrogen deficiency, prior to the implant placement, had no effect on bone to implant contact in the cortical area. Nevertheless, the bone area with the limits of the threads of the implant in the cortical area, was significantly affected by the hormone deficiency (Table 2). Similarly, all parameters were significantly influenced, in the cancellous bone area, by the decrease in estrogen levels (Table 2). Based on these findings, one may assume that estrogen deficiency may have enhanced bone resorption in the newly formed bone around titanium implants. The data generated by the present study are similar to those reported by other studies [19-21]. However, differently than any other, the implants were placed in rats with early established osteoporosis (21 days).

Since bone quality is an important factor for retaining an implant-supported prostheses (later failure) [6-7], the present study evaluated the influence of estrogen deficiency on the proportion of mineralized bone matrix in a 500 µm-wide zone lateral to the implant surface, besides its influence on the healing process (early failure). Bone density was lower in osteopenic (OVX) than in normal animals in the cancellous, but not in the cortical bone. Therefore, it can be suggested that estrogen deficiency affected bone not only in newly formed bone around the implant, but also in the preexisting cancellous bone, reducing the proportion of mineralized matrix.

The negative effects of estrogen deficiency on newly and preexisting bone around titanium implants, observed in the present study and other studies[16-21], might have been caused by accelerated osteoclastic bone resorption in the OVX animals[10-11,24]. The estrogen protective mechanism apparently involves suppression of bone turnover as a direct effect of estrogen on bone cells, in which this hormone induces cells of the osteoblastic lineage to inhibit osteoclastic bone resorption[10-11,24]. Further, estrogen was found to regulate the synthesis and secretion of some cytokines involved in bone metabolism[12-13].

Alkaline phosphatase has been described as a biochemical marker of high bone turnover used to reflect the changes in bone during the diagnosis of osteoporosis. In this study, the alkaline phosphatase level was significantly elevated in the OVX group, indicating a high bone turnover promoted by estrogen deficiency. This is a similar observation to previous animal studies in which osteopenic rats showed increased levels of alkaline phosphatase[25-26]. In addition, our results are in agreement with clinical studies, which also demonstrated that alkaline phosphatase level is about 20 % higher in the menopausal period [27].

The present study provides pre-clinical information regarding titanium dental implants placed in individuals with osteoporosis induced by estrogen deficiency. However, long-term clinical studies are necessary to compare osteoporotic with non-osteoporotic subject under the effects of occlusion stimulation, and controlling factors such as medical history, diet, smoking and physical activity [28]. Thus, within the limits of this study it can be concluded that estrogen deficient state has a negative influence on bone healing and bone quality around titanium implants. It could suggest that when it comes to elderly patients or menopausal women, clinicians should be aware when placing conventional implants in regions with predominantly cancellous bone.

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Table 1: Mean and standard deviation (grams) of body weight for test and control groups at the beginning of the experiment and before sacrifice.

	INITIAL	SACRIFICE
TEST	203.00 ± 9.75 aA	261.67 ± 14.98 aB
CONTROL	204.40 ± 14.94 aA	256.27 ± 15.70aB

Different letters (lower case - column, capital - line) indicate significant differences (α=0.05)

Table 2: Mean and standard deviation (%) of bone-to-implant contact (BIC), bone area (BA) within the limits of the implant threads and the proportion of mineralised tissue (BD) in a 500 µm-wide zone lateral to the implant, for test (OVX) and control (sham rats) groups in Zone A (cortical bone) and Zone B (medullar bone).

	CORTICAL BONE			MEDULLAR BONE	
	TEST	**CONTROL**		**TEST**	**CONTROL**
BIC	45.61 ± 8.36 A	50.99 ± 15.19 A	**BIC**	40.28 ± 9.52 A	51.75 ± 12.54 B
BA	81.35 ± 3.81 A	84.76 ± 3.74 B	**BA**	41.38 ± 10.82 A	49.26 ± 5.60 B
BD	93.27 ± 3.81 A	95.39 ± 1.34 A	**BD**	8.26 ± 4.77 A	48.39 ± 9.37 B

Different letters (line) indicate significant differences within cortical and medullar bone (α=0.05).

Figure 1: Experiment design.

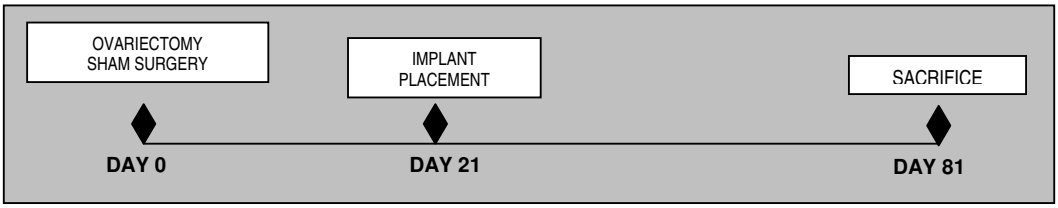


Figure 2: The tibiae surface was exposed by dissection and a titanium implant was inserted until the screw thread had been completely introduced into the bone cortex.

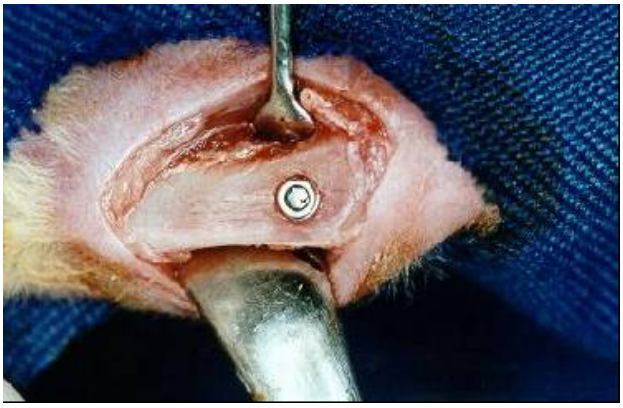
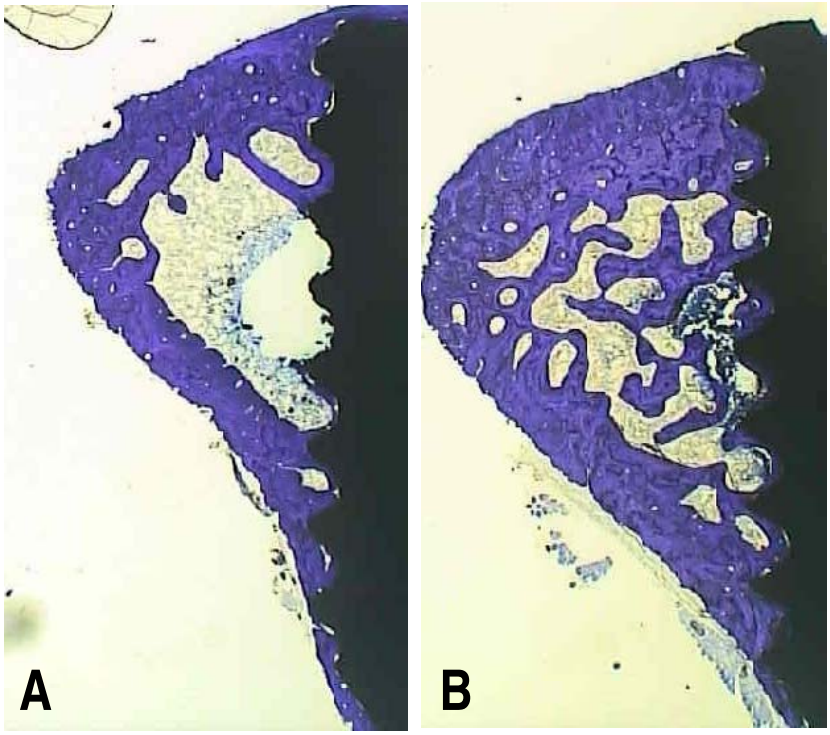


Figure 3: Photomicrographs illustrating the histological aspects observed within the limits of the threads and in a 500 µm-wide zone lateral to the implant surface. Figures A and B illustrate test (OVX) and control (sham) groups, respectively. (Toluidine blue / Original magnification = 6.25x).



CAPÍTULO 2

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EFFECT OF ESTROGEN REPLACEMENT AND CALCITONIN THERAPIES ON BONE AROUND TITANIUM IMPLANTS PLACED IN OVARIECTOMIZED RATS. A HISTOMETRIC STUDY

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ABSTRACT

The aim of the present study was to evaluate whether hormone replacement therapy (HRT) and calcitonin (CT) administration could influence bone healing around implants placed in ovariectomized rats (OVX). One screw-type titanium implant was placed bilaterally in ovariectomized rats. The animals were assigned to one of the following groups: Group 1 (n=15) - sham surgeries; Group 2 (n=15) - OVX rats; Group 3 (n=14) - OVX rats administered with calcitonin (CT) 4 days/week (16 IU/Kg); Group 4 (n=14) - OVX rats administered with 17β estradiol (20μg/Kg). After 60 days, the animals were sacrificed and undecalcified sections obtained. Bone-to-implant contact (BIC) and bone area (BA) around the implants were separately determined for the cortical (Zone A) and cancellous (Zone B) bone areas. In Zone A, intergroup analysis did not reveal significant difference regarding BIC. In contrast, the HRT-group presented a greater BA than groups 2 and 3 ($P<0.05$). Data from

Zone B revealed that HRT nullified the negative effect of the ovariectomy on BIC and BA ($P<0.05$), while CT presented no effect ($P>0.05$). Within the limits of the present study, it may be concluded that HRT may prevent the influence that estrogen deficiency exerts on bone healing around titanium implants.

KEY WORDS: estrogen deficiency, titanium implants, calcitonin, hormone replacement therapy.

INTRODUCTION

Titanium endosseous implants have been increasingly used in various edentulous situations[1-4]. However, because the success of osseointegration depends, in part, on the state of the host bone bed and its healing capacity, concerns have been raised regarding conditions affecting its quality and quantity[5].

Since osteoporosis is a skeletal disorder in which bone density and bone mass decrease as a function of a high rate of bone turnover[6], some studies have investigated the impact of osteoporosis on osseointegrated implant outcomes[5,7]. Recently, the reactions of bone tissues following placement of implants under estrogen-deficient conditions have been studied using experimental animals[8-10]. Generally speaking, estrogen deficiency seems to negatively influence bone quality around titanium implants.

To prevent or "treat" bone loss in postmenopausal women, i.e., estrogen deficient individuals, hormone replacement therapy (HRT) and calcitonin (CT) have been used. An increased trabecular bone volume, unchanged mechanical properties and depressed bone turnover have been reported as benefits of the HRT[11-13]. Similarly, normal bone volume and decreased bone turnover have been reported for estrogen-deficient animals (ovariectomized) treated by osteoclast inhibitors such as CT[14].

To date, no information is available regarding the effect of HRT and CT administration on the bone healing around osseointegrated implants placed in estrogen-deficient animals. Therefore, the present study was designed to evaluate, by histologic analysis, whether HRT and CT administration could prevent the negative influence of estrogen deficiency around titanium implants placed in ovariectomized rats.

MATERIALS AND METHODS

ANIMALS

The experimental animals were 58 female Wistar rats which were 90 days old and weighed 210 g at the beginning of the study. The animals were kept in plastic cages with access to water *ad libitum*. The food consumption of the ovariectomized (OVX) rats was restricted to that of control rats (pair feeding) to minimize the increase in body weight associated with ovariectomy[15]. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee (School of Dentistry at Piracicaba, University of Campinas. Piracicaba, S.P., Brazil).

OVARIECTOMY PROCEDURE

General anesthesia was obtained by intramuscular administration of ketamine (0.5ml/kg). Bilateral ovariectomies were performed in 43 rats from a dorsal approach. The remaining 15 rats were subjected to sham surgeries in which the ovaries were exteriorized and replaced intact. For the sham-ovariectomy, the ovaries were lifted up and returned to the original position.

EXPERIMENTAL DESIGN

On the day after the ovariectomies, the animals were assigned to one of the four treatment groups: **Group 1** (n=15) - sham surgeries (negative control); **Group 2** (n=15) - OVX rats (positive control); **Group 3** (n=14) - each OVX rat in this group was injected subcutaneously with CT (Miacalcic®, Sandoz A.G., Fertigung Schützenstrsse, Ravenburg, Germany) 4 days/week at a dose of 16 IU/Kg body weight; **Group 4** (n=14) - each OVX rat in this group was injected subcutaneously daily with 17β estradiol (Sigma Chemical, St. Louis, MO, USA), dissolved in 100% ethanol and diluted in mineral oil at a dose of 20μg/Kg body weight.

IMPLANT SURGERY

Twenty-one days after the ovariectomies, all animals were anesthetized (ketamine - 0.5ml/kg). The skin was cleansed with iodine surgical soap and an incision of approximately 1 cm in length was made and the bone surface of the tibiae surgically exposed by blunt dissection. Under profuse saline irrigation, bicortical implant beds were drilled at a rotary speed not exceeding 1500 rpm and one screw-type commercially available pure titanium implant (AS Technology, São José dos Campos, S.P., Brazil), of 4.0 mm in length and 2.2 mm in diameter, was placed bilaterally. The soft tissues were replaced and sutured. Postoperatively, the animals received antibiotic (Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil) given as a single intramuscular injection.

SERUM ANALYSES

At the time of sacrifice (60 days after implant placement), blood was drawn to measure the serum level of alkaline phosphatase and calcium. Alkaline phosphatase activity was obtained colorimetrically (Gold Analisa Diagnóstica, Belo Horiaonte, MG, Brazil) and calcium by the ion selective electrode method (Eletrolyte Analyzer, AVL Scientific Corporation, Roswell, Georgia, USA).

CLINICAL ANALYSES

To confirm the success of the ovariectomy, two weeks after the ovariectomy and sham surgeries, the estrous cycle of the rats was daily monitored for one week by collecting vaginal smears. In addition, the success of the ovariectomy was also confirmed at necropsy by marked atrophy of the uterine horns and no histologic evidence of ovarian tissue at the surgical site.

HISTOMETRIC PROCEDURE

After 60 days, the animals were sacrificed; the tibiae were removed and fixed in 4% neutral formalin for 48 hours. Undecalcified sections were prepared as previously described[16], i.e. the blocks were dehydrated by using an ascending series of ethanol (60-100%) and embedded in glycolmethacrylate resin (Technovit 7200® ; Heraeus Kulzer GmbH, Wehrheim, Germany). Subsequently, the sections (20-30 µm) were obtained and stained using toluidine blue 1% staining. The percentage of bone-to-implant contact (BIC) and bone area (BA) within the threads of the implants was determined bilaterally (Image-Pro®; Media Cybernetics, Silver Spring, MD, USA) and arranged separately in cortical (Zone A) and cancellous bone (Zone B) areas.

STATISTICAL ANALYSIS

Data from Zones A and B (cortical and cancellous bone, respectively) were separately averaged. The hypothesis that HRT and CT administration had no influence on the bone healing around the implants placed in the OVX rats was tested using an intergroup analysis (Kruskal-Wallis test - alpha = 0.05). In the case that statistical difference was detected, Dunn´s method was used to isolate the groups that differed from the others. In addition, to test the hypothesis that HRT and CT administration did not influence alkaline phosphatase and calcium serum levels; the one way ANOVA (alpha = 0.05) was used. If statistical difference was detected, a pairwise multiple comparison procedure was used (Bonferroni t-test).

Finally, an intergroup analysis was used to test the hypothesis that there was no difference between the groups with respect to the animals´ body weights at the end of the experimental period (one way ANOVA - alpha = 0.05).

RESULTS

CLINICAL OBSERVATIONS

All animals gained weight during the course of the study. The animals from groups 1, 2 and 3 weighed significantly more than the animals from group 4 ($P<0.05$). The final mean body weights were 256.27g ± 15.70, 261.67g ± 14.98, 255.47g ± 20.25 and 228.57g ± 13.68, for the animals from groups 1, 2, 3 and 4, respectively.

In the present study, macro and microscopic analysis of the uterine horns, and assessment of the estrous cycle of the rats confirmed the success of the ovariectomy surgery. All OVX and CT animals were in the diestrus stage and presented their reproductive organs atrophied, therefore confirming the reduction of serum estrogen levels in these two groups[17]. In contrast, the sham group presented the four stages of the estrous cycle (estrus, metestrus, diestrus and proestrus), while the HRT animals remained in the estrus stage. Furthermore, macro and microscopic analysis showed that the reproductive organs of sham and HRT-groups were intact, assuring that the serum estrogen levels were maintained in both groups.

SERUM ANALYSES RESULTS

The alkaline phosphatase serum level varied between the experimental groups. It was similar for the animals from groups 1 and 4 (29.13IU/l ± 10.93 and 33.29 IU/l ± 14.91, respectively - $P>0.05$), but statistically higher in the animals from groups 2 and 3 (80.47 IU/l ± 20.16 and 98.20 IU/l ± 14.27, respectively - $P<0.05$).

Regarding the calcium serum level, the animals, which were ovariectomized and did not receive either HRT or CT (group 2), presented higher values than the animals from groups 1, 3 and 4 ($P<0.05$). The mean calcium serum levels were 1.10mmol/l ± 0.07, 1.24 mmol/l ± 0.08, 1.10 mmol/l ± 0.14 and 1.07 mmol/l ± 0.13 for groups 1, 2, 3 and 4, respectively. Figures 1a and 1b illustrate the results observed for alkaline phosphatase and calcium serum levels, respectively.

HISTOMETRIC RESULTS

Intergroup analysis did not reveal significant differences regarding BIC in Zone A ($P=0.64$). Therefore, in the cortical bone area, estrogen deficiency did not appear to influence osseointegration around titanium implants. On the other hand, in Zone B, data analysis showed that estrogen deficiency may result in a lower percentage of BIC than the animals not submitted to ovariectomy ($P<0.05$). However, this negative effect was restored in the OVX, which received 17β estradiol, to a

similar percentage as that of the sham surgery animals. In addition, data analysis revealed a slight positive effect of CT administration on the percentage of BIC in the OVX animals, but this difference was not statistically significant (P>0.05) (Table 1).

Data analysis also showed that in the cortical and cancellous bone regions (Zones A and B), a slight difference in the BA was noted between groups 1, 2 and 3, but this was not statistically significant (P>0.05). In contrast, the OVX animals administered with 17β estradiol (group 4) presented a statistically higher percentage of BA than animals from groups 2 and 3 (P<0.05) (Table 1). Figures 2 to 5 illustrate the histologic findings.

DISCUSSION

Risk factors for osteoporosis have been extensively studied[18-19]. Estrogen levels present prior to menopause are protective against the bone loss resulting from osteoporosis. Therefore, early menopause, naturally occurring, drug- or surgically-induced predisposes individuals to osteoporosis[18].

The influence that osteoporosis exerts on the implant outcome has been investigated. Some authors have suggested on the basis of clinical observations, that osteoporosis is not always a risk factor in osseointegration[7,20]. On the other hand, studies based on histologic observations have reported some negative effects of estrogen deficiency on the bone healing around titanium implants (induced osteoporosis)[8-10].

Although the present study did not aim to investigate the effect of ovariectomy on the bone around titanium implants, the present results showed that estrogen deficiency may slightly influence bone healing around titanium implants in the cortical area (Zone A) and significantly decrease the BIC in the cancellous bone region (Zone B). Extrapolation of the observations found in animal models to a clinical situation is not possible. However, the indication provided by the experimental studies that estrogen deficiency may influence BIC and BA around titanium implants should be investigated on a long-term basis.

Most bone diseases, including osteoporosis, are caused by increased bone resorption, rendering its inhibition a primary therapeutic objective. Indeed, most bone therapies currently available belong to this category. Inhibition of bone resorption can be accomplished by reducing either osteoclast generation (for example, with estrogens) or osteoclast activity (with CT). Estrogen replacement therapy has long been considered the first line therapy for preventing osteoporosis. Treatment with

estrogens clearly inhibits bone loss as well as bone turnover and increases bone mineral density[21]. The molecular mechanism of action of estrogen on bone, as well as on other tissues, is under investigation[22].

Calcitonin (CT) is a polypeptide hormone that has been used to treat or prevent bone metabolism disorders, because of its capacity to inhibit bone resorption[23] and because of its analgesic properties[24]. Because no information is available regarding the effect of HRT and CT administration on the bone healing around osseointegrated implants placed in estrogen deficient animals, the present study aimed to evaluate whether HRT and CT administration could prevent the negative influence of estrogen deficiency on bone around titanium implants placed in ovariectomized rats. The results of the present study showed that estrogen administration immediately after ovariectomy, may neutralize the negative effects of estrogen deficiency on both parameters analyzed, i.e., bone-to-implant contact and bone area around the implants placed in the tibiae of the rats. The OVX rats which daily received 17β estradiol (group 4) present similar levels of BIC and BA to the sham operated animals (group 1). On the other hand, in the present study, although data analysis revealed a slight positive effect of CT administration after ovariectomy, this was not statistically significant.

The biochemical serum analysis supported the histologic results of this study. HRT resulted in a similar level of alkaline phosphatase and calcium to the control group (sham), indicating that estrogen administration might has controlled the high bone turnover promoted by the ovariectomy (increased alkaline phosphatase levels) and blocked its influence on the bone tissue around the implants. In contrast, although CT may have presented its biologic properties, decreasing the level of serum calcium, it did not decrease bone turnover in the OVX animals. Calcitonin-induced loss of CT receptors, resulting in hormone-induced resistance, has been previously reported[25-26]. Whether this was the case in the present investigation remains to be investigated. Thus, the bone tissue around the implants in the CT-treated animals was negatively affected by estrogen deficiency.

The results of the present study are in agreement with previous studies regarding the benefits of HRT to the bone metabolism in estrogen-deficient animals[11-13,27]. Moreover, regarding the effect of CT on bone metabolism in ovariectomized rats, the results of the present study are closely related to the study by Shen et al. (1995)[28] who showed that CT partially prevented bone loss in ovariectomized rats. Results, though, are not in agreement with the report by Wronski et al. (1991)[14] who observed

that CT treatment depressed bone turnover and prevented the development of osteopenia in OVX rats.

Therefore, within the limits of the present investigation, it can be suggested that HRT immediately after ovariectomy may be important to prevent the influence that estrogen deficiency may exert on bone healing around titanium implants. Nevertheless, further studies should be considered to clinically confirm the present data and to investigate whether other inhibitors of osteoclast activity, such as diphosphonates, may influence bone around titanium implants in estrogen-deficient animals. In addition, whether HRT is able to revert, not prevent, the effect of estrogen deficiency on bone healing around titanium implants should also be studied.

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Figure 1a: Mean and standard deviation (IU/l) of alkaline phosphatase serum levels.

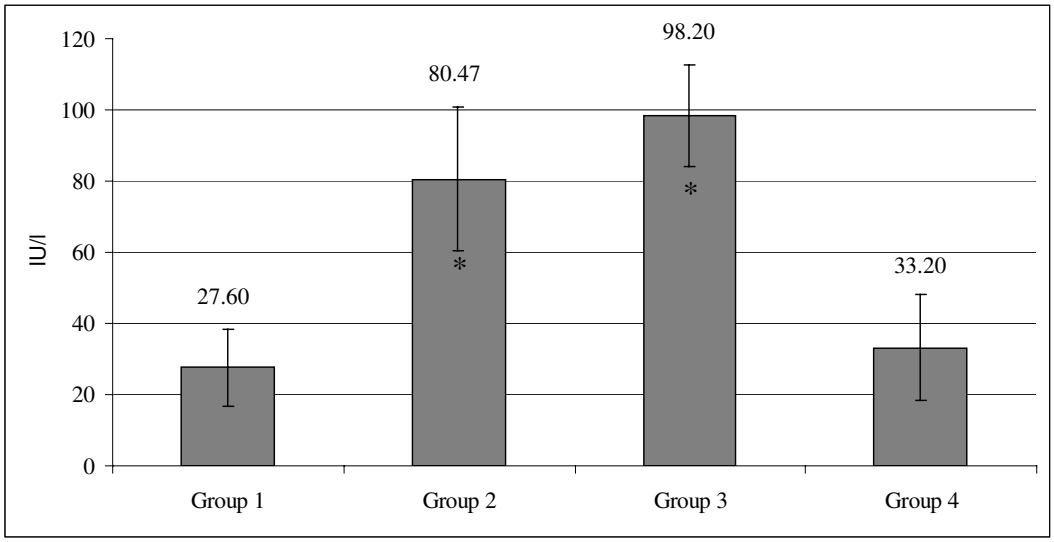

Figure 1b: Mean and standard deviation (mmol/l) of calcium serum levels.

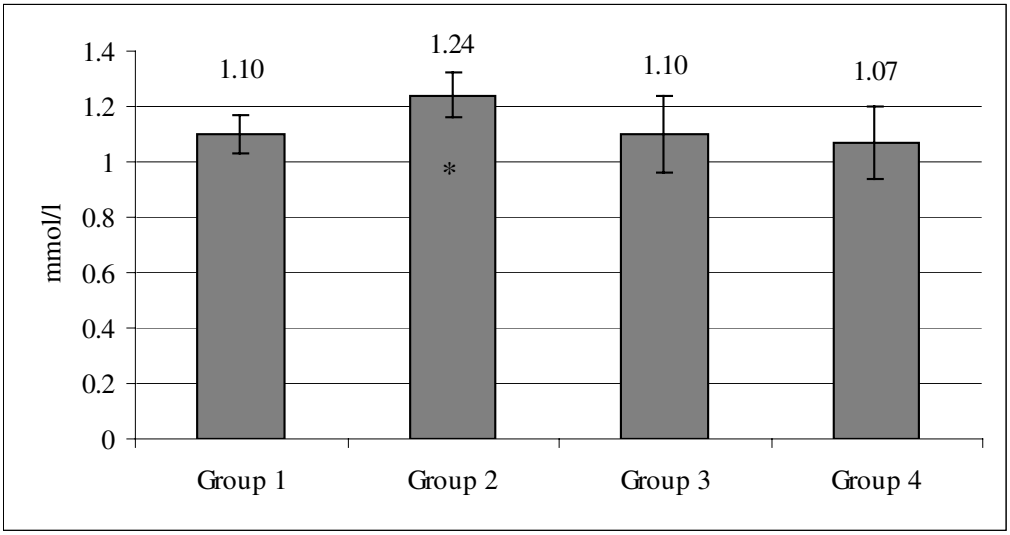

Figure 2: Micrograph of a ground section showing the histologic findings for the animals submitted to sham surgery (group 1). Note that after 60 days, in the cortical zones the bone is in a close contact with the implant surface (➡), and in the cancellous bone area (✱), it is also observed some bone proliferation on the implant surface (Toluidine blue / Original magnification = 6.25x).

Figure 3: Photomicrograph illustrating the histologic aspect observed around the implants placed in the animals that were submitted to ovariectomy (group 2). Note that estrogen deficiency negatively influenced the bone tissue around the implant, especially in the cancellous bone compartment (✱) (Toluidine blue / Original magnification = 6.25x).

Figure 4: Photomicrograph that, histologically, illustrates the bone tissue around the implants placed in the animals that were submitted to ovariectomy and received daily injections of calcitonin (group 3). Observe that calcitonin was not able to prevent the negative effect of estrogen deficiency on bone around the implant, especially in the cancellous bone area (✱), when compared to the control group (figure 2) (Toluidine blue / Original magnification = 6.25x).

Figure 5: Micrograph illustrating the histologic findings observed for the animals that were submitted to ovariectomy and treated by the hormone replacement therapy. A significantly positive effect of the treatment was observed either for the cortical or cancellous bone as compared to groups 2 and 3 (Toluidine blue / Original magnification = 6.25x).

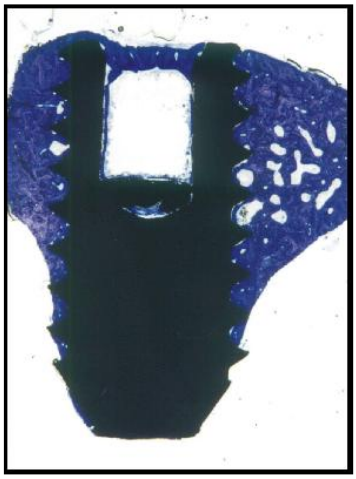

Table 1: Mean and standard deviation (%) of bone-to-implant contact (BIC) and bone area (BA) within the limits of the implant threads for groups 1 to 4 at Zones A and B.

	Zone A			**Zone B**	
	BIC	BA		BIC	BA
Group 1	50.98 ± 15.19**a**	84.76 ± 3.74 **ab**	Group 1	51.75 ± 12.53**a**	49.26 ± 5.60**ab**
Group 2	46.00 ± 7.94 **a**	81.35 ± 3.81**a**	Group 2	40.28 ± 9.51**b**	41.38 ± 10.82**a**
Group 3	49.79 ± 14.81**a**	82.65 ± 5.15**a**	Group 3	48.60 ± 10.16**ab**	42.64 ± 5.49**a**
Group 4	50.45 ± 12.76**a**	88.36 ± 3.39**b**	Group 4	52.99 ± 10,27**a**	58.56 ± 12.22**b**

Non-capital letters should be considered in columns.

CAPÍTULO 3

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EFFECT OF ESTROGEN AND CALCITONIN THERAPIES ON THE BONE DENSTIY IN A LATERAL AREA ADJACENT TO IMPLANTS PLACED IN THE TIBIAE OF OVARIECTOMIZED RATS.

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ABSTRACT

Background: This study evaluated the influence of estrogen and calcitonin administration on tibial bone density in a lateral area adjacent to implants placed in ovariectomized rats (OVX).

Methods: One screw-type titanium implant was placed bilaterally in the ovariectomized rats, and the animals assigned to one of the following groups: Group 1 (n=15): sham surgeries; Group 2 (n=15): OVX; Group 3 (n=14): OVX subcutaneously administered with calcitonin (CT) 4 days/week (16 IU/kg); Group 4 (n=14): OVX rats daily administered with 17β estradiol (20μg/Kg). After sixty days, the animals were killed and undecalcified sections obtained. Blood samples were collected to measure serum levels of alkaline phosphatase and calcium at the time of sacrifice. Bone density was measured in a 500μm-wide mineralised zone lateral to the implant.

Results: Alkaline phosphatase levels in groups 2 and 3 (P>0.05) were statistically higher than groups 1 and 4 (P<0.05) and calcium serum level was higher in group 2 than the other groups (P<0.05). Regarding bone density, the data were arranged separately for cortical (Zone A) and cancellous (Zone B) bone. In Zone A, intergroup analysis revealed no significant difference among groups (P>0.05). However, in Zone B the animals that received estrogen administration (group 4) presented a higher bone density than groups 2 and 3 (P<0.05).

Conclusion: Thus, it appears that estrogen therapy may prevent the negative influence of endogenous estrogen deficiency on bone density around titanium implants placed in ovariectomized rats.

KEY WORDS: estrogen deficiency, ovariectomy, titanium implants, calcitonin, osteoporosis

INTRODUCTION

An increasing number of titanium endosseous dental implants has been used to replace and restore function and esthetic in edentulous patients. Successful outcomes can be expected when dental implants are placed in good bone quality and quantity[1-3]. However, local and systemic conditions, which may impair bone healing or may interfere with the stability of the implants, have been described[4-5].

Osteoporosis is a systemic skeletal disease characterized by low bone mass, widened trabecular space and thin cortical bone due to a high rate of bone turnover[6]. The ovarian hormone deficiency (estrogen) results in increased bone turnover and imbalance in favor of resorption, causing osteoporosis[7-10]. These osteoporotic changes have been observed in the oral bone[11-12], however; only a few studies have investigated the impact of osteoporosis on osseointegrated implant outcomes. In summary, clinical reports have been contradictory[13-14] in correlating osteoporosis as risk factor for dental implants, and experimental studies, using animal models, have demonstrated that estrogen deficiency may influence bone healing around titanium implants[15-18].

Estrogen plays an important role in the regulation of bone turnover in adult bone. In the menopause, estrogen deficiency induces cancellous as well as cortical bone loss[19]. At a cellular level, estrogen inhibits differentiation of osteoclasts thus decreasing their number and reducing the amount of active remodeling units[20]. At a molecular level, estrogen regulates the expression of IL-1, IL-6 and TNF-α, cytokines that regulate bone resorption[21-22].

The administration of estrogen is the most currently and widely treatment used in the prevention of bone loss in postmenopausal women and estrogen deficient individuals[19]. In fact, many observations in animal and clinical studies show that after estrogen hormone replacement therapy (HRT), skeletal remodeling is reduced and bone loss is halted[23-25].

Calcitonin (CT), a peptide hormone, has the property of inhibiting bone resorption by acutely blocking osteoclast activity. Studies in humans and animal models have demonstrated its usefulness in treating estrogen deficiency-induced osteoporosis[26-27].

Although, it has been demonstrated, in animal models, that estrogen deficiency impairs bone quality around titanium implants, no information is available regarding the effect of HRT (estrogen) and CT administration on the bone density around osseointegrated implants placed in estrogen-deficient animals. Therefore, the present study was designed to evaluate, by histological analysis, whether estrogen and CT administration could prevent the negative influence that endogenous estrogen deficiency exerts on bone density around titanium implants placed in the tibiae of rats.

MATERIALS AND METHODS

ANIMALS

The experimental animals were 58 female Wistar rats that were 90 days old and weighed an average of 210g at the beginning of the study. During the period of the experiment, the animals were housed in groups of six in plastic cages. Food and water were given *ad libitum* to all animals, except ovariectomized (OVX) rats not on estradiol treatment (pair feeding)[28]. This protocol was approved by University of Campinas Institutional Animal Care and Use Committee.

OVARIECTOMY PROCEDURE

All rats were anesthetized with intramuscular administration of ketamine (0.5ml/kg). Skin was cleansed with iodine surgical soap. Bilateral ovariectomies were performed in 45 rats from a dorsal approach. The remainders were subjected to sham surgeries in which the ovaries were lifted up and returned intact to the original position.

EXPERIMENTAL DESIGN

Twenty-four hours after ovariectomies, the animals were randomly assigned to one of the four groups: Group 1 (n=15): sham surgeries (negative control); Group 2 (n=15): OVX rats (positive control); Group 3 (n=14) OVX rats and 4 days/week subcutaneous injections of CT† at a dose of 16

IU/Kg body weight; Group 4 (n=14): OVX rats and daily subcutaneous injections of 17β estradiol‡, dissolved in 100% ethanol and diluted in mineral oil at a dose of 20μg/Kg body weight.

IMPLANTS SURGERY

Twenty-one days after ovariectomies, general anesthesia was performed using the ovariectomy protocol (ketamine - 0.5ml/kg). The skin was cleansed with iodine surgical soap, an incision of approximately 1cm in length was made and the bone surface of the tibiae surgically exposed by blunt dissection. Under profuse saline solution irrigation, bicortical implant beds were drilled at a rotary speed not exceeding 1500 rpm. A screw-shaped commercially available pure titanium implant, of 4.0 mm in length and 2.2 mm in diameter, was placed bilaterally until the screw thread had been completely introduced into the bone cortex. Finally, soft tissues were replaced and sutured. Postoperatively, the animals received antibiotic§ given as a single intramuscular injection.

CLINICAL ANALYSES

In order to confirm the success of ovariectomy and efficiency of estrogen administration on ovariectomized animals, estrous cycle was monitored. The changes in the vaginal smear during 4-5 days of the estrus cycles were observed in each group. At autopsy, success of the ovariectomy was confirmed by absence of ovaries and atrophy of uterine horns in rats not submitted to estrogen therapy.

BIOCHEMICAL SERUM ANALYSES

Blood samples were collected to measure plasma concentration of alkaline phosphatase and calcium at the time of sacrifice. Using automated laboratory techniques, alkaline phosphatase activity was obtained calorimetrically‖ and calcium, by the ion selective electrode method ¶.

HISTOMETRIC PROCEDURE

After 60 days, animals were killed and tibiae were removed and fixed in 4% neutral formalin for 48 hours. Undecalcified sections were prepared as previously described[26], i.e. the blocks were dehydrated by using an ascending series of ethanol (60-100%) and embedded in glycolmethacrylate#. Subsequently, the sections (20-30μm) were obtained and stained using 1% toluidine blue. The bone density, i.e., the proportion of mineralized matrix, in a 500 μm-wide zone lateral to the implant was separately recorded for both sides of the implant** in the cortical (Zone A) and cancellous bone (Zone B) areas (figure 1).

STATISTICAL ANALYSIS

Data from Zones A and B (cortical and cancellous bone, respectively) were separately averaged. The hypothesis that estrogen and CT administration had no influence on the bone denstiy around the implants placed in the OVX rats was tested using an intergroup analysis (Kruskal-Wallis test - alpha = 0.05). In the case that statistical difference was detected, Dunn´s method was used to isolate the groups that differed from the others. In addition, to test the hypothesis that HRT and CT administration did not influence alkaline phosphatase and calcium serum levels; the one-way ANOVA (alpha = 0.05) was used. If statistical difference was detected, a pairwise multiple comparison procedure was used (Bonferroni t-test).

Finally, an intergroup analysis was used to test the hypothesis that there was no difference between the groups with respect to the animals´ body weights at the end of the experimental period (one way ANOVA - alpha = 0.05).

† Miacalcic®, Sandoz A.G., Fertigung Schützenstrsse, Ravenburg, Germany.

‡ Sigma Chemical, St. Louis, MO, USA.

§ Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil.

| Gold Analisa Diagnóstica, Belo Horizonte, MG, Brazil.

¶ AVL Scientific Corporation, Roswell, GA, USA.

Technovit 7200®; Heraeus Kulzer GmbH, Wehrheim, Germany.

** Image-Pro®; Media Cybernetics, Silver Spring, MD, USA.

RESULTS

CLINICAL ANALYSES

All animals gained weight during the course of the study. The animals from groups 1, 2 and 3 weighted significantly more than the animals from group 4 ($P<0.05$) (table 1).

The success of ovariectomy and efficiency of estrogen administration were confirmed by estrus cycles and macro analysis of the uterine horns and absence of ovaries. The *diestrous* smear, which consisted mainly of leucocytes and some epithelial cell, were found in all OVX and CT animals. In contrast, the animals that received estrogen remained in the *estrus* stage, in which the smear consists entirely of large cornified epithelial cells. Finally, the sham group presented changes in the kind of cells in the vaginal smears, clearly identifying the four stages of the estrous cycle (*estrus, metestrus, diestrus and proestrus*).

Regarding the autopsy, macroscopic analysis showed that the uterus of the group-1 and -4 animals were pink and fluid filled, assuring that the serum estrogen levels were kept normal in both groups. On the other hand, OVX and CT rats presented their reproductive organs thin and anemic, confirming the reduction of serum estrogen levels in both groups.

BIOCHEMICAL SERUM ANALYSIS

Serum concentrations of alkaline phosphatase and calcium at the time of sacrifice are summarized in table 1. The alkaline phosphatase serum level was similar in groups 1 and 4 ($P>0.05$), but was statistically higher in groups 2 and 3 ($P<0.05$). Regarding the calcium serum level, the animals that were ovariectomized and did not receive either HRT or CT (group 2) presented higher values than the animals from groups 1, 3 and 4 ($P<0.05$).

HISTOMETRIC RESULTS

In zone A, intergroup analysis revealed no significant difference regarding the bone density ($P=0.64$ – figure 2). However, data analysis demonstrated that estrogen deficiency resulted in a lower bone density in zone B than the sham animals ($P<0.05$). This negative influence was overcome when the animals were administered with 17β estradiol ($P<0.05$). CT administration resulted in a slight increase in bone density, but no significant difference was detected ($P>0.05$ - figure 3). Figures 4A to 4D illustrate the histological findings.

DISCUSSION

Treatment with dental implants has become a routine procedure for rehabilitation of edentulous patients with high predictability[1-3]. Postmenopausal osteoporosis is a heterogeneous bone disorder characterized by a progressive loss of bone tissue due to estrogen deficiency after natural or surgical cessation of the ovarian function[29], and some concern has been raised on the implant outcome in such patients.

Since the prognosis of an implant-supported prostheses may be influenced by the proportion of mineral bone around the implant[15], the present study aimed to evaluate the influence of estrogen and calcitonin on bone quality (PMM) in a 500 µm-wide zone lateral to the implant surface in ovariectomized rats, instead of its influence on osseointegration (early failure). Results indicated that ovariectomy (group 2) decreased bone density around titanium implants in cancellous bone, but not in cortical bone. Therefore, it is likely that, besides affecting the new-formed bone as previously

described[15-17,30], the accelerated osteoclastic bone resorption resulting from estrogen deficiency, may also affect the pre-existing cancellous bone in an adjacent area lateral to the implant surface.

Estrogen replacement therapy has long been considered the first line therapy for preventing osteoporosis[19]. Estrogen administration clearly inhibits bone loss as well as bone turnover and increases bone mineral density[24]. To date, the present study is the pioneer in evaluating the effect of HRT (estrogen) and CT on the proportion of mineralized bone in a 500μm-wide zone lateral to the implant surface. The results of this study demonstrated that estrogen treatment immediately after ovariectomy provided complete protection against the negative effects of estrogen deficiency. The OVX rats which daily received 17β estradiol (group 4) presented bone density levels similar to sham animals (group 1). The mechanism for this protective effect apparently involves suppression of bone turnover as a direct effect of estrogen on bone cells[19-20, 31] and cytokines regulation[21-22]. Such an observation is in agreement with previous studies regarding the benefits of estrogen to the bone metabolism in estrogen-deficient animals[23-24].

On the other hand, the present study showed that treatment of OVX rats with CT, immediately after ovariectomy, revealed a slight positive effect to prevent the development of cancellous osteopenia, but this was not statistically significant. The data of the present study are closely related to the study reported by Shen et al.[32], who showed that CT partially prevented bone loss in ovariectomized rats when administrated during 90 days. These trends suggest that the skeletal response to CT therapy may decline along the treatment. This phenomenon has been reported in some clinical trial involving long-term CT treatment of postmenopausal osteoporotic patients[33-34]. Calcitonin induces loss of CT cell receptors, resulting in hormone-induced resistance, and induces a progressive down-regulation of bone binding sites for the hormone [35-36]. If these same phenomena occurred in this investigation is still unknown, but it is clear that CT treatment was not able to completely revert the effects of estrogen deficiency on bone adjacent to the implant surface.

The biochemical serum analyses support the histometric results. Alkaline phosphatase may assist in the diagnosis of osteoporosis, indicating high bone turnover. In the present study, the serum alkaline phosphatase was significantly elevated in the OVX group. Estrogen administration resulted in a level of alkaline phosphatase similar to control group (sham), indicating that this therapy controlled the high bone turnover promoted by ovariectomy. These results were in agreement with previous animal studies[37-38]. In addition, Christiansen et al.[39] also demonstrated that alkaline phosphatase level

is 20 % higher in the menopausal period and decreases to a normal level with estrogen administration. Furthermore, estrogen therapy resulted in a level of calcium similar to negative control group (sham) and CT group, demonstrating that CT might have presented its biological properties, decreasing the levels of serum calcium [40-41], even though it did not decrease bone turnover in OVX animals.

In conclusion, experimental studies indicate that estrogen deficiency might influence bone around titanium implants. These effects should be seriously investigated on a long-term clinical trial, controlling as confounding variables as possible. Further studies should be considered in order to complement the present study and to investigate whether other inhibitors of bone resorption, such as diphosphonates, may influence bone around titanium implants in estrogen-deficient animals. Extrapolation of the observations found in animal models to a clinical situation is not possible. However, it could suggest, at least, that when it comes to elderly patients or menopausal women, without estrogen treatment, the choice between conventional and different alternatives of implants-supported prostheses should be carefully considered. Finally, within the limits of this investigation, it can be suggested that estrogen administration, immediately after ovariectomy, may prevent the negative influence of estrogen deficiency on bone density around titanium implants.

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Table 1. Body weight, serum levels of alkaline phosphatase (IU/l) and calcium serum levels (mmol/l) at the time of sacrifice of sham, OVX (ovariectomized rats), CT (OVX and calcitonin administration) and E (OVX and estradiol administration) groups.

Group	Body Weight (kg)	Alkaline phosphatase(IU/l)	Calcium serum (mmol/l)
Sham (1)	256,27g $\pm$ 15,7a	29.13 $\pm$ 10.93[a]	1.10 $\pm$ 0.07b
OVX (2)	261,67g $\pm$ 14,98a	80.47 $\pm$ 20.16b	1.24 $\pm$ 0.08a
CT (3)	255,47g $\pm$ 20,25a	98.20 $\pm$ 14.27b	1.10 $\pm$ 0.14b
E (4)	228,57g $\pm$ 13,68b	33.29 $\pm$ 14.91a	1.07 $\pm$ 0.13b

Data represents the mean $\pm$ SD. Different letters indicate significant statistical differences (α=0.05), within each column. Body Weight determined by One Way analyses of variance and Turkey test. Alkaline phosphatase and calcium serum determined by One Way analyses of variance (ANOVA) and Bonferroni t-test.

Figure 1. Schematic illustration of the histometric parameters evaluated in a 500 µm-wide zone lateral to the implant surface in the cortical (Zone A) and cancellous bone (Zone B) areas.

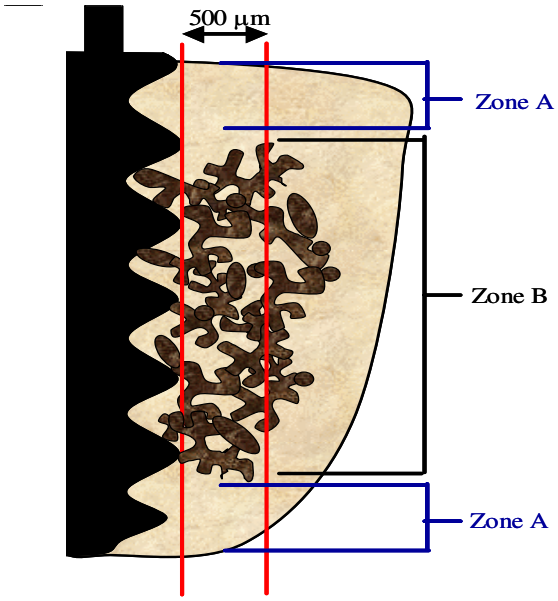


Figure 4. Photomicrographs illustrating the histological aspects within the limits of the threads and in a zone lateral to the implant. Note cortical and cancellous bone. Figures A, B, C and D illustrate groups 1(sham), 2 (OVX), 3 (OVX and calcitonin) and 4 (OVX and estrogen), respectively. (Toluidine blue / Original magnification = 12.5x).

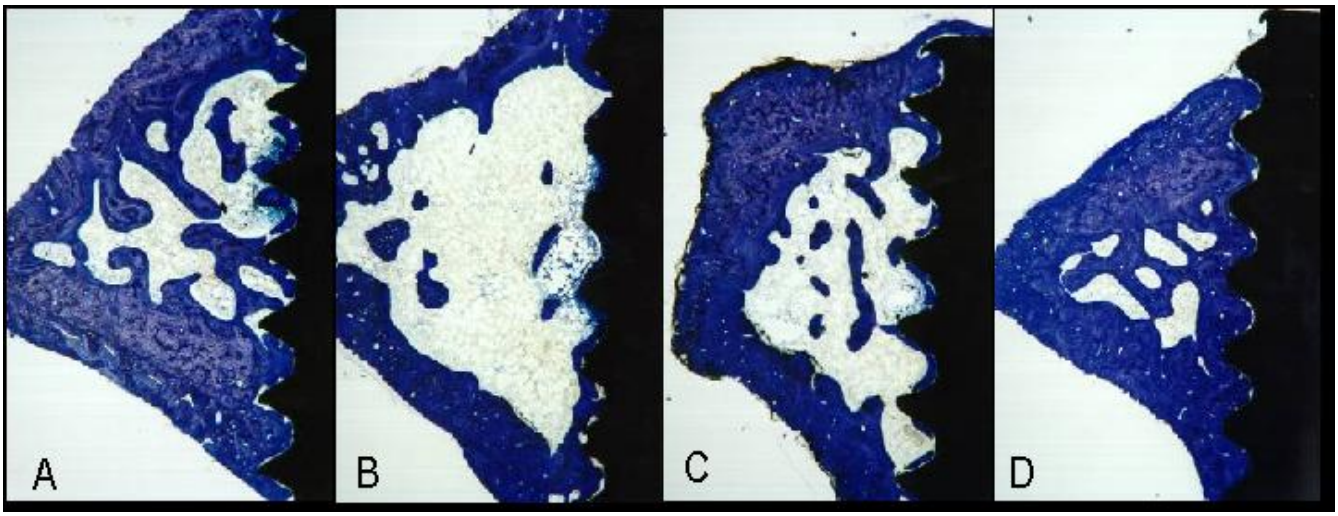


Figure 2. Means and standard deviation (%) of PMM in zone A (cortical bone) for all experimental groups: sham, OVX (ovariectomized rats), CT (OVX and calcitonin administration) and E (OVX and estradiol administration). Bone density means and standard deviations in this area were 95.39 ± 1.34%; 93.27 ± 3.81%; 94.90 ± 2.16 % and 95.84 ± 1.75% for Sham, OVX, CT and E groups, respectively.

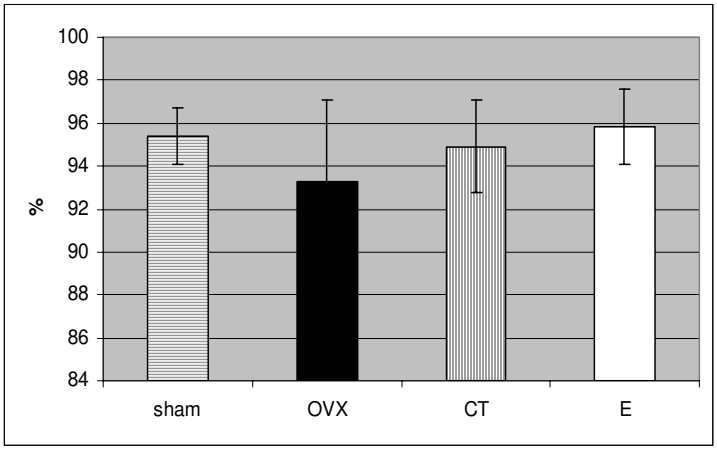
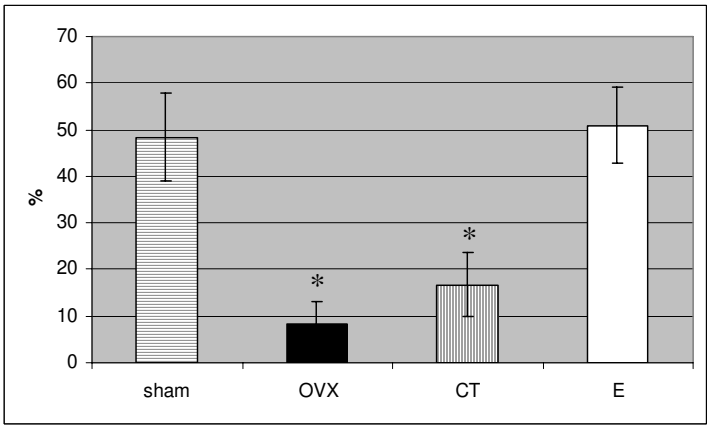


Figure 3. Means and standard deviation (%) of PMM in zone B (cortical bone) for all experimental groups: sham, OVX (ovariectomized rats), CT (OVX and calcitonin administration) and E (OVX and estradiol administration). The bone density means and standard deviations in zone B were 48.39 ± 9.37%; 8.26 ± 4.77%; 16.97 ± 6.87% and 50.97 ± 8.25% for Sham, OVX, CT and E groups, respectively.



CAPÍTULO 4

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ALENDRONATE THERAPY MAY BE EFFECTIVE TO PREVENT BONE LOSS AROUND TITANIUM IMPLANTS INSERTED IN ESTROGEN DEFICIENT RATS.

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ABSTRACT

Background: A negative impact of estrogen deficiency on bone healing around titanium implants has been reported. This study evaluated whether the alendronate (ALD) therapy would influence bone healing and density around titanium implants inserted in ovariectomized rats, and whether ALD therapy would provide a residual effect after its withdrawal.

Methods: Twenty-one days before implant placement, bilateral ovariectomies were performed. One screw-shaped titanium implant was placed in the tibiae. The animals were assigned to one of the following groups: Group SHAM (n=15): sham surgeries; Group OVX (n=15): ovariectomy; Group AT (n=15): OVX plus alendronate administration for 80 days; Group AW (n=14): OVX plus alendronate administration for 40 days; Group ET (n=14): OVX plus 17β estradiol administration for 80 days;

Group EW (n=14): OVX plus 17β estradiol administration for 40 days. After sixty days, the animals were sacrificed and undecalcified sections obtained. Bone-to-implant contact (BIC), bone area (BA) within the limits of implants threads and bone density in a 500µm-wide zone lateral to the implant (BD) were obtained and arranged for the cortical (Zone A) and cancellous (Zone B) regions.

Results: In zone A, data analysis showed no significant differences among the groups regarding BIC and BD ($P>0.05$), and a slight beneficial effect of estradiol on the BA when compared with the OVX, EW and AW groups ($P<0.05$). In zone B, OVX negatively impacted bone healing around the implants, resulted in reduced BA and BD ($P<0.05$). ALD (continuous/interrupted) and estradiol (only continuous) positively affect BIC, BA and BD, resulting in values at the same level as the control group (SHAM).

Conclusion: ALD may prevent the negative influence of estrogen deficiency on bone density and healing around titanium implants inserted in OVX rats. Furthermore, the ALD positive effect, in contrast to estradiol, is sustained in BA and BD following its withdrawal.

KEY WORDS: estrogen deficiency, titanium implants, alendronate, estradiol, osteoporosis.

INTRODUCTION

Currently, osseointegrated titanium implants has became an important alternative to conventional prosthesis in edentulous situations[1-2]. Implant integration into bone involves not only implant-related factors, such as material, topography, shape, and surface chemistry, but also mechanical loading, surgical technique, and patient variables, such as bone quantity and quality[3]. Since osteopenia and osteoporosis are estrogen deficiency-related bone diseases[4], which results low bone mass, some studies have investigated the impact of this diseases on dental implants[5-10]. Although clinical studies have demonstrated no evidence suggesting that osteoporosis is a risk factor for dental implants[5], animal studies have showed that bone volume, bone area and bone contact were significantly decreased around the implant placed in estrogen- deficient animals[6-10].

Estrogen deficiency after ovariectomy or menopause plays a major role in the early changes in the turnover of cancellous bone, leading to a reduction in bone mass and disruption of the trabecular network[11]. Estrogen replacement therapy is commonly used as a prophylactic and therapeutic measure in order to prevent bone loss in postmenopausal women and estrogen deficient individuals[12]. However, the possibility of estrogen side-effects, such as breast swelling and tenderness, bloating, bleeding and spotting, lead postmenopausal women often discontinue this

therapy[13]. In addition, the possibility of clinical contra-indications to the estrogen treatment requires development of therapeutic alternatives.

Bisphosphonates are synthetic compounds extensively used for the treatment of systemic bone loss due to estrogen depletion[14]. Although, the bisphosphonates mechanism of action is not yet fully understood, it has been demonstrated that it inhibits osteoclast-mediated bone resorption[15]. It has been shown that alendronate is a potent inhibitor of bone resorption without significantly affecting bone formation[16]. Despite some patients discontinue ALD therapy because of upper gastrointestinal symptoms[17], it accumulates in the bone, having very long biological half-lives[18].

Therefore, the present study was designed to evaluate, by histometric analysis, whether ALD therapy would prevent the negative influence of endogenous estrogen deficiency around titanium implants placed in ovariectomized rats. Furthermore, little is known about the effects of discontinuation of both ALD and estradiol (E) therapies on bone healing and density around titanium implants, and therefore, the present study also aimed to investigate the impact of both therapies withdrawal.

MATERIALS AND METHODS

ANIMALS

The experimental animals were 87 female Wistar rats that were 90 days of age and weighed an average of 210g at the beginning of the study. During the period of the experiment, the animals were kept in plastic cages with access to food and drinking water *ad libitum,* except the ovariectomized (OVX) rats not on estradiol treatment (pair feeding)[19]. This protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

OVARIECTOMY

The animals were anesthetized with intramuscular administration of ketamine (0.5ml/kg). Ovariectomy or sham surgeries were performed at the beginning of the study. Bilateral ovariectomies were performed in 72 rats from a dorsal approach. The remaining 15 animals were subjected to sham surgeries in which the ovaries were lifted up and returned intact to the original position. Postoperatively, the animals received antibiotic[1] given as a single intramuscular injection.

EXPERIMENTAL DESIGN

After ovariectomies, the animals were randomly assigned to one of the following six groups: Group SHAM (n=15): sham surgery; Group OVX: (n=15): ovariectomy; Group AT (n=15): OVX plus 4

days/week subcutaneous injections of ALD[2] at a dose of 5mg/Kg body weight for 80 days; Group AW (n=14) OVX plus 4 days/week subcutaneous injections of ALD at a dose of 5mg/Kg body weight for 40 days; Group ET (n=14): OVX and daily subcutaneous injections of 17β estradiol[3], dissolved in 100% ethanol and diluted in mineral oil at a dose of 20μg/Kg body weight for 80 days ; Group EW (n=14): OVX and daily subcutaneous injections of 17β estradiol, dissolved in 100% ethanol and diluted in mineral oil at a dose of 20μg/Kg body weight for 40 days (figure 1).

IMPLANTS SURGERY

Twenty-one days after ovariectomies, general anesthesia was performed (ketamine - 0.5ml/kg). Skin was cleansed with iodine surgical soap. An incision of approximately 1 cm in length was made and the bone surface of the tibiae surgically exposed by blunt dissection. Under profuse saline solution irrigation, bicortical implant beds were drilled at a rotary speed not exceeding 1,500rpm. A screw-shaped commercially available pure titanium implant, of 4.0 mm in length and 2.2 mm in diameter, was placed until the screw thread had been completely introduced into the bone cortex. Finally, soft tissues were replaced and sutured. Postoperatively, the animals received antibiotic[4] given as a single intramuscular injection.

CLINICAL ANALYSES

In order to confirm the success of ovariectomy and estrogen administrations, the estrous cycle was monitored two weeks after the ovariectomy surgeries, and two weeks after the withdrawal of the drugs. Changes in the vaginal smear during 4-5 days of the estrus cycles were observed in each group. At autopsy, success of the ovariectomy was also confirmed by absence of ovaries and atrophy of uterine horns in ovariectomized rats. In addition, the presence of normal uterine horns was also used to support the success of estrogen administrations.

ALKALINE PHOSPHATASE ANALYSIS

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[1] Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil.
[2] Teva Pharmaceutical Ltda. , Petach Tikva, Israel
[3] Sigma Chemical, St. Louis, MO, USA.
[4] Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil.

Blood samples were collected to measure plasma concentration of alkaline phosphatase at the time of sacrifice. Using automated laboratory techniques, alkaline phosphatase activity was obtained colorimetrically[5].

HISTOMETRIC PROCEDURE

Sixty days after implant placement (figure 1), the animals were sacrificed, the tibiae removed and fixed in 4% neutral formalin for 48 hours. Undecalcified sections were prepared, as previously described[8-10]. Briefly, the blocks were dehydrated by using an ascending series of ethanol (60-100%) and embedded in glycolmethacrylate[6]. Subsequently, the sections (20-30 µm) were obtained and stained using toluidine blue 1% staining. The percentage of bone-to-implant contact (BIC) and bone area (BA) within the limits of the threads of the implants and bone density (BD), i.e., the proportion of mineralized matrix in a 500µm-wide zone lateral to the implant surface was separately recorded for both sides of the implant in the cortical (Zone A) and cancellous bone (Zone B) areas[7].

MORPHOLOGICAL ANALYSIS

In order to parallel the histometric data with the morphological aspect of the contralateral tibiae, specimens were observed on the scanning electron microscopy (SEM) level. Samples were fixed in 2,5% glutaraldehyde in 0.05mol/l cacodylate buffer, pH 7,4. Subsequently, specimens were washed in five changes of tap water for 15 min to remove the smear layer, fixed, post-fixed, dehydrated in ascending acetone concentrations up to 100%, critical point dried[8], sputter-coated with gold[9], and observed under a SEM[10]. The sections were obtained in a transversal direction. Representative areas of cortical and cancellous bone were photographed at 35X magnification.

STATISTICAL ANALYSIS

Data from Zones A and B were separately averaged. Intergroup analysis was used to test the hypothesis that the treatments had no influence on BIC, BA and BD. (Kruskal-Wallis test - alpha = 0.05). In the case that statistical difference was detected, the Dunn's method was used to isolate the groups that differed from the others. Furthermore, One Way ANOVA (alpha = 0.05) was used to test

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[5] Gold Analisa Diagnóstica, Belo Horizonte, MG, Brazil
[6] Technovit 7200®; Heraeus Kulzer GmbH, Wehrheim, Germany
[7] Image-Pro®; Media Cybernetics, Silver Spring, MD, USA
[8] CPD 030 - BAL-TEC, Furstentum, FL, Liechtenstein
[9] MED 010 - BAL-TEC, Furstentum, FL, Liechtenstein
[10] LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, United Kingdom

the hypothesis that the treatments did not influence the alkaline phosphatase serum levels. If statistical difference was detected, a pairwise multiple comparison procedure was used (Bonferroni t-test). Finally, an intergroup analysis was used to test the hypothesis that there was no difference among the groups with respect to the animal's body weight at the end of the experimental period (One Way ANOVA - alpha = 0.05). A pairwise multiple comparison procedure was used (Bonferroni t-test), if statistical difference was detected.

RESULTS

CLINICAL OBSERVATIONS

All animals gained weight during the study. The animals from SHAM, OVX, AW and EW groups weighed significantly more than the animals from group AT and ET groups ($P<0.05$). The final mean body weight was 256.26g $\pm$ 16.08; 261.66g $\pm$ 17.16; 244.18g $\pm$ 20.4; 266.56g $\pm$ 23.89; 228.57g $\pm$ 15.76 and 269.44g $\pm$ 35.85 for the animals from SHAM, OVX, AT, AW, ET, EW groups, respectively.

Clinical appearance of uterine horns, absence of ovaries and assessment of the estrous cycle confirmed the success of ovariectomy and estrogen replacement. Groups OVX, AT and AW presented *diestrous* smear and their reproductive organs atrophied, confirming the reduction of serum estrogen levels. In contrast, the animals submitted to SHAM surgery presented the four stages of estrous cycle *(estrus, diestrus, proestrus and metaestrus)* and a pink and fluid filled uteri. The animals administered for 80 days with estradiol (ET) remained in the *estrus* stage and presented normal uteri, assuring that the serum estrogen levels were kept normal in these animals. Finally, animals in which the estradiol therapy was interrupted by day 40 presented *diestrous* smear after the withdrawal and, their reproductive organs were atrophied at sacrifice.

MORPHOLOGICAL ANALYSIS

Similarly to the histometric observation, morphological analysis confirmed the striking differences in the cortical and cancellous bone regions among the groups. SHAM, AT, AW, ET presented a much more dense cancellous bone than OVX and EW. Figures 2A to 2C illustrate the morphological results.

ALKALINE PHOSPHATASE ANALYSIS

Alkaline phosphatase serum concentrations (UI/L) and standard deviation, performed at the time of sacrifice, were 27.6 $\pm$ 11.30; 80.46 $\pm$ 18.72; 40.93 $\pm$ 11.05; 44.28 $\pm$ 13.28; 33.2 $\pm$ 14.90 and 91.23 $\pm$ 23.28 for SHAM, OVX, AT, AW, ET and EW groups, respectively. Alkaline phosphatase levels were

statistically higher for the OVX and EW groups (P<0.001) and, therefore, confirmed the high bone turnover in the animals in an estrogen-deficient state.

HISTOMETRIC RESULTS

BONE-TO-IMPLANT CONTACT (BIC):

Intergroup analysis did not reveal significant difference regarding BIC in Zone A (P>0.05). On the order hand, in Zone B, data analysis revealed a statistically significant positive effect of ALD and E therapies on the percentage of BIC, even after their withdrawal (P< 0.05) (table 1).

BONE AREA WITHIN THE LIMITS OF THE IMPLANT THREADS (BA):

In Zone A, although there was a slight difference, estrogen deficiency did not significantly influence BA around the implants (P>0.05). Furthermore, statistical analysis demonstrated that continuous estradiol administration to OVX animals promoted a significantly higher BA than OVX, EW and AW groups (P<0.05). On the other hand, in Zone B, data analysis showed that estrogen deficiency may result in a lower percentage of BA than the animals not submitted to ovariectomy (P<0.05). However, this negative effect was restored in the OVX rats, which were submitted to AT, ET and AW, but not to EW (table 1).

BONE DENSITY IN A 500µm WIDE ZONE LATERAL TO THE IMPLANT SURFACE (BD):

Data analysis showed that, in Zone A, there was no significant difference regarding the percentage of mineralized tissue in lateral region to the implant surface (P>0.05). Means and standard deviations were 95.39% $\pm$ 1.34; 93.27 % $\pm$ 3.8; 94.44% $\pm$ 1.59; 94.34% $\pm$ 1.55; 95.84% $\pm$ 1.75 and 92.81% $\pm$ 6.89 for SHAM, OVX, AT, AW, ET and EW groups, respectively (Figure 3). In Zone B, the results revealed that estrogen deficiency may result in a lower BD than the SHAM group (p<0.05). Furthermore, AT, AW and ET, but not EW, were able to prevent against the negative influence of estrogen deficiency. Means and standard deviations were 48.39% $\pm$ 9.37; 8.2% $\pm$ 4.7; 56.91% $\pm$ 8.7; 57.12 % $\pm$ 5.2; 50.97% $\pm$ 8.2 and 32.81% $\pm$ 10.7 for SHAM, OVX, AT, AW, ET and EW groups, respectively (Figure 4). Figures 5A to 5F illustrate the histological results

DISCUSSION

Postmenopausal osteoporosis and osteopenia are characterized by a progressive bone loss, which begins after natural or surgical cessation of the ovarian function[4]. These conditions are strongly supported by the estrogen role in bone metabolism[20-24].

Since dental implant outcome is dependent on bone quality, estrogen deficiency has been investigated with respect to its impact on the osseointegration process[5-10]. Although clinical data are not conclusive[5], animal studies have suggested that estrogen deficiency may negatively impact on bone around titanium implants [6-10]. In the present study, as previously reported [8-10] data analysis support the observation that bone is affected by an estrogen-deficient state. At this stage, however, caution should be used since further studies are requires to determine if implant supported prostheses would also be involved.

Pharmacological interventions for osteoporosis include mainly estrogen replacement therapy, calcitonin and parathyroid hormones and bisphosphonate administrations[25]. In the present study the impact that alendronate therapy (AT) and its withdrawal (AW) would exert on bone healing around titanium implants placed in ovariectomized rats was investigated. In addition, continuous and interrupted estrogen administrations were also investigated with respect to their effect on preventing the influence of endogenous estrogen deficiency on bone around the implants.

Estrogen replacement is commonly used as a first line therapy for preventing and treating osteoporosis. The protective effect of estrogen replacement has been demonstrated in clinical and animal studies with respect to both bone mass loss and fracture incidence[12, 26-27]. The results of the current study, as in our previous studies[8-10], showed that ET after ovariectomy may neutralize the negative effects of estrogen deficiency, mainly in cancellous bone, on all parameters analyzed, i.e., BIC, BA and BD. Although the mechanisms involved remain to be investigated, currently, it has been demonstrated that bone resorption is characterized by two key molecules, receptor activator of NF-kappa ligand (RANKL) and osteoprotegerin (OPG) [24]. Bone resorption is significantly reduced by RANKL function inhibition via its decoy receptor OPG[24,28]. Since estrogen has been reported to control bone resorption acting on OPG, it may be suggested that estrogen deficiency induces an unbalance in the RANKL/OPG system favoring bone resorption, and its replacement may revert this mechanism. However the precise mechanisms remain to be clarified.

In addition, for the first time, we demonstrated that the interruption in the treatment with estrogen resulted in BA and BD, in the cancellous bone, similar to the OXV group. Although, little is known about the effects of discontinuation of estrogen therapy on bone around titanium implants, these observations are in agreement with those studies that demonstrated that EW causes marked increase in bone turnover and physiological changes, i.e., loss of bone density and increased risk of

fracture [29-30]. Even though our findings do not support the hypothesis that bone loss might continue to be accelerated long after estrogen treatment cessation, we cannot fully observe residual benefit of estradiol on bone after its withdrawal. Therefore, the pattern of bone loss observed after estrogen withdrawal seems to be comparable to that which occurs in rats after ovariectomy without treatment.

ALD is a bisphosphonate compound that has been described to inhibit bone loss and has been extensively applied in the treatment of osteoporosis [14]. Several mechanisms have been investigated including osteoclasts development and activity inhibition, osteoclasts apoptosis induction and stimulation of an osteoclast inhibitory factor proctuction[15, 31]. In the current study, alendronate immediately after ovariectomy prevented bone loss and depressed bone turnover around titanium implants. The OVX rats which received alendronate (AT), immediately after ovariectomy, present similar levels of BIC, BA and BD to the sham operated animals (SHAM) in the cortical and cancellous bone (Zone A and B, respectively). These findings are consistent with the skeletal effects of bisphosphonates in early postmenopausal and ovariectomized women[32]. Our results are also in agreement with that reported by Narai *et al.*[33], which demonstrated that removal torque was lower for the osteoporotic rats than ALD-treated rats and, there was no significant difference between the ALD-treated and sham groups.

ALD side effects, including sensitivity to phosphates and gastrointestinal upset, often lead postmenopausal women to discontinue therapy[17]. Thus, in the present study, we also investigated the effects of alendronate withdrawal (AW) on bone healing and density around titanium implants in OVX rats. Data analysis revealed that all the histometric parameters (BIC, BA and BD), in cancellous and cortical bone, for the animals that received an interrupted ALD therapy, were similar to control group (SHAM). Thus, in contrast to estrogen withdrawal, OVX rats maintained normal bone mass and low levels of bone turnover after alendronate treatment withdrawal. These findings are in agreement with studies showing that accelerated bone loss is seen after withdrawal of estrogen therapy but not after withdrawal of alendronate or combination therapy [34]. The prolonged residual skeletal effects of bisphosphonates are probably a consequence of a strong affinity to the hydroxyapatite crystals[35]. Bisphosphonates bound to bone mineral are released during bone resorption by osteoclasts. This could lead to a localized accumulation of the drug, which could directly perturb osteoclasts as explained above [31,36].

Alkaline phosphatase has been described as a biochemical marker of high bone turnover used to reflect the changes in bone metabolism during the diagnosis of osteoporosis[37-38]. Serum levels of alkaline phosphatase were significantly higher in the OVX and EW groups, demonstrating a high bone turnover as a consequence of an estrogen-deficient state. On the other hand, ET, AT and AW demonstrated an alkaline phosphatase level similar to the control group (SHAM), indicating that these treatments were able to control the high bone turnover induced by low level estrogen, thus providing support to the histometric and morphological findings.

Furthermore, the assessment of estrus cycle and the presence of atrophic uterine horns in OVX rats not submitted to ET confirmed the success of the ovariectomy and estrogen administrations.

Therefore, within the limits of the present investigation, it can be suggested that ET and AT, immediately after ovariectomy may provide complete protection against the negative influence of endogenous estrogen deficiency on bone healing and density around titanium implants. Furthermore, our findings suggest that the effects of EW and AW were found to be widely dissimilar. EW may be a high risk for subsequent bone loss around titanium implant, while AW maintained relatively normal cancellous bone. Nevertheless, further studies should be considered in order to clinically evaluate the relevance of these findings.

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Table 1: Mean and standard deviation (%) of bone-to-implant contact (BIC) and bone area (BA) within the limits of the implant threads for all groups at Zones A and B.

GROUPS	BIC		BA	
	ZONE A	***ZONE B***	***ZONE A***	***ZONE B***
SHAM	52.4 ± 11.74 a	51.08 ± 12.54 a	84.79 ± 3.70 ab	49.64 ± 5.60 ab
OVX	42.08 ± 8.20 a	36.75 ± 8.80 b	80.21 ± 3.80 a	36.59 ± 7.90 c
AT	55.66 ± 17.66 a	52.56 ± 9.38 a	83.85 ± 3.40 ab	56.84 ± 13.80 a
AW	57.97 ± 12.65 a	55.05 ± 15.04 a	82.99 ± 5.70 a	59.59 ± 7.80 a
ET	50.45 ± 12.76 a	52.99 ± 10.27 a	88.36 ± 3.39 b	58.54 ± 12.19 a
EW	51.08 ± 18.17 a	52.14 ± 13.66 a	81.04 ± 6.80 a	40.73 ± 7.05 bc

Different letters indicate significant statistical differences (α=0.05), within each column.

BIC and BA determined by Kruskal-Wallis and Dunn's tests.

Figure 1: Schematic experimental design

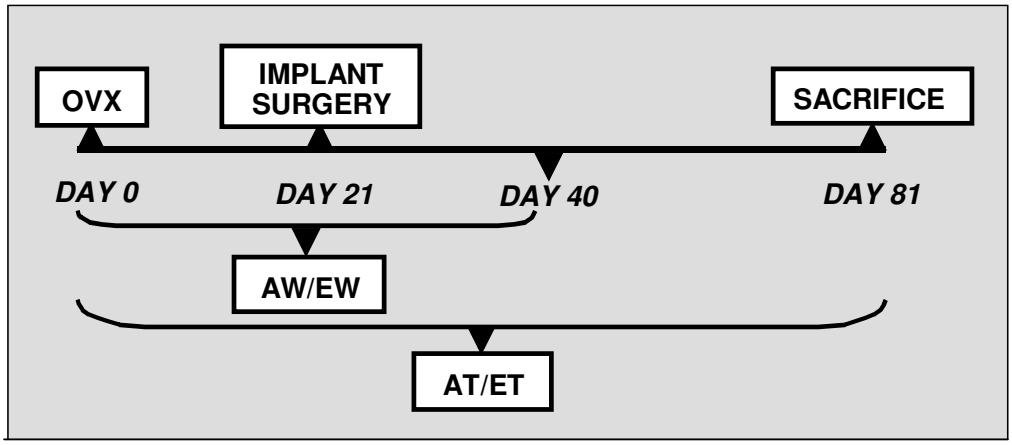


Figure 2. Transversal sections of the proximal tibial diaphysis from SHAM (A); AT (B) and OVX (C) observed at the SEM level. Note the reduced amount of trabecular bone (TB) in the OVX rats. Note also that the AT provided complete protection against bone loss. The trend for increased trabecular bone in SHAM and AT is very similar. (Magnification =35X).

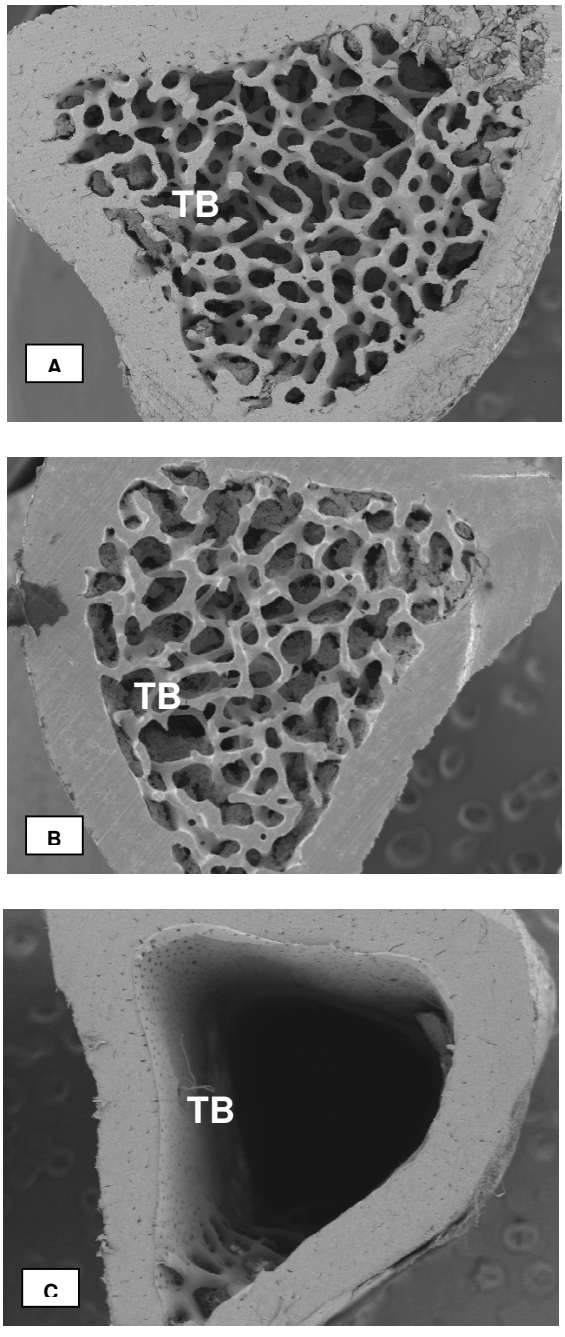


Figure 3. Means and standard deviation (%) of BD in zone A (cortical bone) for all experimental groups: sham, OVX (ovariectomized rats), AT (alendronate therapy), AW (alendronate withdrawal), ET (estrogen therapy), EW (estrogen withdrawal).

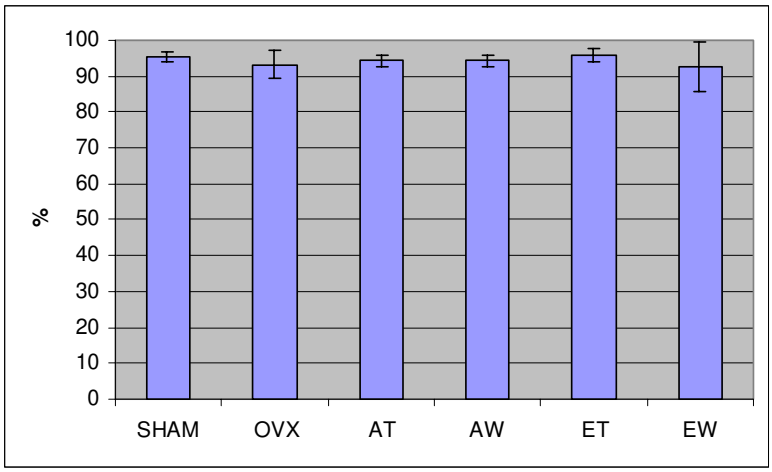
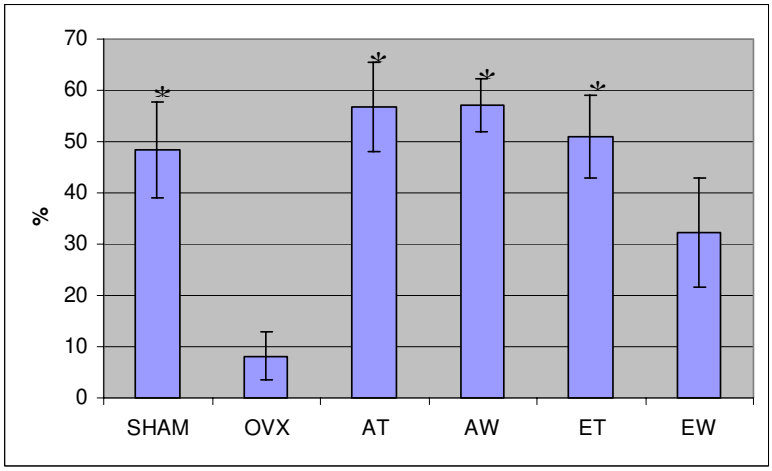
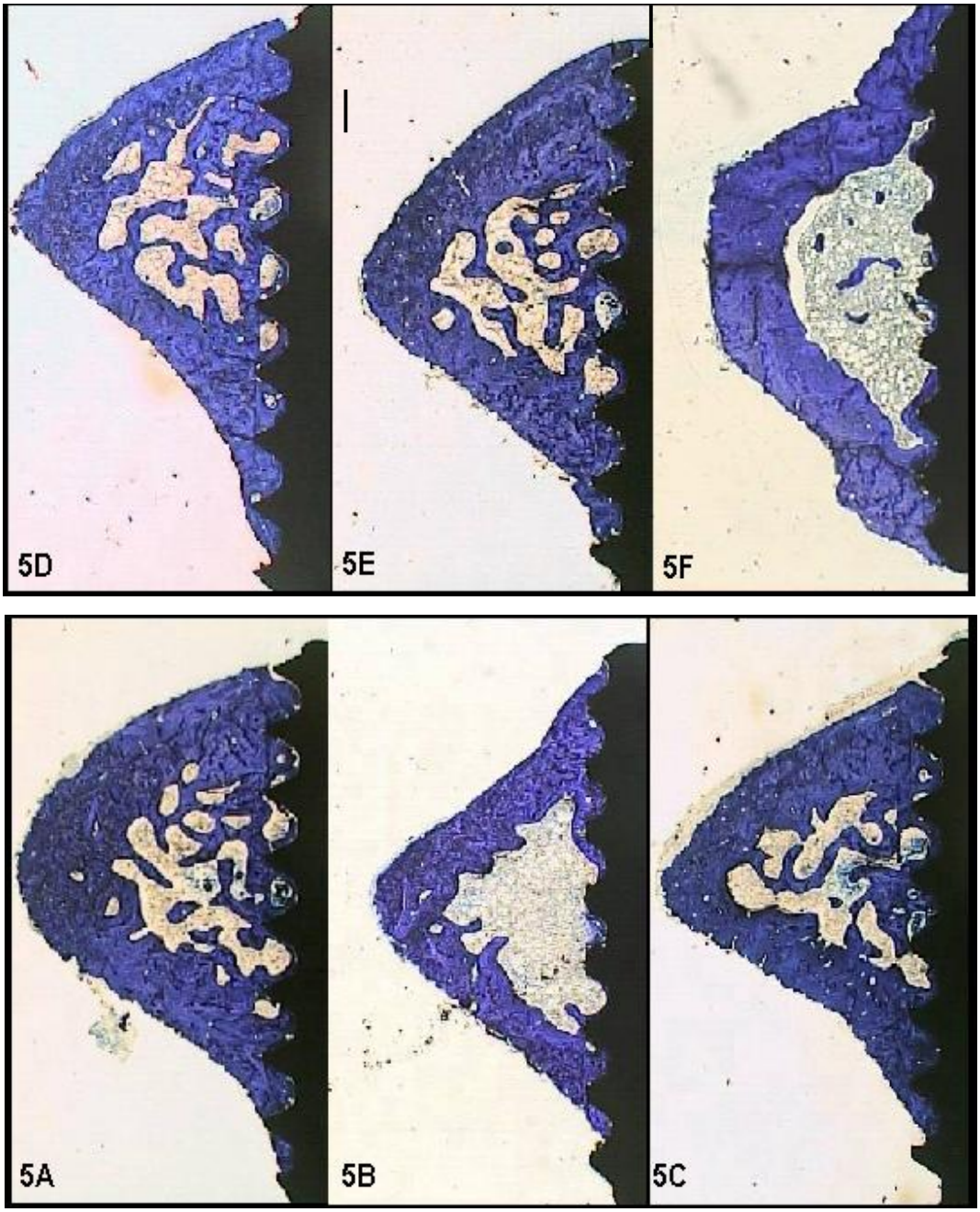


Figure 4. Means and standard deviation (%) of BD in zone B (medullar bone) for all experimental groups: sham, OVX (ovariectomized rats), AT (alendronate therapy), AW (alendronate withdrawal), ET (estrogen therapy), EW (estrogen withdrawal).



* Significantly different when compared with the OVX group (P<0.05) (Kruskal-Wallis and Dunn's tests).

Figure 5. Photomicrographs 5A to 5 F illustrating the histological aspects observed within the limits of the threads and in a 500 µm-wide zone lateral to the implant surface in sham, OVX (ovariectomized rats), AT (alendronate therapy), AW (alendronate withdrawal), ET (estrogen therapy) and EW (estrogen withdrawal) groups, respectively. (Toluidine blue / Original magnification = 6.25x).



CAPÍTULO 5

Enviado para a publicação no Journal of Periodontology

AGE-RELATED AND SURGICALLY INDUCED ESTROGEN DEFICIENCIES MAY DIFFERENTLY AFFECT BONE AROUND TITANIUM IMPLANTS INSERTED IN RATS.

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ABSTRACT

Background: Valuable information has been generated by using the ovariectomy model, however clinical studies have indicated that such a model may not be the most appropriate to parallel with the post-menopausal condition and titanium implants. Thus, the aim of this study was to comparatively evaluate, by histometric analysis, the influence of age-related (ARED) and surgically-induced (OVX) estrogen deficiencies on bone healing and density around titanium implants inserted in rats.

Methods: Single screw-shaped titanium implants were placed in rat tibiae and animals were then assigned to one of the following groups: **Group SHAM** (n=15): bilateral sham ovariectomies in 90 days old rats, twenty-one days before implant placement; **Group OVX** (n=15): bilateral ovariectomies in 90 days old rats, twenty-one days before implant placement. **Group ARED** (n=15): implant placement in reproductive aged rats (22 months old). After sixty days, the animals were sacrificed and undecalcified sections obtained. Bone-to-implant contact (BIC) and bone area (BA),

within the limits of implants threads; and bone density (BD), in a 500μm-wide zone lateral to the implant, were obtained and arranged for cortical (Zone A) and cancellous (Zone B) bone regions.

Results: For Zone A, data analysis showed no significant differences among the groups regarding BIC and BA ($P>0.05$). In contrast, ARED negatively influenced BD around the implants ($P<0.05$). In Zone B, OVX negatively affected BIC and BA ($P<0.05$), and both ARED and OVX groups demonstrated lower BD than the control group ($P<0.05$).

Conclusion: Within the limits of this study, it can be concluded that ARED mainly affects pre-existing bone while OVX more significantly affects both newly-formed and pre-existing bone.

KEY WORDS: estrogen deficiency, titanium implants, aging, osteoporosis.

INTRODUCTION

The osseointegrated implant is becoming an increasingly used for the rehabilitation of partially or fully edentulous patients[1-2]. The biological principle of osseointegration may be influenced either by implant materials, designs, and surface characteristics, or by patient variables, such as bone quantity and quality[3]. Postmenopausal osteoporosis and osteopenia have been defined as estrogen deficiency-related diseases characterized by gradual bone loss following the cessation of ovarian function either naturally or surgically induced[4-6]. The accelerated bone loss associated with estrogen deficiency appears to be caused by a direct and indirect action of estrogen in the regulation of bone turnover[7]. It has been shown that estrogen presents an important role in controlling bone resorption through its action on OPG and RANKL[8] molecules and on cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)[9-10]. Moreover, it has been reported that estrogen deficiency leads to increased osteoclast formation and enhanced resorption in cancellous as well as cortical bone[7].

For these reasons, ovariectomized rats have been widely used in order to simulate an estrogen-deficient state, and histologically document its impact on bone around titanium implants[11-12]. These studies have shown that bone volume, bone area and bone contact are significantly decreased around the implant placed in estrogen-deficient animals induced by ovariectomy[11-12]. On the other hand, clinical studies have, in general, reported that implant failures do not seem to depend clinically on the osteoporotic condition of the patients[13]. While the significant effect of ovariectomy on bone results almost exclusively from reduced estrogen levels, the more gradual bone loss associated with post-menopause seems to result from mechanisms other than only the reduced

estrogen level[14]. These mechanisms include age-related diminution in transforming growth factor-β levels[14], reduced osteoclast recruitment[15] and increased parathyroid hormone secretion[16].

Based on these considerations, it seems clinically relevant to study whether age- related estrogen deficiency may affect bone around titanium implants in a similar manner to that observed with the surgically-induced model (ovariectomy). Thus, the present study was designed to evaluate, by histometric analysis, whether age-related estrogen deficiency may affect bone healing and density around titanium implants inserted in aged rats with intact ovaries, comparing results with the ovariectomy model.

MATERIALS AND METHODS

ANIMALS

The experimental animals were 45 female Wistar rats that weighed an average of 211g (208-214g) at the beginning of the study. During the period of the experiment, the animals were kept in plastic cages with access to food and drinking water *ad libitum,* with the exception of the ovariectomized (OVX) rats (pair feeding)[17]. This protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

OVARIECTOMY AND SHAM SURGERY

Thirty 90 day-old rats were anesthetized with intramuscular administration of ketamine (0.5ml/kg). Bilateral ovariectomies were performed in 15 rats from a dorsal approach and sham surgeries, in which the ovaries were lifted up and returned intact to the original position, were performed on the remaining 15 rats. Ovariectomy or sham surgeries were performed at the beginning of the study. Postoperatively, the animals received antibiotic• given as a single intramuscular injection (1ml/Kg).

ESTROUS CYCLE AND AGING

In order to verify the success of ovariectomy and the hormonal status in the aged animals, the estrous cycle was monitored two weeks after the ovariectomy surgeries and, in the older rats, monthly after they were 14 months old. Rats were considered irregularly cycling when vaginal smears showed disordered estral cycle phases for at least 15 days. Only OVX animals (90 days old) which presented *diestrus* smears and reproductive aged (22 months old) animals with irregular estral cycles, with repeated pseudopregnancies, were included in the current study.

IMPLANTS SURGERY

In order to place the implants, general anesthesia was performed (ketamine - 0.5ml/kg) and the skin was cleansed with iodine surgical soap. An incision of approximately 1.0cm in length was made and the bone surface of the tibiae surgically exposed by blunt dissection. Under profuse saline solution irrigation, bicortical implant beds were drilled at a rotary speed not exceeding 1,500rpm. A screw-shaped commercially available pure titanium implant, of 4.0mm in length and 2.2mm in diameter, was placed until the screw thread had been completely introduced into the bone cortex. Finally, soft tissues were replaced and sutured. Postoperatively, the animals received antibiotic[∞] given as a single intramuscular injection (1ml/Kg).

EXPERIMENTAL DESIGN

The animals were randomly assigned to one of the following groups: **Group SHAM** (n=15): bilateral sham ovariectomies in 90-day-old rats, twenty-one days before implant placement; **Group OVX** (n=15): bilateral ovariectomies in 90 days old rats, twenty-one days before implant placement; **Group ARED** (n=15): implant placement in ovaries intact reproductive aged rats (22 months old).

ALKALINE PHOSPHATASE ANALYSIS

Blood samples were collected to measure serum concentration of alkaline phosphatase at the time of sacrifice. Using automated laboratory techniques, alkaline phosphatase activity was colorimetrically obtained[f].

HISTOMETRIC PROCEDURE

Sixty days after implant placement, the animals were sacrificed and the tibiae removed and fixed in 4% neutral formalin for 48 hours. Undecalcified sections were prepared, as previously described[12]. Briefly, the blocks were dehydrated by using an ascending series of ethanol (60-100%) and embedded in glycolmethacrylate[ϒ]. Subsequently, the sections (20-30µm) were obtained and stained using 1% toluidine blue staining. A blinded examiner obtained the percentages of bone-to-implant contact (BIC) and bone area (BA) within the limits of the threads of the implants, and bone

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• Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil.

∞ Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil.

f Gold Analisa Diagnóstica, Belo Horizonte, MG, Brazil

ϒ Technovit 7200®; Heraeus Kulzer GmbH, Wehrheim, Germany

density (BD), i.e., the proportion of mineralized matrix in a 500µm-wide zone lateral to the implant surface for both sides of the implant in the cortical (Zone A) and cancellous bone (Zone B) areas[∠].

STATISTICAL ANALYSIS

Data from Zones A and B were separately averaged. Intergroup analysis was used to test the hypothesis that age-related and surgically-induced estrogen deficiency had no influence on BIC, BA and BD. (Kruskal-Wallis test - alpha = 0.05). In the case that statistical difference was detected, the Dunn's method was used to isolate the groups that differed from the others. Additionally, One Way ANOVA (alpha = 0.05) was used to test the hypothesis that age-related and surgically induced estrogen deficiency did not influence the alkaline phosphatase levels and the animal's body weight at the end of the experimental period. If statistical difference was detected, a pairwise multiple comparison procedure was used (Tukey Test). Finally, an intragroup analysis (paired t-Test; alpha = 0.05) was performed to determine whether the body weight was affected over-time.

RESULTS

CLINICAL OBSERVATIONS

Intragroup analysis demonstrated that all animals gained weight during the period of the study. Moreover, an intergroup analysis showed that the aged animals weighed significantly more than the animals from group SHAM and OVX ($P<0.05$), and both SHAM and OVX were statistically similar (209.2g $\pm$ 12.92 / 256.26g $\pm$ 16.08; 209.87g $\pm$ 12.08 / 261.66g $\pm$ 17.16 and 214.34g $\pm$ 13.7 / 341.45g $\pm$ 37.58, at the beginning and the end of the experiment, for SHAM, OVX and ARED groups, respectively).

Changes in the vaginal smear during 4-5 days of the estrus cycles were observed for the OVX and SHAM groups. Animals in the OVX group presented *diestrous* smear and atrophied reproductive organs, confirming the reduction of serum estrogen levels. In contrast, the animals submitted to SHAM surgery presented the four regular stages of estrous cycle *(estrus, diestrus, proestrus and metaestrus),* and a pink and fluid filled uteri. Finally, reproductive aged animals (ARED group) were characterized by irregular estrous cycles. By the age of 22 months most female rats did not present regular hormonal cycles for at least 9 months. These reproductive aged rats presented

[∠] Image-Pro®; Media Cybernetics, Silver Spring, MD, USA

repeated pseudopregnant states, long *diestrous* periods of variable length and uteri with numerous secretory glands.

ALKALINE PHOSPHATASE ANALYSIS

Alkaline phosphatase serum concentrations (UI/L) and standard deviation, performed at the time of sacrifice, were 27.6 ± 11.30; 80.46 ± 18.72 and 64.61± 10.40 for SHAM, OVX and ARED groups, respectively. Statistical analysis showed differences among all the experimental groups, with higher alkaline phosphatase serum levels for the OVX group, followed by the ARED and SHAM groups ($P<0.001$). Thus, although both surgically-induced and age-related estrogen deficient states promoted a high bone turnover, ovariectomy affected bone metabolism significantly more than did naturally-occurring estrogen deficiency.

HISTOMETRIC RESULTS

BONE-TO-IMPLANT CONTACT (BIC) AND BONE AREA (BA):

Intergroup analysis did not reveal significant difference regarding BIC and BA in the cortical bone (Zone A- $P>0.05$). On the other hand, in Zone B, data analysis revealed a statistically significant negative effect of OVX on the percentage of BA and BIC ($P<0.05$), while there was no difference between the both ARED and SHAM groups ($P>0.05$). (Table 1)

BONE DENSITY IN A 500µm WIDE ZONE LATERAL TO THE IMPLANT SURFACE (BD):

Data analysis showed that, in Zone A, age-related estrogen deficiency (ARED group) may affect the pre-existing bone, resulting in a lower percentage of mineralized tissue in a lateral region to the implant surface (BD) ($P>0.05$). Moreover, in Zone B, the results revealed that both surgically induced and age-related estrogen deficiency significantly affected the pre-existing bone, resulting in a lower BD than that of the SHAM group ($p<0.05$). Means and standard deviations are graphically illustrated by figures 1 and 2. Additionally, figures 3A to 3C illustrate the histological results.

DISCUSSION

The ovariectomized rat is considered to be a good animal model of estrogen deficiency induced-osteopenia, since it has been established that ovariectomy induces an imbalance between bone resorption and formation[18]. As such, the model has been widely used to study the impact of estrogen deficiency on the bone around titanium implants[11-12]. Although the precise mechanisms involved remain to be investigated, it has recently been reported that estrogen controls bone resorption acting on the osteoprotegerin (OPG) molecule, and its deficiency induces an imbalance in

the receptor activator of the NF-kappa ligand (RANKL) and the OPG system, favoring bone resorption[8,19]. In general, studies using the ovariectomy model have suggested that estrogen deficiency may negatively affect bone around titanium implants[11-12]. Data from the present investigation advance the concept that ovariectomy significantly hinders the process of healing around implants inserted in rats, as judged by measurements of BIC and BA. In addition, as previously reported[12], the bone density (BD) findings in the present study support the observation that the pre-existing bone may also be affected by this surgically-induced estrogen-deficient state. Contrary to the information provided by the ovariectomized animal studies, the available clinical data do not provide a compelling theoretical or practical basis to indicate that osteoporosis and osteopenia are risk factors for osseointegrated dental implants[13,20]. Moreover, although the ovariectomy model seems to be a suitable method for the study of problems related to postmenopausal bone loss, it is not certain whether the characteristics of the ovariectomy-induced bone loss, and its radical sequels, resemble those found in naturally occurring estrogen deficiency in women. It has been suggested that age-related and surgically-induced estrogen deficiencies appear to initiate cancellous and cortical bone loss by a different cellular mechanism. The first stimulates a more gradual bone loss and limits the bone formation process while the second stimulates a rapid bone resorption[21]. Thus, the aim of the present investigation was to evaluate, by histometric analysis, whether naturally occurring estrogen deficiency may affect bone healing and density around titanium implants inserted in ovaries intact aged rats, comparing results to the ovariectomy model data.

In general, studies have used rats aged at least 18 months to evaluated age-related changes in bone mineral density[14,22]. In order to select natural estrogen-deficient rats (ARED group), in addition to age, the estrous cycle was precisely monitored in the current study. It has been reported that, by the age of 22 months, most female rats do not present regular hormonal cycles, progressing from a constant-estrous syndrome to a series of irregular pseudopregnancies and to an anestrous state[22]. The present study utilized only the 22 months old rats that presented repeatedly pseudopregnant states characterized by long *diestrous* periods and consequently reduced estrogen levels. In the current investigation, data analysis showed that age-related estrogen deficiency resulted in similar levels of BIC and BA to the sham-operated animals (SHAM) in the cortical and cancellous bone (Zone A and B, respectively) and, therefore, demonstrated that bone healing around

titanium implants may not be significantly affected by an age-related estrogen deficiency state, as seen for the ovariectomy model. Although clinical studies have demonstrated no evidence suggesting differences in implant survival between older and younger groups[23], the osteopenia associated with advanced age appears to be a universal phenomenon in humans and animals[14]. In the present study, the pattern of cancellous bone density in the older animals (ARED group) seemed to be comparable to that seen in ovariectomized rats (OVX). Furthermore, data analysis demonstrated that, in contrast to OVX, age-related estrogen deficiency also promoted a reduced cortical bone density in a lateral area to the implant surface, which may be explained by an increased bone turnover and a decreased mineral apposition rate during the aging process[21]. Takeshita et al. (1997)[23] were the first to investigate age-related changes in bone metabolism and its impact on the bone tissue surrounding titanium implants. However, the short experimental period used in this study may not have allowed osseointegration to occur completely. Therefore, to the authors' knowledge, the present study is the first to examine the impact of age-related bone changes on osseointegration and on bone around titanium implants, comparing its results to the surgically induced estrogen deficiency model.

It is not certain whether estrogen deficiency alone is responsible for the decrease in bone mass during the female aging period[21]. This phenomenon is probably a multifactorial problem due to the decline of bio available estradiol and testosterone, altered hormonal status and nutrition[25] and loss of osteogenic stem cells. In addition, it has been demonstrated that the "old" bone tissue presents reduced numbers of osteogenic precursor cells[26] and a change in the pattern of cytokine stimulation of bone [27-28]. The present data suggest that some architectural changes in bone resulting from age-related estrogen deficiency may mirror the one associated to surgically-induced estrogen deficiency, but the second may provide a more rapid and marked bone turnover change. However, it remains to be elucidated whether these physiological aspects occurred in the present study and contributed to these findings.

Thus, within the limits of the present investigation, it can be suggested that OVX may negatively influence the newly-formed and pre-existing bone around titanium implants, especially in the cancellous bone. Furthermore, age-related estrogen deficiency may be more restricted to the pre-existing bone lateral to the titanium implant surface in both cancellous and cortical bone.

Additionally, further studies should be considered in order to explore the clinical implications of these findings on a long-term basis.

ACKNOWLEDGMENT

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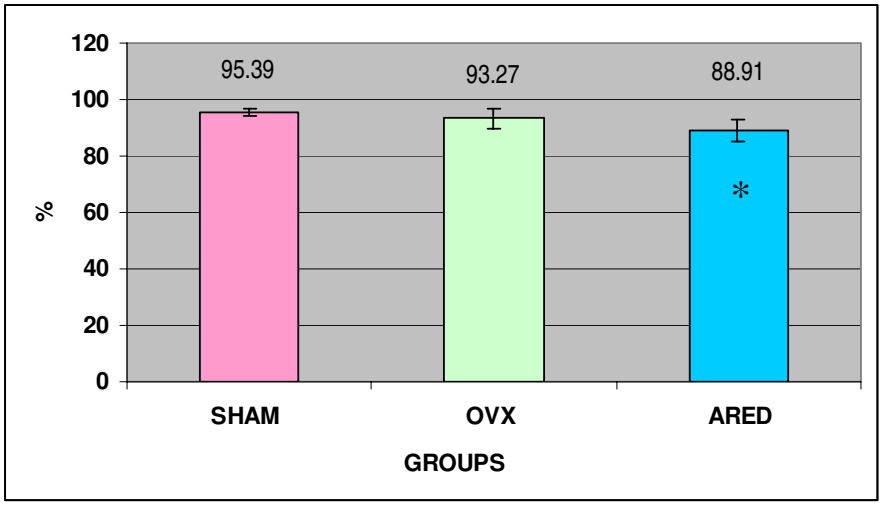
Table 1: Mean and standard deviation (%) of bone-to-implant contact (BIC) and bone area (BA) within the limits of the implant threads for all groups (SHAM: sham surgery; OVX: ovariectomy; ARED: age-related estrogen deficiency) at Zones A (cortical bone) and Zone B (cancellous bone).

GROUPS	BIC		BA	
	ZONE A	***ZONE B***	***ZONE A***	***ZONE B***
SHAM	49.84 ± 15.19**a**	51.08 ± 12.54**a**	84.79 ± 3.70**a**	49.64 ± 5.60**a**
OVX	45.72 ± 8.36**a**	36.75 ± 8.80 **b**	81.61 ± 3.81**a**	36.59 ± 7.90 **b**
ARED	47.49 ± 9.70**a**	43.46 ± 13.68**a**	81.63 ± 5.9**a**	43.62 ± 7.32**a**

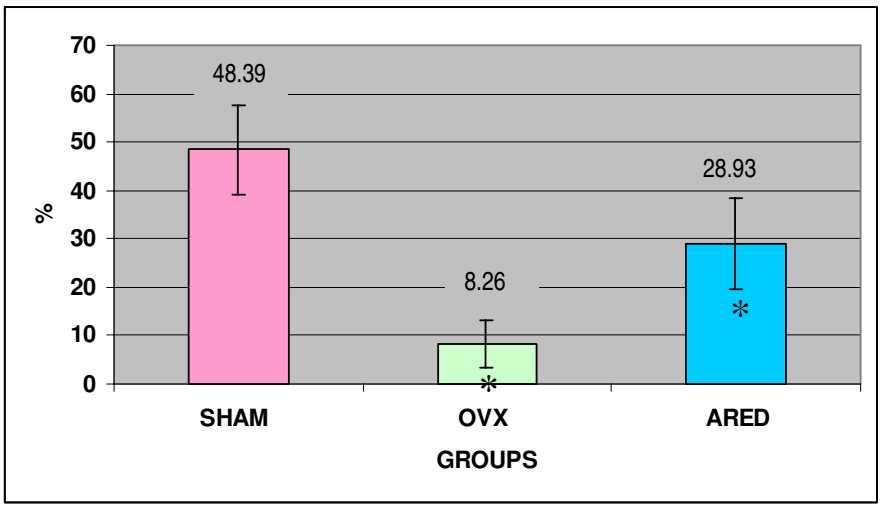
Different letters, within each column, indicate significant statistical differences determined by Kruskal-Wallis and Dunn's tests (α=0.05).

Figure 1. Means and standard deviation (%) of BD in zone A (cortical bone) for all experimental groups: SHAM: sham surgery; OVX: ovariectomy; ARED: age-related estrogen deficiency.



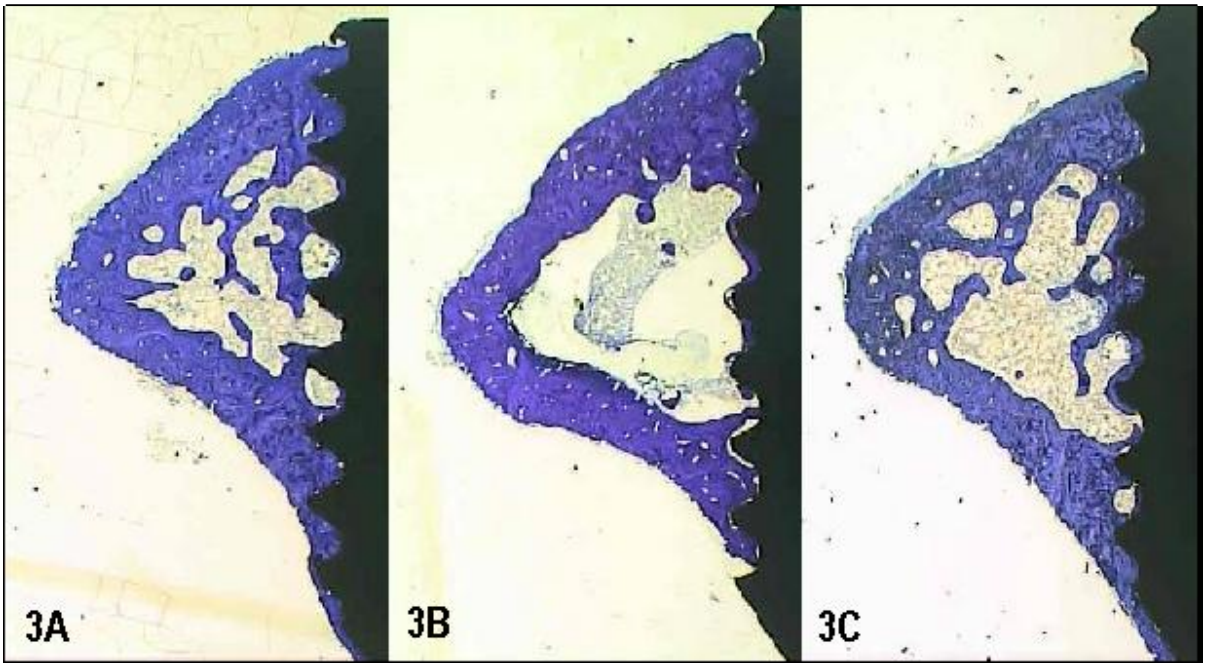
* Statistically different when compared to the SHAM group (P<0.05) (Kruskal-Wallis and Dunn's tests).

Figure 2. Means and standard deviation (%) of BD in zone B (medular bone) for all experimental groups: SHAM: sham surgery; OVX: ovariectomy; ARED: age-related estrogen deficiency.



* Statistically different when compared to the SHAM group (P<0.05) (Kruskal-Wallis and Dunn's tests).

Figure 3. Photomicrographs 3A to 3C illustrate the histological aspects observed within the limits of the threads and in a 500 µm-wide zone lateral to the implant surface in SHAM, OVX (ovariectomy) and ARED (age-related estrogen deficiency), respectively. (Toluidine blue/ Original magnification = 6.25x).



CAPÍTULO 6

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EFFECT OF AN ESTROGEN-DEFICIENT STATE AND ITS THERAPY ON BONE LOSS RESULTING FROM AN EXPERIMENTAL PERIODONTITIS IN RATS.

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Running Title: Osteoporosis and periodontitis

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KEY WORDS: osteoporosis, periodontitis, calcitonin, estrogen.

Abstract

The aim of this study was to evaluate the impact of an estrogen-deficient state (ED) and its therapies (estrogen and calcitonin administration) upon bone loss resulting from an experimental periodontitis. Fifty-eight Wistar rats were divided into four groups: Group 1 (n=15): sham operated; Group 2 (n=15): ovariectomized (OVX); Group 3 (n=14): OVX plus calcitonin administration; Group 4 (n=14): OVX plus estrogen administration. Twenty-one days after ovariectomy or sham surgeries, the ligature was randomly placed. Sixty days later, the animals were sacrificed and the specimens routinely processed. In addition, serum levels of alkaline phosphatase and calcium were assessed. Intergroup analysis

revealed that ED significantly increased bone loss resulting from periodontitis and that such an effect could not be prevented either by estrogen or calcitonin administration (0.34 ± 0.13, 0.65 ± 0.06, 0.63 ± 0.19, 0.67 ± 0.28 for groups 1, 2, 3 and 4; respectively). Furthermore, ED presented a direct effect on the alveolar bone regardless of plaque accumulation and this effect may be significantly reduced by estrogen administration ($p<0.05$). Serum analysis demonstrated a higher bone turnover for the animals with estrogen deficiency and estrogen therapy restored bone metabolism.

In conclusion, estrogen administration may prevent the direct effect of ED on alveolar bone; however, neither estrogen nor calcitonin administration could prevent this effect when associated with a response to a plaque-related inflammatory process.

Introduction

Periodontitis is characterized by inflammation of the supporting tissues of the teeth, resulting in alveolar bone resorption and soft tissue attachment loss (1). The role of systemic factors in the initiation and progression of periodontitis has been proposed (2).

Osteopenia and osteoporosis are age-related metabolic bone diseases that result in low bone mass and susceptibility to fractures (3). Currently, estrogen hormone replacement remains the single most effective treatment of menopausal symptoms and prevention of osteoporosis (4). However, alternative therapies such as calcitonin have also been proposed (5). Since osteoporotic changes have been observed in the oral bone (6) and loss of alveolar bone is a prominent feature in periodontal disease, osteoporosis could be suspected of being an aggravating factor in periodontal disease.

Therefore, the aim this study was to evaluate the impact of an estrogen-deficient state (ED) and its treatments, estrogen and calcitonin administration, on bone loss resulting from an experimental periodontitis in rats.

Material and methods

Experimental design: On the day after the ovariectomies, the animals were randomly assigned to one of four groups: **Group 1** (n=15): sham surgeries (negative control); **Group 2** (n=15): ovariectomy (OVX) (positive control); **Group 3** (n=14): OVX plus 4 days/week subcutaneous injections of CT (Miacalcic®, Sandoz A.G., Fertigung Schützenstrsse, Ravenburg, Germany) at a dose of 16 IU/Kg body weight; **Group 4** (n=14): OVX plus a daily subcutaneous injection of 17β estradiol (Sigma Chemical, St. Louis, MO, USA), dissolved in 100% ethanol and diluted in mineral oil at a dose of 20μg/Kg body weight.

Ligature placement/ clinical and biochemical analyses: Twenty-one days after ovariectomy, one of the mandibular first molars of each animal was randomly assigned to receive cotton ligature and the contralateral tooth was left unligated. Ovariectomy success was confirmed by monitoring the estrous cycle and, at autopsy, by the atrophy of uterine horns in rats not given estrogen therapy. Before the sacrifice, blood samples were collected in order to obtain plasma concentration of alkaline phosphatase (Gold Analisa Diagnóstica, Belo Horizonte, MG, Brazil) and calcium (AVL Scientific Corporation, Roswell, GA, USA).

Histometric procedure: Sixty days after ligature placement, the animals were sacrificed and the specimens routinely processed for decalcified sections in a mesio-distal direction (6µm). Using an image analysis system (Image-Pro®; Media Cybernetics, Silver Spring, MD, USA), the area of bone loss in the furcation region was histometrically determined.

Statistical analysis: Measurements were averaged to allow intergroup and intragroup analysis, using the one-way analysis of variance (ANOVA) (alpha = 0.05). Pairwise multiple comparisons were carried out by Bonferroni test ($\alpha = 0.05$) in the cases where the ANOVA test showed significant differences. In addition, the paired t-test ($\alpha = 0.05$) was used for intragroup comparisons between ligated and unligated teeth.

Results

Clinical observations: Macro analysis of the uterine horns and assessment of the estrous cycle of the rats confirmed the success of the ovariectomy surgery. Groups 2 and 3 presented *diestrous* smears and their reproductive organs were atrophied, confirming the reduction of estrogen levels. Conversely, group 1 presented the four stages of the estrous cycle and group 4 remained in the *estrus* stage. Finally, a pink and fluid filled uteri were clearly identified in groups 1 and 4, confirming that the serum estrogen levels were kept normal in these animals.

Biochemical serum analysis: The alkaline phosphatase level (IU/L) was statistically higher in groups 2 and 3 ($P<0.05$) and, therefore, confirmed a high bone turnover in the animals in the estrogen-deficient state (29.13 ± 10.93, 80.47 ± 20.16 and 98.20 ± 14.27, 33.29 ± 14.91 for groups 1, 2, 3 and 4; respectively). With respect to calcium serum levels, group 2 presented higher values than the other groups ($P<0.05$). The mean calcium serum levels (mmol/L) were 1.10 ± 0.07, 1.24 ± 0.08, 1.10 ± 0.14 and 1.07 ± 0.13 for groups 1, 2, 3 and 4; respectively.

Histometric results: Intragroup analysis showed that cotton ligatures placed around the teeth were able to promote periodontitis ($p<0.05$) (Table 1). In unligated teeth, intergroup analysis showed that ED may have a direct effect on the alveolar bone regardless of plaque accumulation, resulting in some bone loss in the furcation region. However, this negative effect was restored by 17β estradiol administration, but not by CT treatment (Table 1). In ligated teeth, ED resulted in a significant bone loss when compared to estrogen-sufficient animals ($p<0.05$). In addition, none of the treatments (groups 3 and 4) were able to protect against the impact of ED on the alveolar bone in the ligated teeth ($p<0.05$) (Table 1). Figures 2A to 2D illustrate the histological findings.

Discussion

Osteoporosis and periodontal disease are major health problems in older populations (7). Determination of the correlation between these two diseases may be critical for the prevention of morbidity and mortality related to these disorders in elders.

Thus, the present study aimed to investigate the impact of an estrogen-deficient state (ED), and two therapies, on the alveolar bone loss resulting from experimental periodontitis in rats. The results of the present study showed that ED may directly affect alveolar bone regardless of plaque accumulation and may also significantly increase bone loss resulting from ligature-induced periodontitis. Other studies have shown that ED and osteopenia/osteoporosis may increase oral bone resorption, attachment loss and tooth loss (6,8-9), however, this is the first study to show a direct correlation between periodontitis-related bone loss and lower levels of estrogen.

On a molecular basis, bone resorption has been characterised by two key molecules, RANKL (receptor activator of NF-kappaβ ligand) and OPG (osteoprotegerin) (10). Inhibition of RANKL function via the decoy receptor, OPG, has been shown to significantly reduce alveolar bone destruction, as reported by Teng *et al* (2000) (11). It has been shown that estrogen presents an important role in controlling bone resorption through its action on OPG (12). Therefore, in the present study, the synergistic effect observed between ED and plaque accumulation may be explained by an increased level of RANKL and a decreased level of OPG resulting from LPS stimulation and estrogen deficiency, respectively. However, the mechanisms involved remain to be investigated.

In the present study, estrogen therapy immediately after ovariectomy provided protection against the negative effects of ED on the alveolar bone around unligated teeth. These findings are in agreement with previous reports showing that ED induces osteoclastogenesis and osteoporotic changes in the

interradicular septum of rat first molars, and estrogen administration would prevent this effect (13). On the other hand, estrogen therapy was not able to protect the alveolar bone against the negative influence of ED associated with plaque accumulation. To date, to the authors' knowledge, no information is available regarding the effects of estrogen administered to estrogen-deficient individuals upon bone loss resulting from periodontitis. The protective mechanism of estrogen on bone tissue apparently involves suppression of bone turnover as a direct effect on bone cells and an indirect effect on the regulation of cytokine expression (14). Since the effects of estrogen on bone metabolism have also been attributed to its effect on OPG levels (12), estrogen replacement (in the present study) may have not reproduced OPG levels capable of counteracting LPS-stimulated RANKL levels. Caution, however, should be taken when drawing conclusions from these results and further studies should be considered.

Wronski et al., (1991) (15) reported that CT therapy for 41 days depresses bone turnover and prevents the development of osteopenia in OVX rats. Shen et al (1996) (16), however, demonstrated that CT, when administered to OVX rats for 90 days, only partially prevented bone loss. Similarly, our results demonstrated that CT partially affected bone loss around unligated teeth, although this effect was not noted in the presence of dental plaque. As previously reported (17), this phenomenon suggests that the skeletal response to CT may decline in a time-dependent manner, probably due to a down-regulation of bone binding receptors (18).

In conclusion, the present study clearly demonstrated, in rats, a synergistic effect of ED and plaque accumulation. In addition, the administration of estrogen or calcitonin could not protect against the effect of ED on the bone loss resulting from the experimental periodontitis. According to the data presented, a negative influence of ED on the alveolar bone can also be expected regardless of plaque accumulation, but estrogen administration was able to prevent such an effect. Therefore, in addition to the importance of estrogen deficiency in the general health status, it may also constitute a critical state with respect to the periodontium and controlled clinical studies should be considered in order to provide information as to the best approach to deal with this condition.

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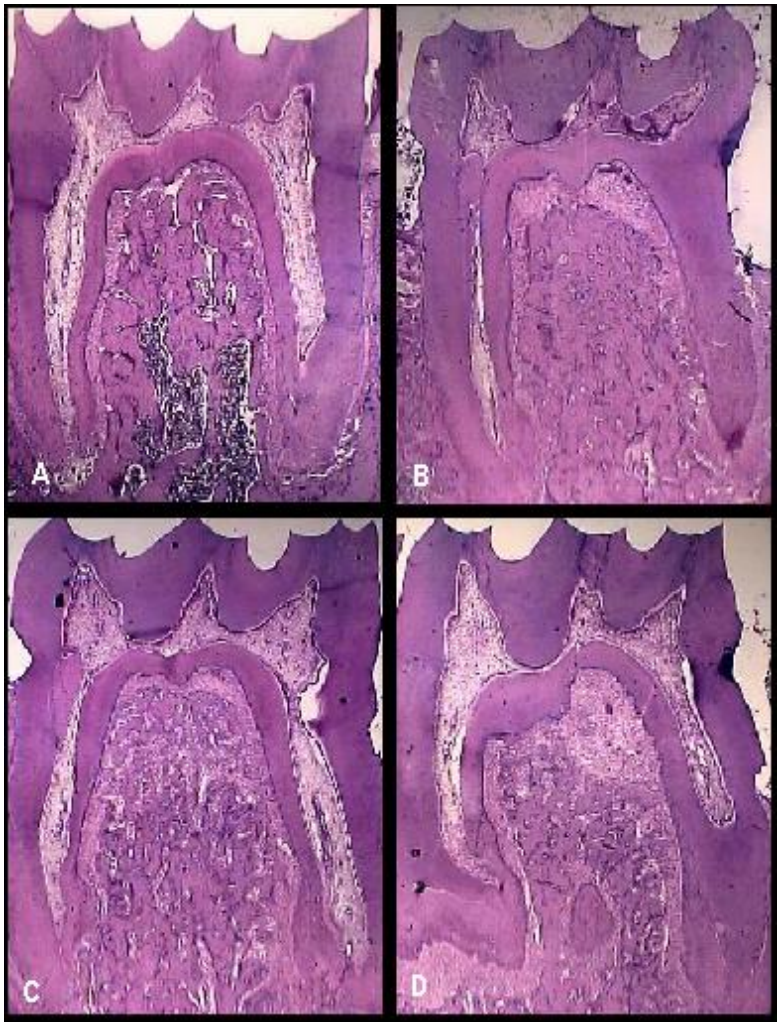
Table 1: Mean and standard deviation (mm^2) of bone loss and periodontal ligament areas around ligated and unligated teeth, according to each group.

	SHAM	**OVX**	**CALCITONIN**	**ESTRADIOL**
UNLIGATED	0.18 ± 0.03 aA	0.32 ± 0.08 aB	0.26 ± 0.04 aB	0.17 ± 0.03 aA
LIGATED	0.34 ± 0.13 bA	0.65 ± 0.06 bB	0.63 ± 0.19bB	0.67 ± 0.28 bB

Capital letters should be considered in lines and non-capital letters in columns.

Figure 2: Photomicrography illustrating periodontal ligament and bone loss areas in the furcation region for unligated and ligated teeth, respectively. Unligated tooth (group 1), ligated tooth (group 1), unligated tooth (group 2) and ligated tooth (group 2) are illustrated for figures A, B, C and D; respectively (Original Magnification 12.5x - H & E).



CAPÍTULO 7

Journal of Periodontology, v.75, n.9, p.1196-1202, 2004

ALENDRONATE PROTECTS AGAINST INCREASED PERIODONTITIS-RELATED BONE LOSS IN ESTROGEN-DEFICIENT RATS

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ABSTRACT

Background: The aim of this study was to evaluate the impact of alendronate (ALD) and estrogen (EST) therapies, and their withdrawal, on bone loss resulting from an experimental periodontitis in ovariectomized rats.

Methods: Eighty-seven Wistar rats were divided into six groups: Group 1 (n=15): sham surgery; Group 2 (n=15): ovariectomy (OVX); Group 3 (n=15): OVX plus alendronate administration for 80 days (AT); Group 4 (n=14): OVX plus alendronate administration for 40 days (AW); Group 5 (n=14): OVX plus 17β estradiol administration for 80 days (ET); Group 6 (n=14): OVX plus 17β estradiol administration for 40 days (EW). Twenty-one days after ovariectomy or sham surgeries, the mandibular molar was randomly assigned to receive a ligature, whilst the contralateral tooth was left unligated. Sixty days later, the animals were sacrificed and the specimens processed.

Results: OVX presented a direct impact on alveolar bone, regardless of plaque accumulation, and significantly increased bone loss resulting from periodontitis ($p<0.05$). The effect of OVX on unligated sites was significantly reduced by AT, AW and ET ($p<0.05$), but not by EW ($p>0.05$). In addition, alendronate administration (AT/AW) significantly reduced the impact of OVX on periodontitis-related bone loss ($p<0.05$), while estradiol did not ($p>0.05$).

Conclusion: Within the limits of this study, alendronate administration, but not estrogen replacement, may protect against the impact of estrogen deficiency on alveolar bone presenting a significant residual effect after its withdrawal.

KEY WORDS: estrogen deficiency, experimental periodontitis, alendronate, estradiol

INTRODUCTION

Periodontitis is characterized by inflammation of the supporting tissues of the teeth, resulting in soft tissue attachment loss and alveolar bone resorption[1]. Although the dental biofilm is the initiator of periodontal disease, animal and clinical studies have demonstrated that systemic factors may play an important role in its initiation and progression [2-3]. Estrogens have long been known to be important for skeletal homeostasis preventing bone loss[4]. The discovery of estrogen receptors in osteoblasts[5] and osteoclasts[6] suggests that a direct skeletal effect may be involved. It has also been shown that estrogen presents an important role in controlling bone resorption through its action on OPG and RANKL mechanism[7-8] and on bone-regulating factors such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF)[9 -10]. Since osteoporotic changes have been observed in the oral bone[11-12], and alveolar bone loss is a prominent feature in periodontal disease, estrogen deficieny could be suspected to be an aggravating factor in periodontal disease[13-14].

Estrogen therapy has been proposed to deal with osteoporosis in order to prevent bone loss in postmenopausal women and estrogen deficient individuals[15]. However, possibility of clinical contra-indications, risk of side effects and doubt about the real effect of estrogen treatment in the advanced years, has prompted the search for alternative approaches[16-17]. Alendronate, a member of the nitrogen-containing bisphosphonate family, has been shown to be an alternative therapy for preventing estrogen deficiency-induced osteopenia in experimental animal models and clinical trials[17-18], and inhibiting resorptive activity of mature osteoclasts[19]. Unlike other bisphosphonates (e.g., etidronate or pamidronate), alendronate does not impair mineralization[20], and in contrast to estradiol therapy, a

residual effect has been reported for alendronate therapy, favoring the maintenance of bone tissues[21-24].

Our group has previously shown that, in rats, estrogen deficiency resulted in an increased bone loss around ligated and unligated teeth, and that estradiol therapy was only able to protect unligated sites[25]. Here, we demonstrate that alendronate administration may protect against the negative impact of estrogen deficiency on unligated and ligated sites, and that such a beneficial effect was maintained even after interrupting alendronate administration. However, estradiol therapy was not able to impede ligature-induced bone loss in the ligated sites.

MATERIALS AND METHODS

ANIMALS

The experimental animals were 87 female Wistar rats that were 90 days of age and weighed an average of 210g at the beginning of the study. During the period of the experiment, the animals were kept in plastic cages with access to food and drinking water *ad libitum,* except ovariectomized (OVX) rats not on estradiol treatment (pair feeding) [26]. This protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

OVARIECTOMY

The animals were anesthetized with intramuscular administration of ketamine (0.5ml/kg). Ovariectomy or sham surgeries were performed at the beginning of the study. Bilateral ovariectomies were performed in 72 rats from a dorsal approach. The remaining animals (15 rats) were subjected to sham surgeries in which the ovaries were lifted up and returned intact to the original position. Postoperatively, the animals received antibiotic• given as a single intramuscular injection.

EXPERIMENTAL DESIGN

On the day after ovariectomies, the animals were randomly assigned to one of the six groups: **Group 1** (n=15): sham surgery (SHAM); **Group 2** (n=15): ovariectomy (OVX); **Group 3** (n=15): OVX plus 4 days/week subcutaneous injections of ALD∂ at a dose of 5mg/Kg body weight for 80 days (AT); **Group 4** (n=14) OVX plus 4 days/week subcutaneous injections of ALD at a dose of 5mg/Kg body

• Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil.

∂ Teva Pharmaceutical Ltda. , Petach Tikva, Israel

weight for 40 days (AW); **Group 5** (n=14): OVX and daily subcutaneous injections of 17β estradiol[∝], dissolved in 100% ethanol and diluted in mineral oil at a dose of 20μg/Kg body weight for 80 days (ET); **Group 6** (n=14): OVX and daily subcutaneous injections of 17β estradiol, dissolved in 100% ethanol and diluted in mineral oil at a dose of 20μg/Kg body weight for 40 days (EW) (figure 1);

LIGATURE PLACEMENT

Twenty-one days after ovariectomies, general anesthesia was obtained as mentioned above (ketamine - 0.5ml/kg). One of the mandibular first molars of each animal was randomly assigned to receive a cotton ligature in a submarginal position to induce experimental periodontitis. The contralateral tooth was left unligated to serve as control.

CLINICAL ANALYSES

In order to confirm the success of ovariectomy and estrogen administration, the estrous cycle was monitored two weeks after the ovariectomy surgeries and two weeks after the withdrawal of the drugs. Changes in the vaginal smear during 4-5 days of the estrus cycles were observed in each group. At autopsy, success of the ovariectomy was also confirmed by absence of ovaries and atrophy of uterine horns in ovariectomized rats. Success of estrogen administration was confirmed by the presence of normal uterine horns.

ALKALINE PHOSPHATASE ANALYSES

Blood samples were collected to measure plasma concentration of alkaline phosphatase at the time of sacrifice (60 days after ligature placement). Using automated laboratory techniques, alkaline phosphatase activity was obtained calorimetrically[ϒ].

HISTOMETRIC PROCEDURE

Sixty days after ligature placement, the animals were sacrificed. The specimens were then fixed in 4% neutral formalin for 48hs, and demineralized in a solution containing equal parts of 50% formic acid and 20% sodium citrate for 45 days. Paraffin serial sections (6μm) were obtained in a mesio-distal direction and stained by hematoxylin and eosin. Using an image analysis system[ƒ], the area of bone loss in the furcation region was histometrically determined by the point counting technique.

[∝] Sigma Chemical, St. Louis, MO, USA.
[ϒ] Gold Analisa Diagnóstica, Belo Horizonte, MG, Brazil
[ƒ] Image-Pro®; Media Cybernetics, Silver Spring, MD, USA

STATISTICAL ANALYSIS

Measurements were averaged to allow intergroup and intragroup analysis, using the one-way analysis of variance (ANOVA) (alpha = 0.05). Pairwise multiple comparisons were carried out by Bonferroni test (alpha = 0.05) in the cases where the ANOVA test showed significant differences. In addition, the paired Student t-test (alpha = 0.05) was used for intragroup comparisons between ligated and unligated teeth. In order to test the hypothesis that estradiol and alendronate administration did not influence alkaline phosphatase level, a one-way ANOVA (alpha = 0.05) was used. If statistical difference was detected, a pairwise multiple comparison procedure was used (Bonferroni t-test).

RESULTS

CLINICAL OBSERVATIONS

Clinical appearance of uterine horns, absence of ovaries and assessment of the estrous cycle confirmed the success of ovariectomy and estrogen replacement. Groups 2, 3 and 4 (OVX, AT and AW, respectively) presented *diestrous* smear and their reproductive organs atrophied, confirming the reduction of serum estrogen levels in these groups. In contrast, animals that were not ovariectomized (SHAM) presented the four stages of estrous cycle and a pink and fluid filled uteri. Animals that were administered for 80 days with estradiol (group 5) remained in the *estrus* stage and presented normal uteri, assuring that the serum estrogen levels were kept normal in these animals. Finally, animals in which the estradiol therapy was interrupted by day 40 presented *diestrous* smear after the withdrawal and their reproductive organs were atrophied at sacrifice.

ALKALINE PHOSPHATASE ANALYSES

Serum concentrations of alkaline phosphatase (UI/L) and standard deviation, performed at the time of sacrifice, were 27.6 $\pm$ 11.30; 80.46 $\pm$ 18.72; 40.93 $\pm$ 11.05; 44.28 $\pm$ 13.28; 33.2 $\pm$ 14.90 and 91.23 $\pm$ 23.28 for SHAM, OVX, AT, AW, ET and EW groups, respectively. Alkaline phosphatase levels were statistically higher for the OVX and EW groups ($P<0.001$) and, therefore, confirmed a high bone turnover in the animals in an estrogen-deficient state.

HISTOMETRIC RESULTS

Intragroup analysis demonstrated that ligature placement induced a significant periodontal bone loss in the furcation area for all the experimental groups when unligated and ligated sites were compared by the paired Student t-test ($p<0.05$). The intergroup comparison between unligated teeth by ANOVA demonstrated that estrogen deficiency affected alveolar bone regardless of biofilm

accumulation. In addition, it was also observed that continuous estradiol/alendronate and interrupted alendronate therapies were able to protect against the direct effect of estrogen deficiency on alveolar bone. Intergroup analysis further showed a significant increase in bone loss around ligated teeth for groups 2, 5 and 6 (OVX, ET and EW) ($p<0.05$), demonstrating that estrogen deficiency may have an effect upon periodontitis progression, and that estradiol therapy (continuous or interrupted) was not able to maintain such an effect. In contrast, statistical analysis demonstrated that continuous alendronate administration to OVX animals resulted in similar bone loss levels to control group (SHAM), and that such an effect was still observed even 40 days after drug withdrawal (Figure2). Figure illustrates the histological findings.

DISCUSSION

Although previous studies have failed to establish a relationship between osteoporosis and periodontal disease, it has been suspected that estrogen deficiency may act as a risk factor for periodontitis[11-14]. Confounding factors such as race, age, gender, or smoking, and the lack of precise methods for assessment of osteoporosis in the jaws have been reported to effect the establishment of a clear interaction between osteoporosis and periodontitis[14]. We previously demonstrated, in rats, that a surgically-induced estrogen deficiency may affect bone loss around unligated and ligated teeth, and that estrogen therapy was not able to reverse this effect[25]. In this study, we hypothesized that alendronate would protect against the deleterious effect of a reduced availability of estrogen, and that a residual effect would be observed after drug withdrawal.

The results of the present study confirm our previous observation[25] that estrogen deficiency might directly affect alveolar bone regardless of plaque accumulation. Data also confirm the fact that the periodontitis progression rate is increased due to an estrogen-deficient state. Although the mechanisms involved remain to be investigated, it has been demonstrated that bone resorption is characterised by two key molecules, RANKL (receptor activator of NF-kappa ligand) and OPG (osteoprotegerin) [27-28], that both RANKL and OPG are present with the periodontium [29-31], and that inhibition of RANKL function via its decoy receptor OPG significantly reduces alveolar bone destruction [29]. Since estrogen has been reported to control bone resorption through its action on OPG [7-8] regulation, we believe that the reduced levels of this hormone, induced in this study, may have promoted an unbalance in the RANKL/OPG system favouring bone resorption. Moreover, a synergic

effect between the biofilm and reduced levels of estrogen, resulting in an increased RANKL:OPG ratio, may be suggested.

In the current study, daily estrogen therapy (ET) immediately after ovariectomy provided protection against the negative effects of estrogen deficiency on alveolar bone around unligated teeth. This observation is in agreement with previous studies showing that ET protects against osteoclastogenesis and osteoporotic changes in the interradicular septum of the rat's first molar [32-34]. In contrast, in the present study, ET was not able to protect against the increased rate of bone loss observed around ligated teeth in ovariectomized animals, also supporting the findings of a previous investigation [34]. Estrogen withdrawal was observed to suppress its protective effect around unligated teeth, confirming the fact that estrogenic protection against osteopenia is not possible after drug removal[21], and as expected, no effect of ET was observed when drug administration was interrupted for ligated teeth.

Similarly to ET, alendronate administration provided protection against the negative effects of estrogen deficiency on alveolar bone around unligated teeth. However, a very different scenario was observed around ligated teeth when ovariectomized animals were treated with alendronate. Both continuous and interrupted alendronate therapies significantly reduced the effect of ovariectomy on the rate of periodontitis-related bone loss. These observations are consistent with the skeletal effects of bisphosphonates in early postmenopausal and ovariectomized women, and also confirm previous studies[22-24,35-36]. Bisphosphonates are synthetic compounds that are absorbed preferentially by the skeleton and suppress osteoclast-mediated bone resorption[37-38]. They inhibit osteoclast-mediated bone resorption by a mechanism that is not yet fully understood[36], although it has been suggested that alendronate, at low intracellular levels, inhibits osteoclast activity[37] and, at higher concentrations, may influence recruitment and differentiation of osteoclasts[39-40].

Data from studies on naturally-occurring and ligature-induced periodontitis[41-42] suggest that alendronate may retard bone loss around periodontitis affected teeth, however, alendronate therapy did not reduce signs of inflammation. The aim of the present study was not to investigate whether alendronate therapy affects bone loss resulting from periodontitis alone, and therefore, caution must be used before comparisons are made. If a synergic effect is occurring, between estrogen deficiency and biofilm accumulation, upon bone loss, the mechanism by which alendronate affects this process is unclear at the moment and further investigation should be considered. An interesting finding,

described herein, was the fact that the bone protective effect of alendronate therapy was maintained after its withdrawal around ligated and unligated teeth. These findings are in agreement with studies showing that the positive bone balance induced by alendronate is sustained for at least 18 months, long after therapy is stopped[22] and that intermittent periods of alendronate administration may be sufficient for long-term protection against estrogen deficiency[24]. The prolonged skeletal effects of diphosphonates after its withdrawal are probably a consequence of the long retention of this drug in bone, due to its strong binding to hydroxyapatite crystals[43].

The analysis of the estrus cycle and the aspect of the uterine horns in ovariectomized rats not submitted to ET confirmed the success of the ovariectomy model. In addition, biochemical serum analyses support the histometric results. Serum levels of alkaline phosphatase were significantly higher in the OVX and EW groups, demonstrating a high bone turnover as a consequence of the low levels of estrogen. On the other hand, ET, AT and AW resulted in an alkaline phosphatase level similar to that of the control group (SHAM), indicating that these therapies controlled the high bone turnover characteristic of low level estrogen.

In conclusion, within the limits of the present study, estrogen deficiency presented a significant effect upon alveolar bone, and also significantly increased bone loss rate resulting from experimental periodontitis in rats. Furthermore, continuous and interrupted alendronate administration may be a very important tool in dealing with periodontitis in estrogen-deficient individuals, whilst estrogen replacement therapy is not. Further studies should be considered in order to determine the potential role of osteoclastogenesis “inhibitors” drugs in dealing with alveolar bone loss in postmenopausal women or any estrogen-deficient individual.

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Figure 1: Schematic experimental design

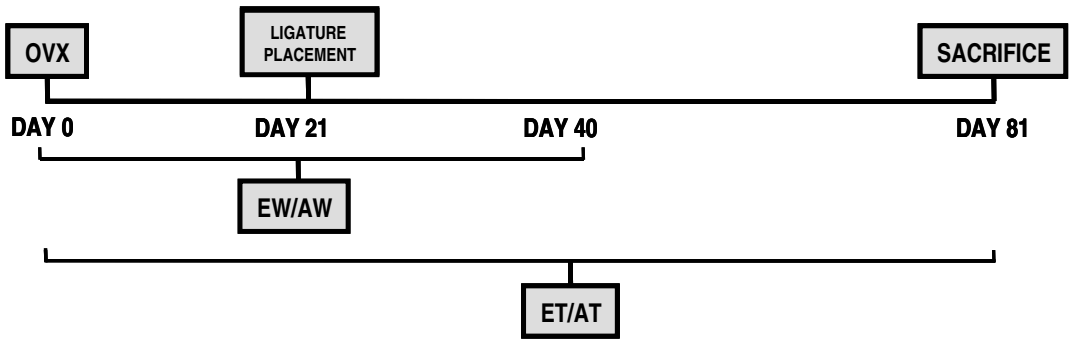


Figure 2: Mean and standard deviation (mm^2) of bone loss and periodontal ligament areas around ligated and unligated teeth, according to each group.

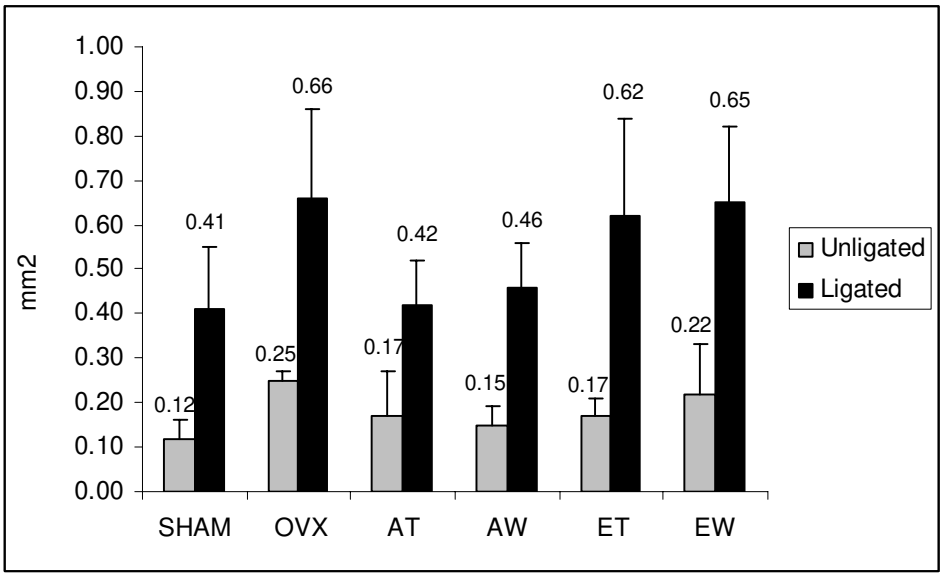
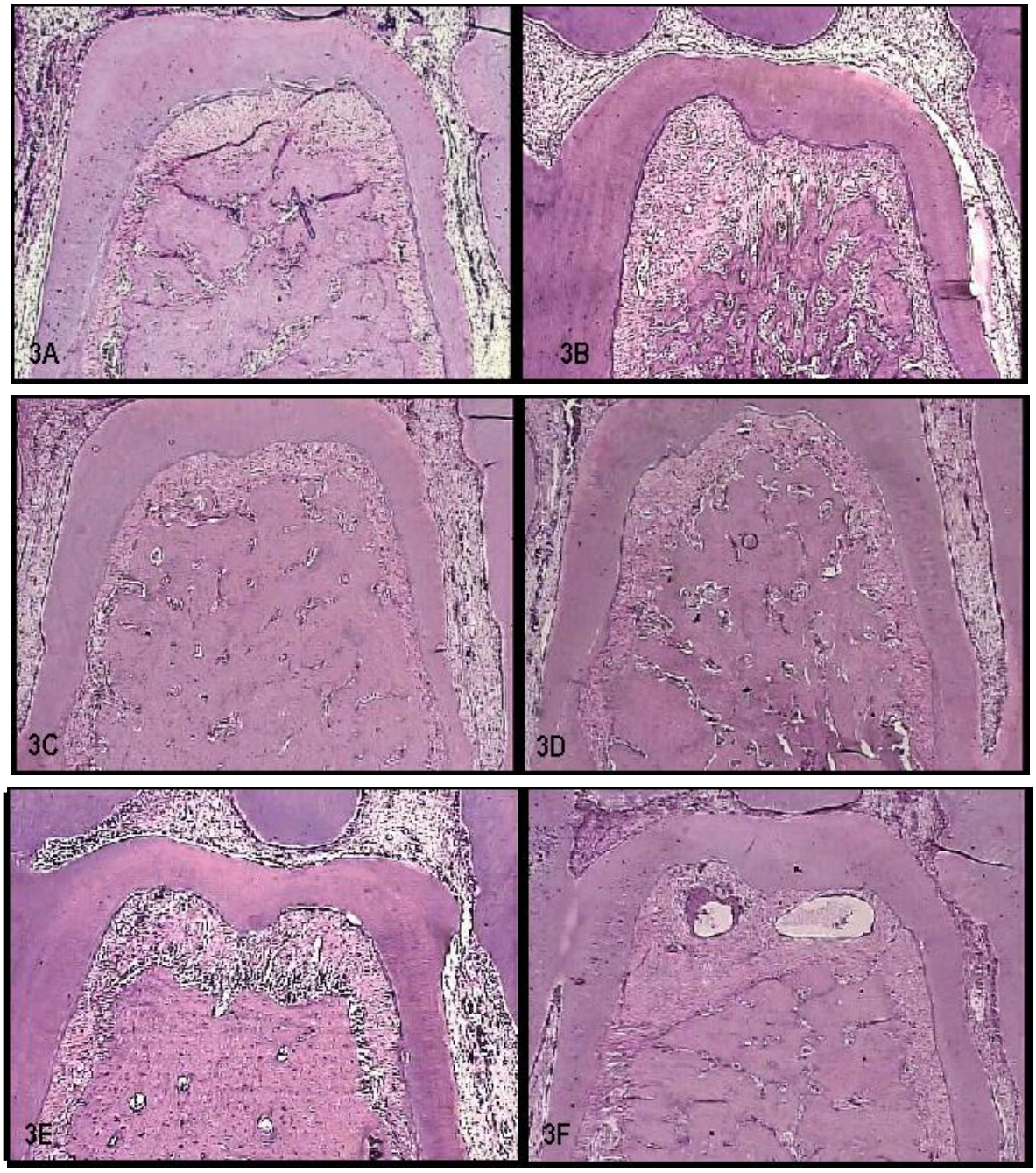


Figure 3: Photomicrography illustrating bone loss in the furcation region. Figures 3A, 3B, 3C, 3D, 3E, 3F illustrate, respectively: sham ligated tooth, OVX ligated tooth, AT ligated tooth, AW ligated tooth, ET ligated tooth, EW ligated tooth (Original Magnification 12.5x - H & E).



DISCUSSÃO GERAL

Considerando que uma extensa porcentagem da população necessita da substituição de dentes perdidos e de tratamento periodontal e que a expectativa de vida está aumentando, podemos esperar que a demanda para a colocação de implantes e para tratamento periodontal em pacientes osteoporóticos esteja sujeita a um aumento significativo. Com base nessa probabilidade, a relação entre osteoporose e implantes dentais bem como osteoporose e doença periodontal tem sido alvo de alguns estudos clínicos e em animais.

Já está bem estabelecido que os implantes dentais de titânio apresentam alta taxa de sucesso para substituição de espaços edêntulos (ADELL *et al.,* 1992), porém, alguns fatores de ordem local e/ou sistêmica podem interferir no processo de osseointegração (ESPOSITO *et al*., 1998). A osteoporose induzida pela deficiência de estrógeno, por ser uma doença sistêmica do metabolismo ósseo, poderia ser um fator prejudicial para o processo de osseointegração. Este processo patológico poderia ainda resultar em um osso alveolar de qualidade pobre (osso tipo IV), que apresentam grande risco para o sucesso de implantes dentais (JAFFIN & BERMAN , 1991). Logo, a primeira proposta deste trabalho foi verificar se a osteoporose induzida pela deficiência de estrógeno poderia afetar o tecido ósseo ao redor de implantes de titânio, utilizando para isso um modelo de ratas ovariectomizadas. Embora relatos de casos clínicos não tenham demonstrado que a osteoporose interfere no sucesso da osseointegração (FRIEBERG, 1994), os resultados deste estudo foram conclusivos em demonstrar que o tecido ósseo é negativamente afetado pela deficiência de estrógeno (capítulo 1).

Alguns estudos avaliaram modificações na superfície e no desenho de implantes para serem utilizados em ossos osteopênicos, com o intuito de melhorar o contato osso-implante (PAN *et al*., 2000). Entretanto, poucas são as informações sobre o efeito que medicamentos utilizados sistemicamente para o tratamento de osteoporose poderiam ocasionar sobre o osso ao redor dos implantes de titânio. Logo, o presente trabalho avaliou o impacto da terapia de reposição estrogênica, da calcitonina e do alendronato ao redor dos implantes inseridos em tíbias de ratas ovariectomizadas. Os resultados demonstraram efeitos benéficos mais evidentes da terapia com estrógeno e com alendronato (capítulos 2, 3 e 4). Uma vez que, por inúmeras razões ambos

tratamentos podem ser interrompidos, foi proposto ainda uma avaliação dos efeitos residuais desses medicamentos. Como discutido no capítulo 4, o alendronato apresentou efeito protetor do tecido ósseo mesmo após sua interrupção, enquanto a descontinuação do estrógeno resultou um uma perda óssea subseqüente.

Uma vez que ainda existem dúvidas se o modelo de ovariectomia reproduz realmente os fenômenos que ocorrem no tecido ósseo num processo natural de perda gradativa de estrógeno em mulheres na pós-menopausa, foi proposta uma comparação entre a deficiência de estrógeno relacionada à idade e a ovariectomia. Os resultados demonstraram que a ovariectomia apresenta um efeito negativo mais radical e rápido que a perda de estrógeno natural, que afeta preferencialmente o osso preexistente (capítulo 5).

Embora não existam evidências clínicas suficientes para contra-indicar a colocação de implantes em mulheres osteoporóticas ou estrógeno-deficientes, os resultados do presente trabalho sugerem que estas devem ser minuciosamente interrogadas sobre seu estado de saúde para a elaboração de um plano de tratamento seguro. Durante a anamnese deve ser observado se o paciente está sendo submetido a algum acompanhamento médico ou não. Ao exame clínico e radiográfico, devem ser analisados as características do leito receptor e o grau de perda óssea local e periférica, considerando que a osteoporose é uma doença osteometabólica progressiva. Esses pacientes poderão ser encorajados a realizar algum controle da doença, através do encaminhamento para um ortopedista, reumatologista ou ginecologista. Finalmente, cirurgiões dentistas devem ser cautelosos em colocar implantes e próteses convencionais em pacientes osteoporóticos sem tratamento prévio, principalmente aqueles que já apresentem um leito receptor desfavorável.

A presença de diversos fatores de confundimento em estudos clínicos tem impossibilitado o estabelecimento concreto da relação entre a osteoporose e a doença periodontal. Através de um modelo animal de osteoporose e periodontite induzidas, o presente trabalho demonstrou primeiramente que a deficiência de estrógeno apresenta um efeito sinérgico com o biofilme dental na perda óssea alveolar (capítulo 6). Subseqüentemente, foi verificado se na presença de alguns tratamentos para a osteoporose (estrógeno, calcitonina e alendronato) este efeito negativo poderia ser minimizado (capítulos 6 e 7). Os resultados demonstraram que apenas o alendronato (contínuo

ou interrompido) apresentou efeito protetor do tecido ósseo na presença de deficiência de estrógeno e de periodontite induzidas. Tais resultados encontraram suporte na literatura no que diz respeito ao benefício da droga na osteoporose e na perda óssea decorrente da doença periodontal, bem como à sua longevidade de ação pela união aos cristais de hidroxiapatita (FLEISCH, 1987; REDDY *et al.*1995) (capítulo 7). Da mesma forma que para o planejamento de implantes, os capítulos 6 e 7 chamam a atenção para a importância do estado de saúde geral do paciente na progressão da doença periodontal e para a possibilidade de minimização dos efeitos negativos da deficiência de estrógeno através do uso de medicamentos.

CONCLUSÃO

Diante dos objetivos do presente estudo, conclui-se que:

- A deficiência de estrógeno induzida pela ovariectomia afetou negativamente o tecido ósseo preexistente e neoformado ao redor de implantes de titânio inseridos em tíbia de ratas, especialmente o osso medular;
- As administrações de estrógeno e de alendronato, imediatamente após a ovariectomia e durante todo período experimental, foram capazes de prevenir a influência negativa da deficiência do estrógeno endógeno tanto no reparo quanto na densidade óssea ao redor dos implantes de titânio;
- A calcitonina, imediatamente após a ovariectomia, não foi capaz de evitar a influência negativa da deficiência de estrógeno no reparo e na densidade óssea adjacente aos implantes de titânio;
- Ao contrário do estrógeno, dentro do tempo avaliado, o efeito benéfico do alendronato sobre o tecido ósseo ao redor dos implantes inseridos em ratas ovariectomizadas foi mantido mesmo após sua interrupção;
- A deficiência de estrógeno relacionada à idade afetou principalmente o tecido ósseo preexistente ao redor dos implantes de titânio, enquanto a deficiência de estrógeno induzida pela ovariectomia que afetou os tecidos ósseos preexistente e neoformado;
- A deficiência de estrógeno induzida apresentou um efeito negativo direto sobre o osso alveolar de ratas mesmo sem o acúmulo de biofilme, que pôde ser reduzido pelas administrações contínuas de estrógeno e alendronato e pelo alendronato interrompido; mas não pela calcitonina e estrógeno interrompido;
- Existe um impacto negativo sinérgico da deficiência de estrógeno induzida e o acúmulo de biofilme sobre o osso alveolar de ratas ovariectomizadas. Nessas condições, o estrógeno (contínuo e interrompido) e a calcitonina não foram capazes de proteger o tecido ósseo. O alendronato (contínuo ou interrompido), por sua vez, protegeu o tecido ósseo alveolar dentro do tempo avaliado.

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* De acordo com a norma da UNICAMP/FOP, baseada no modelo Vancouver. Abreviatura dos periódicos em conformidade com o medline.

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ANEXOS

1: Implant Dent. 2003;12(4):340-6.

Related A

Estrogen deficiency affects bone healing around titanium imp histometric study in rats.

Duarte PM, Cesar Neto JB, Goncalves PF, Sallum EA, Nociti FH.

Department of Prosthodontics and Periodontics, Division of Periodontics, of Dentistry at Piracicaba, UNICAMP, Sao Paulo, Brazil.

The purpose of this study was to evaluate the influence of an estrogen-de state on bone around titanium implants placed in rats. Thirty female Wist were divided into 2 groups: test (n = 15), ovariectomized rats (OVX); anc (n = 15), sham-operated rats. Screw-type titanium implants were placed b in rats 21 days after ovariectomy or sham surgery. After 60 days, the anir killed and undecalcified sections obtained. Blood samples were collected serum levels of alkaline phosphatase at the time of killing. Bone-to-impla (BIC), bone area (BA) around the implants, and bone density (BD) in a 5 microns-wide zone lateral to the implant were obtained and arranged sepa the cortical (zone A) and cancellous (zone B) regions. In zone A, there w significant difference between test and control groups regarding BIC and 0.05). A lower BA was observed in the estrogen-deficient animals ($P < 0.$ zone B, data analysis showed that estrogen deficiency could result in a lo percentage of BIC, BA, and BD ($P < 0.05$). In addition, a higher concentr alkaline phosphatase was observed for the test group. An estrogen-deficie could affect bone healing and bone density around titanium implants plac especially in the cancellous bone area.

PMID: 14752971 [PubMed - indexed for MEDLINE]

1: Int J Oral Maxillofac Implants. 2002 Nov-Dec;17(6):786-92.

Effect of estrogen replacement and calcitonin therapies on bo around titanium implants placed in ovariectomized rats: a histometric study.

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PURPOSE: The aim of the present study was to evaluate whether hormor replacement therapy (HRT) and calcitonin (CT) administration could infl bone healing around implants placed in ovariectomized (OVX) rats. MA AND METHODS: One screw-type titanium implant was placed bilaterall rats. The animals were assigned to one of the following groups: group 1 (sham surgeries; group 2 (n = 15), OVX rats; group 3 (n = 14), OVX rats administered CT 4 days/week (16 IU/kg); group 4 (n = 14), OVX rats adr 17beta estradiol daily (20 microg/kg). After 60 days, the animals were sa and undecalcified sections obtained. Bone-to-implant contact (BIC) and b (BA) around the implants were determined separately for the cortical (zor cancellous (zone B) bone areas. RESULTS: In zone A, intergroup analys reveal a significant difference regarding BIC. In contrast, the HRT group presented greater BA than groups 2 and 3 ($P < .05$). Data from zone B rev HRT eliminated the negative effect of the ovariectomy on BIC and BA (I while CT had no effect ($P > .05$). DISCUSSION: It was the first study to and demonstrate the impact of HRT and CT on bone around titanium imp estrogen-deficient model. CONCLUSION: Within the limits of the prese may be concluded that HRT may prevent the influence that estrogen defi exerts on bone healing around titanium implants.

PMID: 12507237 [PubMed - indexed for MEDLINE]

1: J Periodontol. 2003 Nov;74(11):1618-24.

Comment in:

- J Periodontol. 2003 Nov;74(11):1692-3.

Effect of estrogen and calcitonin therapies on bone density in lateral area adjacent to implants placed in the tibiae of ovariectomized rats.

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BACKGROUND: This study evaluated the influence of estrogen and calc administration on tibial bone density in a lateral area adjacent to implants ovariectomized rats (OVX). METHODS: One screw-type titanium implan placed bilaterally in the ovariectomized rats, and the animals assigned to following groups: group 1 (n = 15): sham surgeries; group 2 (n = 15): OV 3 (n = 14): OVX subcutaneously administered with calcitonin (CT) 4 day (16 IU/kg); group 4 (n = 14): OVX administered daily with 17beta estrad microg/kg). After 60 days, the animals were sacrificed and undecalcified obtained. Blood samples were collected to measure serum levels of alkali phosphatase and calcium at the time of sacrifice. Bone density was measu 500 microm wide mineralized zone lateral to the implant. RESULTS: All phosphatase levels in groups 2 and 3 ($P > 0.05$) were statistically higher t groups 1 and 4 ($P < 0.05$), and calcium serum levels were higher in group the other groups ($P < 0.05$). Regarding bone density, the data were group separately for cortical (zone A) and cancellous (zone B) bone. In zone A, intergroup analysis revealed no significant difference among groups ($P >$ However, in zone B, the animals that received estrogen administration (g presented a higher bone density than groups 2 and 3 ($P < 0.05$). CONCLU appears that estrogen therapy may prevent the negative influence of endo estrogen deficiency on bone density around titanium implants placed in ovariectomized rats.

PMID: 14682658 [PubMed - indexed for MEDLINE]

To: nociti@fop.unicamp.br
Subject: Journal of Periodontology - JOP-04-0053.R1
From: rjgenco@buffalo.edu
CC: nociti@fop.unicamp.br
Date: Thu, 10 Jun 2004 09:35:28 -0400 (EDT)

06 10 2004

Dear Prof. Nociti,
I am pleased to inform you that your manuscript, ALENDRONATE THERAPY MAY BE EFFECTIVE IN THE PREVENTION OF BONE LOSS AROUND TITANIUM IMPLANTS INSERTED IN ESTROGEN-DEFICIENT RATS. (JOP-04-0053.R1), is accepted for publication in the Journal of Periodontology, with the following acceptance date (the date your most recent revision was submitted):

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Subject: Manuscript Submitted - JOP-04-0353

October 27th, 2004

Dear Prof. Nociti,

Thank you for submitting your manuscript, AGE-RELATED AND SURGICALLY INDUCED ESTROGEN DEFICIENCIES MAY DIFFERENTLY AFFECT >BONE AROUND TITANIUM IMPLANTS INSERTED IN RATS (JOP-04-0353), to the Journal of Periodontology. Your manuscript will be forwarded to Dr. Genco or an Associate Editor and placed in the review process. *If this is a revised manuscript, indicated by an "R" after the manuscript number, a decision will be* rendered shortly. As a reminder, the Journal of Periodontology requires a cover letter containing the signatures of all authors. If you have not yet done this, please e-mail or fax this letter to my attention (e-mail: julie@perio.org; fax: 312.573.3225). If you have any questions, or if I can provide further information, please do not hesitate to contact me at julie@perio.org. It will be most helpful if you would include both your manuscript title and reference number in any correspondence.

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1: J Periodontal Res. 2004 Apr;39(2):107-10.

Effect of an estrogen-deficient state and its therapy on bone l resulting from an experimental periodontitis in rats.

Duarte PM, Goncalves PF, Sallum AW, Sallum EA, Casati MZ, Hun Nociti F Jr.

Department of Prosthodontics and Periodontics, Division of Periodontics. of Dentistry at Piracicaba, UNICAMP, Piracicaba, Sao Paulo, Brazil.

OBJECTIVE: The aim of this study was to evaluate the impact of an estr deficient state and its therapies (estrogen and calcitonin administration) u loss resulting from an experimental periodontitis. METHODS: Fifty-eigh rats were divided into four groups: group 1 (n = 15): sham operated; grou 15): ovariectomized; group 3 (n = 14): ovariectomized plus calcitonin administration; group 4 (n = 14): ovariectomized plus estrogen administra Twenty-one days after ovariectomy or sham surgeries, the ligature was ra placed. Sixty days later, the animals were killed and the specimens routin processed. In addition, serum levels of alkaline phosphatase and calcium assessed. RESULTS: Intergroup analysis revealed that an estrogen-defici significantly increased bone loss resulting from periodontitis and that suc could not be prevented either by estrogen or calcitonin administration (0. 0.13, 0.65 +/- 0.06, 0.63 +/- 0.19, 0.67 +/- 0.28 for groups 1, 2, 3 and 4, respectively). Furthermore, an estrogen-deficient state presented a direct the alveolar bone regardless of plaque accumulation and this effect may b significantly reduced by estrogen administration ($p < 0.05$). Serum analys demonstrated a higher bone turnover for the animals with estrogen defici estrogen therapy restored bone metabolism. CONCLUSION: Estrogen administration may prevent the direct effect of an estrogen-deficient state alveolar bone; however, neither estrogen nor calcitonin administration co prevent this effect when associated with a response to a plaque-related inflammatory process.

PMID: 15009518 [PubMed - indexed for MEDLINE]

September 2004 (Vol. 75, No.

Alendronate May Protect Against Increased Periodontitis-Related Bon Loss in Estrogen-Deficient Rats

Poliana Mendes Duarte, Daniel Roberto de Assis, Marcio Zaffalon Casati, Antonio Wilson Sallum, Enilson Antonio Sallum, and Francisco H. Nc Jr.

Abstract

Background: The aim of this study was to evaluate the impact of alendronate (ALD) and estrogen (EST) therapies and their withdrawal on bone loss in experimental periodontitis in ovariectomized rats.

Methods: Eighty-seven Wistar rats were divided into six groups: group 1 (N = 15): sham surgery; group 2 (N = 15): ovariectomy (OVX); group 3 (N = 15): OVX plus alendronate administration for 80 days (AT); group 4 (N = 14): OVX plus alendronate administration for 40 days (AW); group 5 (N = 14): OVX plus 17β estradiol administration for 80 days (ET); and group 6 (N = 14): OVX plus 17β estradiol administration for 40 days (EW). Twenty-one days after ovariectomy or sham surgery, one mandibular molar was randomly assigned to receive a ligature, while the contralateral tooth was left unligated. Sixty days later, the animals were sacrificed and the specimens processed.

Results: OVX presented a direct impact on alveolar bone, regardless of plaque accumulation and significantly increased bone loss resulting from periodontitis ($P < 0.05$). The effect of OVX on unligated sites was significantly reduced by AT, AW, and ET ($P < 0.05$), but not by EW ($P > 0.05$). In addition, alendronate administration (AT/AW) significantly reduced the impact of OVX on periodontitis-related bone loss ($P < 0.05$), while estradiol did not ($P > 0.05$).

Conclusion: Within the limits of this study, alendronate administration, but not estrogen replacement, may protect against the impact of estrogen deficiency on alveolar bone presenting a significant residual effect after its withdrawal. *J Periodontol 2004;75:1196-1202.*

KEY WORDS

Alendronate/therapeutic use; alveolar bone loss/therapy; animal studies; estradiol/therapeutic use; estrogen deficiency.

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **469-2**, sobre "**Efeito da Deficiência Natural e Induzida de Estrógeno e do seu Tratamento sobre o Tecido Ósseo ao Redor de Implantes de Titânio: Estudo Histométrico em Ratos**", sob a responsabilidade de **Prof. Dr. Enilson Antonio Sallum** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de **21 de Março de 2003**.

CERTIFICATE

We certify that the protocol nº **469-2**, entitled **"Effect of Induced and Natural Estrogen Deficiency and its Treatment Around Titanium Implants: a Histometric Study in Rats",** is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on **March 21, 2003**.

Campinas, 21 de Março de 2003.

Profa. Dra. Liana Verinaud
Presidente
CEEA/IB/UNICAMP

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