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Gabriela Giro

# "Avaliação da influência da osteoporose induzida em ratas, e seu tratamento com alendronato e estrógeno, sobre o tecido ósseo ao redor de implantes."

Tese apresentada ao Programa de Pós-graduação em Odontologia - Área de Periodontia, da Faculdade de Odontologia de Araraquara, Universidade Estadual Paulista, para a obtenção do Título de Doutor em Odontologia.

Orientadora: Profª Drª Silvana Regina Perez Orrico

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*"Avaliação da influência da osteoporose induzida em ratas, e seu tratamento com alendronato e estrógeno, sobre o tecido ósseo ao redor de implantes."*

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Presidente e orientadora: Profª. Drª. Silvana Regina Perez Orrico

2° examinador: Prof. Dr. Carlos Rossa Junior

3° examinador: Prof. Dr. Luis Carlos Spolidorio

4° examinador: Profª. Drª. Márcia Pinto Alves Mayer

5° examinador: Prof. Dr. Marcelo de Faveri

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# Dados Curriculares

## Gabriela Giro

NASCIMENTO 24.09.1979 – Nova Europa / SP, Brasil.

FILIAÇÃO João Giro Filho
Belmira Conceição da Costa Giro

1998/2002 **Curso de Graduação em Odontologia**
Faculdade de Odontologia de Araraquara – UNESP

2003/2005 **Curso de Especialização em Periodontia**
Escola de Aperfeiçoamento Profissional EAP – APCD

2004/2006 **Curso de Pós-Graduação em Periodontia,**
Nível Mestrado
Faculdade de Odontologia de Araraquara UNESP.

2006/2010 **Curso de Pós-Graduação em Odontologia, Área de concentração em Periodontia,** Nível Doutorado
Faculdade de Odontologia de Araraquara UNESP

2009/2011 **Curso de Especialização em Implantodontia**
Fundação Araraquarense de Ensino e Pesquisa em Odontologia (FAEPO)

# O gladiador

Sucesso, reconhecimento, fama, glória. Muitos de nós lutamos por motivos assim. Mas não se constrói um bom nome da noite para o dia. É preciso trabalhar muito. Ainda que haja tropeços e quedas, é preciso superar os obstáculos. É preciso ter motivação, perseverar, insistir... A vida é uma sucessão de batalhas.

Emprego, família, amigos: todos nós temos um status atual e temos também expectativas com relação ao futuro.

No entanto, as reviravoltas do destino nos surpreendem. Nem sempre dá para se fazer só o que gostamos. Mas aquele que gosta do que faz e sente orgulhoso em fazer melhor, a cada dia vai mais longe. Há momentos de calmaria e há momentos agitados, decisivos, em que a boa intenção não basta. É quando a vida nos cobra coragem, arrojo, criatividade e um inabalável espírito de luta.

A verdade é que os problemas e os reveses ocorrem com maior frequência do que gostaríamos. Os tempos mudam. Surgem novos desafios e novos objetivos. Os guerreiros olham nos olhos do futuro sem medo e sem arrogância, mas com a confiança de quem está pronto para o combate.

Viver é também estar preparado para as situações difíceis. O modo como encaramos as dificuldades é que faz a diferença.

Às vezes nos perguntamos: como enfrentar as mudanças radicais que se apresentam diante de nós? Como atuar num novo cenário, onde coisas que fazíamos tão bem precisam ser reaprendidas? Como lutar sem

deixar para trás valores fundamentais? Como saber a medida exata a ser tomada no momento certo?

O mais incrível é que justamente diante de situações adversas, muitos redescobrem o que têm de melhor: a ética, a amizade, a capacidade de criar novas estratégias fundamentadas na experiência, o talento para promover alianças positivas, o espírito de liderança, a consciência da força que reside no verdadeiro trabalho em equipe. Tudo isso aflora quando as circunstâncias exigem, quando se sabe que existe um objetivo maior a ser alcançado.

Claro que não é fácil abandonar hábitos, costumes... Não é fácil adaptar-se aos novos meios, ou usar recursos aos quais não estávamos familiarizados. Mas todo guerreiro sabe que pessimismo e insegurança nessa hora só atrapalham. Ainda que a ameaça venha de vários lados, com agilidade, força e determinação podemos alcançar o resultado.

A combinação de energia e inteligência, assim como o equilíbrio entre a razão e a emoção, são fundamentais para o sucesso. É uma sensação extremamente agradável chegar ao fim de uma etapa com a consciência do dever cumprido. E obter a consagração, o respeito de todos, o reconhecimento dos colegas, a admiração das pessoas que amamos... Ouvir o próprio nome com orgulho. Aquele orgulho de quem viu nos obstáculos a oportunidade de crescer. O orgulho de quem soube enfrentar as turbulências da vida e crescer... O orgulho de ser um vencedor que não abriu mão dos seus valores fundamentais: excelência, ética, criatividade, comprometimento, responsabilidade, respeito.

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# Sumário



Gabriela Giro

# Resumo

Giro G. Avaliação da influência da osteoporose induzida em ratas, e seu tratamento com alendronato e estrógeno, sobre o tecido ósseo ao redor de implantes. [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2010.

## Resumo

O presente projeto teve como objetivo avaliar a influência da deficiência de estrógeno, e as implicações dos tratamentos com alendronato e estrógeno, sobre o tecido ósseo ao redor de implantes. Para isso foram realizados dois estudos distintos, visando elucidar as questões em torno desse tema. No primeiro estudo, foram utilizadas ratas Wistar, com idade aproximada de 60 dias, submetidas à colocação de um implante na metáfise tibial com o objetivo de avaliar a influência da deficiência estrogênica e dos tratamentos com alendronato e estrógeno sobre o tecido ósseo em implantes que já se encontravam osseointegrados. Os resultados mostraram efeitos deletérios da referida deficiência, com diminuição da força de torque necessária para remoção dos implantes, menor quantidade de osso em contato com a superfície e área de osso entre as roscas, aumento nos marcadores de remodelação óssea e menor taxa de aposição mineral no período avaliado. Por outro lado, os animais que receberam a administração do alendronato apresentaram os melhores resultados para todas as análises realizadas, apesar do tecido ósseo mostrar-se mais compacto e sem sinais de remodelação. Concluiu-se que a deficiência estrogênica decorrente da ovariectomia (OVX) acarretou alterações sistêmicas, como perda de massa

óssea, e alterou as características do tecido ósseo ao redor dos implantes. Esse quadro pôde ser revertido com os tratamentos instituídos, tendo o alendronato apresentado os melhores resultados.

O segundo estudo visou avaliar os efeitos da deficiência de estrógeno e de diferentes dosagens de alendronato, sobre implantes instalados na maxila de ratas e, portanto, sob influência da carga mastigatória. Nesse estudo foram avaliadas também as implicações da instalação de implantes em animais que estavam sob terapia com diferentes dosagens de alendronato por um período prolongado. Os resultados desse estudo mostraram que, embora tenham sido constatadas alterações sistêmicas advindas da supressão estrogênica, não houve diferença para os achados histométricos em comparação ao grupo com função ovariana normal (SHAM). Em relação aos grupos que receberam administração sistêmica do alendronato após a osseointegração dos implantes, apesar de também não apresentarem diferença em relação aos parâmetros histométricos avaliados, pôde-se observar um aumento no numero de células em apoptose e acúmulo de lacunas de osteócitos vazias. Também foram observados sinais de estagnação da remodelação do tecido ósseo, como a diminuição das linhas de reversão. Por outro lado, os animais que receberam a administração da droga antes da instalação dos implantes apresentaram menor porcentagem de contato do tecido ósseo com as roscas dos implantes, menor área ocupada por tecido ósseo entre as roscas e extensas áreas de necrose ao longo do implante. Outro achado bastante interessante refere-se à deposição de uma matriz amorfa entre as roscas dos implantes, em conseqüência de uma provável deficiência na fase de

mineralização da matriz secretada. A administração do alendronato também estimulou a expressão de citocinas anti-inflamatórias, pró-inflamatórias e inibiu a expressão dos marcadores de formação e reabsorção óssea. O tratamento com estrógeno não apresentou diferença, em comparação ao grupo controle, para nenhum dos marcadores avaliados. Tais achados nos levam à conclusão de que a deficiência estrogênica não deve ser considerada um fator de contra-indicação do tratamento com implantes, desde que estes estejam em função. Por outro lado, uma atenção especial deve ser dada aos pacientes que fazem uso crônico de alendronato, uma vez que a medicação pode alterar seriamente a cicatrização do tecido ósseo ou modificar este tecido ao redor de implantes osseointegrados.

**Palavras-chave:** implante dentário; ovariectomia; osteoporose; alendronato; bifosfonatos; terapia de reposição hormonal.

# Abstract

Giro G. Effects of induce osteoporosis, and its treatment with alendronate and estrogen, on bone tissue around implants [tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2009.

## Abstract

This study aimed the evaluation of the influence of bone mass loss and its therapies on bone tissue around osseointegrated implants. For that reason two major projects were conducted. First of all, two implants were placed in 66 female rats tibiae. The animals were assigned to 5 groups: control (CTL), sham, ovariectomy (OVX), oestrogen (EST) and alendronate (ALE). While CTL was sacrificed 60 days after implant placement, other groups were subjected to ovariectomy or sham surgery according to group and euthanized after 90 days. Blood and urine samples were collected at sacrifice day for osteocalcin and deoxypyridinoline quantification. Densitometry of femur and lumbar vertebrae were performed in order to evaluate rats' skeletal impairment. One implant was subjected to the removal torque test, while the remaining one was referred to non-decalcified sections and analysed by fluorescent and light microscopy for analyses of mineral apposition rate (MAR), eroded and osteoclastic surfaces, bone to implant contact (BIC), and bone area fraction occupancy (BAFO). The results of this study showed that OVX presented significantly lower values for all parameters analysed compared to other groups. ALE increased bone mineral density and the removal torque of the implants, but also reduced osteocalcin and deoxypyridinoline concentrations, MAR, osteoclastic and eroded surfaces, and no difference was in BIC and

BAFO relative to SHAM. EST and CTL showed similar results to SHAM for measurements. The results lead the authors to conclude that oestrogen deficiency exerted a negative influence on bone tissue around implants, while oestrogen replacement therapy and alendronate were effective against its effects. Although alendronate therapy maintained the amount of bone tissue around implants, studies evaluating bone turnover kinetics are warranted.

Thus, in order to evaluate the bone turnover kinetics regarding alendronate therapy, the second project targeted the influence of alendronate administration during healing and after osseointegration on bone tissue surrounding endosseous implants in a jaw model. This study included 56 female Wistar rats, randomly divided into 7 groups. All animals had the upper first molars extracted. After 30 days, sham surgery (SHAM), ovariectomy (OVX), estrogen replacement therapy (EST), and the groups that received alendronate therapy after implant integration (ALE 50µg after osseointegration and ALE 1mg after osseointegration) received 2 implants in order to replace the missing teeth. Sixty days after implant placement these animals were submitted to the respective treatment. Two groups (ALE 50 µg prior to osseointegration and ALE 1mg prior to osseointegration) started receiving alendronate 90 days prior to implant placement (implants kept in place for 60 days). The ovariectomy by itself did not affect the integrity of bone tissue around implants. Histomorphometric analysis showed a decrease of bone to implant contact and bone area fraction occupancy between implant threads for animals that received alendronate treatment previously to the implant placement. Alendronate administration also increased the amount

of empty osteocyte lacunae and apoptotic cells in proximity with the implant surface. The expression patterns of skeletal remodeling factors and cytokine production evaluated in the serum and gingival tissue around implants. The levels of RANKL and TNF-$\alpha$ were elevated in rats receiving ALE either prior to, or after, implant placement. However, elevated levels of IL-6 and osteopontin were only characteristic to the peri-implant necrotic tissue of rats receiving ALE prior to implant placement. Irrespective of diminished expression of osteopontin in bone tissue, the increased level of osteopontin on gingival tissue appeared to be derived from the infiltrating CD5+ T cells or activated macrophages. Whitin the findings of this study it could be concluded that alendronate treatment negatively affected the bone tissue healing and establishment of osseointegration of dental implants. These results also suggest that increased osteopontin-expressing cells to play a key role in inhibition of dental implant integration under bisphosphonate therapy.

**Key words:** ovariectomy, osteoporosis, dental implants, alendronate, bisphosphonate, rats.

# Introdução

Branemark et al.[8] (1969) definiram a osseointegração como o contato direto do tecido ósseo vivo com o metal de um implante em função. A obtenção e a manutenção da osseointegração de forma previsível é uma importante questão na prática clínica. Apesar de ser uma técnica com alta previsibilidade de sucesso, estudos têm demonstrado que algumas condições sistêmicas podem alterar severamente o resultado do tratamento com implantes dentais (Duarte et al.[13], 2003; Margonar et al.[28], 2003; Sakakura et al.[49], 2003; van Steenberghe et al.[63], 2003).

Dentre essas alterações sistêmicas tem-se a osteoporose, uma condição caracterizada pela redução da densidade mineral óssea e pela deterioração da microarquitetura do tecido ósseo, levando a um aumento dos espaços medulares, gerando maior fragilidade e conseqüente aumento do risco a fraturas (Consensus Development Conference on Osteoporosis[11], 1993; Marcus[27], 1996). A osteoporose atinge principalmente mulheres após a menopausa, devido à perda de função ovariana e conseqüente redução dos níveis de estrógeno, culminando com alterações neurovegetativas, psicológicas, tróficas e metabólicas (Aldrighi[2], 1972). A deficiência do hormônio resulta em alta taxa de remodelação óssea na qual a reabsorção excede a formação, apresentando, dessa forma, uma perda de massa óssea corpórea.

A análise da literatura demonstra a existência de uma relação positiva entre a perda de massa óssea esqueletal e o comprometimento da cavidade bucal através de achados como maior número de dentes perdidos (Inagaki et al.[21], 2001; Kribbs[23], 1990; Norderyd et al.[39], 1993), maior perda

de inserção periodontal (Duarte et al.[15], 2004; von Wowern et al.[65], 1994), diminuição do número de trabéculas e volume ósseo na região interradicular (Tanaka et al.[57], 2002), menor densidade mineral óssea na mandíbula (Elovic et al.[16], 1995; Jacobs et al.[22], 1996; Kuroda et al.[24], 2003), maior reabsorção óssea alveolar pós-exodontia (Hsieh et al.[20], 1995), severa reabsorção do rebordo residual (Daniell[12], 1983; von Wowern, Kollerup[66], 1992) e alterações condilares (Okuda et al.[40], 1996; Tanaka et al.[56], 2000).

**Osteoporose e osseointegração**

Com relação à instalação de implantes dentais em pacientes com diminuída massa óssea corpórea, a literatura mostra-se bastante contraditória.

A análise dos resultados de diversos estudos sobre osteoporose e osseointegração em animais (Duarte et al.[14], 2005; Giro et al.[19], 2007; Sakakura et al.[48], 2006; Treguerres et al.[61], 2002) e humanos (August et al.[6], 2001; Becker et al.[7], 2000; Shibli et al.[52], 2008) demonstram que a perda de massa óssea provocada pela privação hormonal pode prejudicar o tecido ósseo ao redor de implantes dentais.

Inúmeros estudos envolvendo a osseointegração de implantes dentais em animais com osteoporose foram conduzidos empregando como modelo animal, ratas e coelhas (Shirota et al.[53], 2003; Tokugawa et al.[59], 2003; Treguerres et al.[61], 2002). Entretanto, o rato parece ser o modelo preferido pela possibilidade de uma avaliação completa do tecido ósseo (arquitetura, força e massa óssea) e da utilização de um número maior de

animais em comparação ao coelho. Esse modelo apresenta ainda algumas vantagens como baixo custo, reduzido tamanho o qual possibilita fácil manejo, padronização, curto período de observação e fácil manutenção. Além disso, o rato é um animal geneticamente bem definido, assemelhando-se ao ser humano em vários aspectos, como a presença de osso cortical e trabecular e alterações ósseas similares às observadas na menopausa, envelhecimento ou decorrentes da ovariectomia (Wronski et al.[69], 1989).

Mori et al.[35], em 1997, realizaram um estudo em coelhos com o objetivo de, primeiramente, estabelecer um modelo de experimentação em animais para obtenção de baixa densidade óssea, para então investigar a interface de contato osso/implante. Foram utilizadas 36 coelhas divididas em 3 grupos: ovariectomia, ovariectomia associada à dieta pobre em cálcio e controle. Cada animal recebeu um implante de titânio na tíbia, um mês após a realização da ovariectomia. Através da análise microrradiográfica de contato foi observado que o grupo ovariectomia e dieta pobre em cálcio apresentou menor espessura e maior porosidade da cortical óssea da tíbia quando comparado aos demais grupos, sendo que esses parâmetros tenderam a agravar-se com o passar do tempo. Os achados histológicos na interface osso/implante evidenciaram, no grupo controle, nova formação óssea duas semanas após a instalação dos implantes, sendo que após oito semanas a superfície do implante encontrava-se quase que totalmente coberta pelo osso neoformado. No grupo ovariectomia e dieta pobre em cálcio, foi observado um atraso na formação óssea, com quantidade considerável de osso em contato com o implante somente após 12 semanas.

Motohashi et al.[36], (1999) investigaram as alterações ósseas ao redor de implantes de titânio, inseridos em ratas ovariectomizadas. Os implantes foram instalados na metáfise tibial três semanas após a realização das cirurgias fictícia e de ovariectomia (OVX). Os animais foram sacrificados 7, 14, 28, 56, 84 e 169 dias após a colocação dos implantes para a realização da análise histológica e histomorfométrica. Os resultados foram similares durante todo o tempo experimental, apesar do grupo OVX apresentar menor quantidade de osso formado. Esse estudo demonstrou que a deficiência de estrógeno não alterou os parâmetros analisados na região de osso cortical, porém o volume ósseo formado e o contato osso/implante são reduzidos na região de osso medular.

Yamazaki et al.[70] (1999) propuseram um estudo para investigar o comportamento do tecido ósseo após a colocação de implantes na tíbia de ratas, distribuídas nos grupos OVX e SHAM (cirurgia fictícia). Os implantes foram instalados 168 dias após a excisão dos ovários. O contato osso/implante e as mudanças no tecido ósseo ao redor dos implantes foram analisados histomorfometricamente 7, 14, 28 e 56 dias após a colocação dos implantes. Os autores encontraram uma porcentagem maior de osso neoformado para o grupo SHAM, em relação ao grupo OVX, em todos os períodos analisados. Quanto à interface de contato osso/implante, os resultados mostraram-se superiores para o grupo SHAM somente na observação do período de 56 dias. Baseado nesses dados, os autores sugeriram que a diminuição da massa óssea causa uma redução na área de

contato osso/implante e da quantidade de osso ao redor dos implantes na região medular.

Lugero et al.[26] (2000) estudaram a influência da osteoporose na cicatrização de implantes dentais inseridos em tíbias de ratas. A osteoporose foi induzida pela ovariectomia e comprovada por densitometria óssea vertebral, quatro meses após a remoção dos ovários. Após esse período, os implantes foram instalados na epífise proximal tibial e mantidos por um período de oito semanas. Os resultados desse estudo demosntraram menor formação óssea ao redor dos implantes dentais no animais submetidos à ovariectomia.

Pan et al.[42], em 2000, realizaram um estudo em ratas para avaliar os efeitos da deficiência de estrógeno na remodelação óssea ao redor de implantes de titânio. Os implantes foram instalados e, após 168 dias, realizada a excisão dos ovários. Os animais foram sacrificados 28, 84 e 168 dias após a realização da OVX. Os autores encontraram um percentual significativamente maior de massa óssea neoformada ao redor dos implantes do grupo controle, em todos os períodos avaliados. Em relação ao contato osso/implante na região medular, não foi encontrada diferença entre os grupos para o período de 28 dias. Porém, nos períodos de 84 e 168 dias, o grupo OVX apresentou valores significativamente menores, em relação ao grupo SHAM. Os resultados desse estudo levaram os autores à conclusão de que a deficiência do estrógeno leva a uma progressiva perda de massa óssea, diminuindo a área de contato osso/implante, principalmente na região medular.

Ozawa et al.[41] (2002) avaliaram, biomecânica e histomorfometricamente, a interferência da ovariectomia no processo inicial da integração osso/implante. Cinqüenta e seis ratas Sprague-Dawley foram divididas em 2 grupos: OVX e cirurgia fictícia. Cada animal recebeu dois implantes na epífise distal do fêmur, após duas semanas da realização das cirurgias. Os animais foram sacrificados duas e quatro semanas após a instalação dos implantes, sendo então realizadas análises histomorfométrica e biomecânica. Os resultados mostraram que no período de duas semanas o grupo OVX apresentou menor volume de osso formado, comparado ao grupo SHAM, igualando-se no período de quatro semanas. Os valores de resistência ao teste biomecânico apresentaram diferenças significantes somente em relação ao tempo, com menores valores para o menor período. Não houve diferença entre os grupos no mesmo intervalo de tempo.

Duarte et al.[13] (2003) avaliaram a influência da deficiência de estrógeno sobre o tecido ósseo ao redor de implantes por meio de análise do percentual de contato osso/implante (BIC), da densidade óssea lateral à superfície do implante (BD) e da área de osso entre as roscas (BA), além dos níveis de fosfatase alcalina plasmática. Para isso foram utilizadas 30 ratas Wistar, que receberam um implante de titânio na tíbia, 21 dias após a realização da ovariectomia (grupo teste) ou da cirurgia fictícia (grupo controle). Os resultados mostraram uma concentração de fosfatase alcalina estatisticamente maior para o grupo teste, em relação ao grupo controle. A análise histométrica da região de osso cortical não mostrou diferença significante entre os grupos para BIC e BD, enquanto foi constatada uma

redução da porcentagem de BA entre as roscas. Para a região de osso medular pode-se observar uma diminuição de BIC, BD e BA no grupo teste. Os autores concluíram que a deficiência de estrógeno exerce um efeito negativo tanto no reparo quanto na qualidade do tecido ósseo ao redor de implantes de titânio.

Cho et al.[9], em 2004, desenvolveram um estudo com o objetivo de avaliar o efeito negativo da osteoporose sobre a osseointegração de implantes dentais, utilizando 35 ratas Sprague-Dawley, divididas em 5 grupos experimentais. Os grupos 1 e 2 serviram como controle da OVX e da osseointegração, respectivamente. No grupo 3 a OVX foi realizada 12 semanas após a instalação dos implantes, no grupo 4 a OVX foi realizada 12 semanas antes da colocação dos implantes, enquanto no grupo 5 os implantes foram instalados no momento da realização da OVX. Os resultados da análise histomorfométrica mostraram que o grupo 2 apresentou uma maior porcentagem de área de osso entre as roscas do implante enquanto todos os demais grupos apresentaram valores significativamente menores. Não houve diferença significativa quando comparados os grupos submetidos à ovariectomia. O grupo 2 também apresentou maiores valores na avaliação do contato osso/implante. Com exceção do grupo 4, todos os outros grupos, comparados ao grupo 2, apresentaram valores significativamente menores. Os autores concluíram que a ovariectomia influencia negativamente na osseointegração, porém ainda são necessários estudos avaliando as características biomecânicas para verificar se essa diminuição da quantidade

de tecido ósseo ao redor dos implantes é capaz de comprometer seu uso ao longo do tempo.

Em humanos, August et al.[6] (2001) avaliaram a hipótese de que mulheres na pós-menopausa apresentam menores níveis de osseointegração, em comparação aos homens e às mulheres no período pré-menopausa. A amostra foi constituída de pacientes que possuíam implantes osseointegrados, instalados nos cinco anos anteriores ao estudo, sendo: 1) mulheres na pós-menopausa que não faziam uso da terapia de reposição hormonal (n=168); 2) mulheres na pós-menopausa que faziam uso da terapia de reposição hormonal (n=75); 3) mulheres na pré-menopausa (n=114); 4) homens com idade inferior a 50 anos (n=59); 5) homens com idade superior a 50 anos (n=110). A estabilidade do implante foi o parâmetro adotado para a verificação do sucesso da osseointegração e foi avaliada por meio de teste biomecânico e imagens radiográficas. Os implantes instalados na mandíbula e maxila foram avaliados independentemente. Os resultados mostraram que o insucesso dos implantes instalados na maxila, para o grupo de mulheres na pós-menopausa que não faziam uso da terapia de reposição hormonal, foi significativamente maior do que nos grupos de mulheres na pré-menopausa e de homens com idade superior a 50 anos. Não houve diferença para os implantes instalados na mandíbula. Baseados nesses resultados, os autores concluíram que a deficiência estrogênica pode ser um fator de risco para o sucesso de implantes osseointegrados.

Por outro lado, outros estudos em humanos não encontraram uma associação entre a diminuição da densidade óssea do esqueleto axial e a perda de implantes.

Friberg et al.[18] (2001) realizaram um estudo retrospectivo em uma amostra de 16 pacientes portadores de osteoporose que haviam recebido implantes em ambos os maxilares e relataram uma taxa de sucesso de 97.0% para maxila e 97.3% para a mandíbula. A perda óssea marginal após um ano de acompanhamento não diferiu dos achados presentes na literatura, levando os autores a concluírem que a colocação de implantes em pacientes com osteoporose pode ser realizada com uma alta taxa de previsibilidade.

Shibli et al.[52] (2008) compararam, em pacientes saudáveis e com diagnóstico clínico de osteoporose, o grau de contato do osso com o implante em função. Seus achados também corroboraram com o estudo acima descrito, já que não houve diferença entre os grupos quanto à análise histomorfométrica do tecido ósseo presente entre as roscas dos implantes.

Embora tenha sido mostrado em alguns estudos clínicos que a osteoporose não deve ser considerada uma contra-indicação formal à realização do tratamento com implantes dentários, deve-se ter em mente que os implantes dos estudos citados encontravam-se em função.

## Tratamentos para Osteoporose

Além de prevenir os fatores de risco relacionados à perda de massa óssea, existem alguns tratamentos específicos para a doença. Os

agentes farmacológicos empregados, atualmente, no tratamento da osteoporose são classificados em dois grupos: os anti-reabsortivos (cálcio, vitamina D, estrógenos, calcitonina, bifosfonatos, entre outros), que levam a inibição do processo normal de reabsorção óssea, e os formadores ósseos (esteróides anabólicos, fragmentos de paratormônio (PTH), alguns fatores de crescimento como IGF e TGF e a prostaglandina E2), que atuam estimulando a formação óssea (Consensus Development Conference on Osteoporosis[11], 1993).

O método mais efetivo na prevenção da osteoporose pós-menopausa, sem dúvida nenhuma, é a reposição hormonal com estrógeno. Porém, estudos têm demonstrado que a mesma apresenta algumas contra-indicações e oferece riscos, como o aumento da incidência de neoplasias e doenças cardiovasculares (Rossouw et al.[46], 2002). Dado esse fato, muitos estudos têm buscado uma alternativa terapêutica para a osteoporose pós-menopausa, sendo que a utilização dos bifosfonatos tem apresentado resultados bastante satisfatórios.

1. Estrógeno

A fração estrogênica mais importante é o 17 β-estradiol. A síntese desse composto ocorre principalmente nas células da camada granulosa dos ovários, embora na menopausa, outros tecidos, tais como o adiposo, ganham importância na síntese dos estrogênios.

Além de suas funções na regulação do crescimento, dos ciclos menstruais e do desenvolvimento sexual, o estrógeno exerce papel

importante na manutenção da massa óssea, indiretamente pela inibição da osteoclastogênese e, diretamente, pela inibição da função dos osteoclastos (Monroe, Spelsberg[34], 2003).

Na menopausa, quanto mais precocemente o tratamento é iniciado, ou seja, até os três anos consecutivos ao início do processo, melhores serão as condições da manutenção do metabolismo ósseo. Quando a administração do estrógeno ocorre em um período tardio, a quantidade de osso perdida até o momento não é recuperada, porém futuras perdas ósseas advindas da deficiência do estrógeno passam a ser prevenidas (Turner et al.[62], 1994).

Em relação à reposição hormonal com estrógeno, nenhum estudo mostra os efeitos dessa terapia em implantes que já se encontravam osseointegrados quando foram submetidos a este tipo de deficiência. Os estudos em animais encontrados na literatura relatam que este é um agente efetivo na prevenção da perda óssea induzida pela deficiência hormonal, proporcionando adequada cicatrização dos implantes instalados em ratas ovariectomizadas, mantendo a densidade e parâmetros histomorfométricos do tecido ósseo semelhante ao de animais controle (Duarte et al.[14], 2003; Nociti et al.[38], 2002; Qi et al.[44], 2004).

## 2. Alendronato

Os bifosfonatos são uma classe de medicamentos que tem como principal característica o poder de inibir a reabsorção óssea. Em 1969, Fleisch et al.[17], utilizando como modelo de osteoporose ratos imobilizados,

demonstrou pela primeira vez que os bifosfonatos diminuíam a osteopenia presente nesses animais.

Tais medicamentos são análogos sintéticos do pirofosfato inorgânico e possuem grande afinidade pelo cálcio (Migliorati et al.[33], 2005). São potentes inibidores da ação osteoclástica (Licata[25], 2005) e seus componentes aderem à matriz mineral óssea, onde na dependência da duração do tratamento e do bifosfonato utilizado, este pode permanecer durante muitos anos. Durante o processo de reabsorção óssea, os bifosfonatos são liberados do tecido ósseo, tornando-se disponíveis para uma nova associação com o osso formado ou para ser fagocitado por osteoclastos. Este processo resulta na perda da capacidade de reabsorção óssea pelo osteoclasto e na apoptose celular (Migliorati et al.[33], 2005).

O alendronato (4-amino-1-hidroxibutano bifosfonato de sódio) é o representante da terceira geração de bifosfonatos. Os primeiros compostos, tais como o etidronato, promoviam defeito na mineralização óssea, comprovado por estudos clínicos e experimentais (Fleish et al.[17], 1969; Masarachia et al.[30], 1996). A introdução do radical amina fez com que o alendronato ganhasse característica especial para o uso clínico quando comparado aos compostos iniciais (Schenk[51], 1996).

O desenvolvimento do alendronato como opção terapêutica mais segura e eficaz no tratamento da osteoporose deu um novo impulso à compreensão do mecanismo de ação dos bifosfonatos. A localização preferencial da droga no tecido ósseo é na superfície de reabsorção (Masarachia et al.[30], 1996; Sato et al.[50], 1991), atuando na redução da

atividade reabsortiva dos osteoclastos e acelerando seu processo de apoptose, o que resulta em um aumento na densidade mineral óssea e diminuição dos marcadores de remodelação óssea (Watts[67], 2003).

Alguns estudos (da Paz et al.[43], 2001; Andersson et al.[3], 2002) demonstraram que o tratamento com alendronato é efetivo na prevenção da perda de massa óssea causada pela depleção dos níveis estrogênicos, mostrando que o alendronato possui efeitos iguais, ou em muitos casos superiores, aos encontrados com o tratamento com estrógeno e PTH.

Quando avaliado o efeito do alendronato sobre a osseointegração de implantes, pode-se observar que o alendronato também é efetivo contra a deterioração do tecido ósseo ao redor de implantes, aumentando o torque necessário para a remoção dos mesmos (Giro et al.[19], 2007; Narai, Nagahata[37], 2003), mantendo a área de contato osso/implante com padrões semelhantes ao de animais saudáveis (Narai, Nagahata[37], 2003; Duarte et al.[14], 2005, Viera-Negron et al.[64], 2008) e o osso medular dentro dos parâmetros de normalidade, mesmo após a retirada do medicamento (Duarte et al.[14], 2005).

Portanto, a análise dos resultados dos diversos estudos citados nos permite levantar a hipótese de que a osteoporose provocada pela menopausa pode prejudicar a cicatrização de implantes dentais e que os diversos tratamentos empregados podem proporcionar resultados favoráveis. Entretanto, apenas um estudo correlacionou os possíveis efeitos deletérios da osteoporose provocada por ovariectomia sobre implantes já osseointegrados (Pan et al.[42], 2000). Essa relativa ausência de estudos chama a atenção uma

vez que muitas pacientes portadoras de implantes dentais possivelmente entrarão na menopausa com implantes instalados e já reparados.

Outro fator importante a ser levantado diz respeito aos tratamentos propostos para prevenção da perda de massa óssea advinda do período pós-menopausa, em especial o alendronato. Como foi possível observar, todos os estudos apresentados anteriormente mostram resultados bastante promissores em relação ao uso dessa medicação.

Entretanto, a partir de 1995, foram relatados na literatura casos de perda de implantes osseointegrados e em função na cavidade bucal, em pacientes fazendo uso do alendronato (Stark, Epeker[55], 1995). A partir de 2003, estudos demonstraram que o uso dos bifosfonatos poderia ser associado ao aparecimento de osteonecrose nos maxilares em pacientes que faziam uso da droga, após serem submetidos a tratamento dentário (Marx[29], 2003; Merigo et al.[31], 2006; Migliorati[32], 2003; Ruggiero et al.[47], 2004; Tarassoff, Csermark[58], 2003). Apesar da grande maioria dos relatos ocorrerem concomitantemente à administração de altas doses do medicamento, utilizadas no tratamento de metástases ósseas (Marx[29], 2003; Migliorati[32], 2003; Merigo et al.[31], 2006), a possibilidade de ocorrência desse efeito colateral em pacientes utilizando o alendronato para o tratamento da osteoporose não pode ser descartada.

Algumas teorias foram levantadas na tentativa de explicar o mecanismo para esta complicação. A principal delas sugere que a osteonecrose é causada pela estagnação da remodelação do tecido ósseo devido ao efeito inibidor da droga sobre os osteoclastos, uma vez que este é

utilizado para impedir a perda de massa óssea ou para impedir que células cancerígenas se espalhem pelo tecido ósseo. Levando-se em conta que a remodelação óssea é um processo fisiológico necessário para o reparo de injúrias e para a manutenção de um tecido ósseo sadio, também após a instalação de implantes, entende-se que há a necessidade da realização de novos estudos avaliando o efeito da droga sobre o metabolismo do tecido ósseo, antes e após a instalação de implantes dentais.

# Proposição

## Objetivos gerais:

Este estudo teve como objetivo avaliar a influência da osteoporose induzida, e seu tratamento com alendronato e estrógeno, sobre o tecido ósseo ao redor de implantes.

## Objetivos específicos:

✓ Avaliar os efeitos da privação estrogênica sobre a manutenção de implantes osseointegrados, submetidos ou não à carga mastigatória;

✓ Avaliar as implicações do tratamento com alendronato na remodelação, quantidade e qualidade do tecido ósseo ao redor de implantes instalados previamente e após a implementação da terapia;

✓ Avaliar padrões de formação e reabsorção frente aos diferentes tratamentos propostos.

# Capítulo 1

# Effect of 17 β-estradiol and alendronate on the removal torque of osseointegrated titanium implants in ovariectomized rats.*

Gabriela Giro[1], Celso Eduardo Sakakura[1], Daniela Gonçalves[1], Rosa Maria Rodrigues Pereira[2], Elcio Marcantonio Junior[1], Silvana R. P. Orrico[1].

[1] Department of Oral Diagnosis and Surgery, School of Dentistry of Araraquara, State University of São Paulo, UNESP, Araraquara, SP, Brazil.

[2] Department of Rheumatology, School of Medicine, University of São Paulo, USP, São Paulo, SP, Brazil.

Author responsible for correspondence:

Silvana Regina Perez Orrico. Faculdade de Odontologia de Araraquara, UNESP, Rua Humaitá, 1680, 14801-903, Araraquara, SP, Brazil, Phone: 55 (16) 3301-6377 Fax: 55 (16) 3301-6369 e-mail: s-orrico@foar.unesp.br

**Key words**: dental implants, ovariectomy, osteoporosis, alendronate, estrogen replacement therapy, rats.

**Running-Title:** Estrogen deficiency and dental implants.

**One-sentence summary:** Ovariectomy influenced the biomechanical properties of bone around titanium implants.

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**ABSTRACT**

BACKGROUND: This study investigated the influence of estrogen deficiency and its treatment with estrogen and alendronate on the removal torque of osseointegrated titanium implants.

MATERIAL AND METHODS: Fifty-eight female Wistar rats received a titanium implant in the tibia metaphysis. After 60 days, needed for implant osseointegration, the animals were randomly divided into five groups: control (CTLE, n=10), sham surgery (SHAM, n=12), ovariectomy (OVX, n=12), ovariectomy followed by hormone replacement (EST, n=12), and ovariectomy followed by treatment with alendronate (ALE, n=12). The CTLE group was then sacrificed to confirm osseointegration while the remaining groups were submitted to sham surgery or ovariectomy according to their designations. After 90 days these animals were also sacrificed. Densitometry of femur and lumbar vertebrae was performed by dual energy X-ray absorptionmetry (DXA) to confirm systemic impairment of the animals. All implants were subjected to removal torque.

RESULTS: Densitometric analysis of the femur and lumbar vertebrae confirmed a systemic impairment of the animals disclosing lower values of bone mineral density for OVX. Analysis of the removal torque of the implants showed statistically lower values ($p<0.05$) for the OVX group in relation to the other groups. The group treated with alendronate, ALE, however, presented significantly higher torque values when compared to the others.

CONCLUSIONS: According to this study estrogen deficiency was observed to have a negative influence on the removal torque of osseointegrated implants

while treatment with alendronate increased the torque needed to remove the implants.

## INTRODUCTION

Osseointegration was originally defined as the direct contact of live bone tissue with the metal of the implant in question.[1] The achievement and maintenance of osseointegration in a predictable way is an important aspect in clinical practice.

Osteoporosis is defined as a generalized skeletal disease characterized by reduced bone mass and by deterioration of the micro-architecture of the bone tissue caused by increase of the marrow spaces resulting in fragility of the bone tissue with subsequent greater risk of fractures.[2] Studies[3-5] has demonstrated that osteoporosis may result in a smaller quantity of bone tissue in contact with implant surfaces. Therefore, osteoporosis is now being reported as a systemic alteration possibly related to osseointegration damages. It has been suggested that predictability of dental implant success may be seriously impaired when patients have this type of systemic alteration.[6]

Hormone replacement therapy and bisphosphonates have been used for treatment of osteoporosis in an effort to control bone reabsorption.[7]

Studies have shown that estrogen plays an important role in the maintenance of bone mass, both indirectly by inhibition of osteoclastogenesis and directly by inhibition of the osteoclast function.[8] Although hormone replacement therapy with estrogen is considered the most effective method of preventing post menopause osteoporosis, studies have revealed objections and serious risks such as greater incidence of neoplasia and cardiovascular diseases.[9] Therefore therapeutic alternatives have been sought for post menopause

osteoporosis such as the bisphosphonates, of which the most commonly used now is alendronate, considered to be a 3rd generation bisphosphonate. Alendronate is bound to the reabsorption surface[10,11] reducing osteoclastic reabsorption and accelerating apoptosis resulting in an increased bone mineral density and a decrease of bone turnover labels.[12]

Various studies have clarified the role of osteoporosis in dental implant osseointegration. However, little has been published regarding the effect of osteoporosis on implants already osseointegrated and the benefits of treating systemic alterations on the bone tissue around these implants. Therefore the aim of this study was to evaluate, by means of the removal torque of implants, the influence of induced osteoporosis and its treatment with estrogen and alendronate on the bone tissue around osseointegrated implants.

## MATERIALS AND METHODS

### Animals

This study included 58 adults female Wistar rats approximately 60 days old, weighing from 180 to 220g. The animals, kept in propylene cages, were fed a standard laboratory diet and water *ad libitum*. The ambient was controlled at 25°C temperature and 55% humidity. They were exposed to 12 hours and 30 minutes of light alternating with 11 hours and 30 minutes of darkness. The research protocol was approved by the Institutional Experimentation

Committee of the School of Dentistry, Araraquara, SP, Brazil, under protocol nº 15/2004.

**Experimental design (Fig. 1)**

The animals were submitted to surgery for implant placement in the tibia proximal metaphysis. Anesthesia was administered intramuscularly with a combination of ketamine chlorohydrate (Ketamina Agener®; Agener União Ltda, São Paulo, SP, Brazil.) at a concentration of 0.08 ml/100g and xylazine 2% (Rompum ® Bayer S.A. São Paulo, SP, Brazil) at 0.04 ml/100g of body weight. An incision of approximately 20 mm was made on the internal side of the tibia. After careful dissection the bone tissue was exposed. Bicortical implant beds were prepared by using a progressive sequence of drills cooled with saline solution. A titanium micro-implant (AS Technology, São José dos Campos, SP, Brazil) with a sand blasted and acid etched surface, with 4.0 mm in length by 2.2 mm in diameter, was placed in the left tibia. After implant placement the soft tissue was sutured and the animals received a single intramuscular injection of penicillin associated with streptomycin (Pentabiótico Pequeno Porte – Fort Dodge®, Campinas, SP, Brazil).

After a bone healing period of 60 days, necessary to obtain the osseointegration of the implants[13], the animals were randomly divided into five groups (Table 1).

The control group (CTL) was then sacrificed to obtain a standard of implant osseointegration. Animals of the ovariectomy (OVX), ovariectomy followed by hormone replacement (EST) and ovariectomy followed by treatment with alendronate (ALE) groups were submitted to ovariectomy. After skin incisions,

the tissues were separated to remove the ovaries. The SHAM group was submitted to sham surgery, where procedures similar to those of OVX were carried out, with the difference that the ovaries were exposed and replaced in their original position in order to simulate surgical stress.

The EST group, after a five day post ovariectomy period, received daily subcutaneous injections of 20μg/kg[3,14] of 17 β-estradiol (Sigma Chemical Co, St. Louis, MO, USA). The ALE group, five days post-ovariectomy, received 50 μg/kg doses[15,16] of alendronate every other day. Animals of SHAM, OVX, EST and ALE groups were sacrificed 90 days after ovariectomy with a lethal intraperitoneal injection of chloral hydrate at 20%.

**Densitometric Evaluation by DXA**

In order to evaluate the systemic bone mass loss, the analysis of the bone mineral density (BMD) of the femur and lumbar vertebrae was made using Dual energy X-ray Absorptiometry (DXA) with a densitometer (QDR 2000 Hologic – Bedford, MA, USA) and the small animal software supplied by the equipment manufacturer. The high resolution mode was used with a standardized technique for femur and lumbar vertebrae (Fig. 2).

Global BMD was measured for the femur and three sub regions designated as: distal epiphysis (R1); proximal epiphysis (R2) and diaphysis (R3). Global BMD was evaluated in the region of the lumbar vertebrae and of the L2, L3 and L4 vertebrae.

DXA precision in determining BMD was evaluated by measuring the coefficient of variation, expressed as a percentage of the mean value.[17, 18] For

this, five consecutive measurements of each anatomic region of the same sample were made. The coefficient of variation of the densitometer achieved for the femur sample was of 0.6% and of 1.2% for the lumbar vertebrae.

**Analysis of removal torque of implants**

Analysis of the removal torque was made after sacrifice of the animals. The tibia was dissected to expose the implant and attach a suitable device. A torquemeter (Tohnichi, model ATG24CN-S, Shangai, China). with a scale range of 3 to 24 N.cm and divisions of 0.05 N.cm was used for the test. A wrench was adapted to the implant head to apply torque in the reverse direction of implant placement, until complete rupture of the bone/implant interface was signaled by rotation of the implant. This reading was considered to be the torque necessary for rupture of osseointegration.

**Statistical analysis**

The Kolmogorov - Smirnov test was used for testing the normal distribution of data. For densitometric data analysis of the femur and the lumbar vertebrae Kruskal-Wallis and multiple comparison tests were used. The ANOVA and Tukey tests were used for comparison of data related to the removal torque of implants. The Spearman correlation test was utilized to verify the correlation between data on the torque of implant removals and BMD of the femur and lumbar vertebrae.

## RESULTS

### Densitometric Analysis

Densitometric analysis of the femur (Fig.3) showed that, for all the regions evaluated, group OVX presented the lowest values while group ALE showed the highest values, which were statistically different from the other evaluated groups. For lumbar vertebrae (Fig. 4), similar to the femur analysis, the OVX induced a reduction of BMD, with values differing statistically from the other groups, for all regions. The ALE group presented highest values in relation to the other groups, although for some regions, with no statistically significant difference when compared to CTLE and SHAM groups. The EST group had values statistically different from the OVX group, without a statistical difference in relation to groups CTLE and SHAM.

### Analysis of removal torque of implants

The removal torque analysis showed a statistically significant difference when comparing groups OVX and ALE, respectively having the smallest and largest values, in relation to the other groups evaluated. CTLE, SHAM and EST groups did not show a significant difference among them (Fig. 5).

No correlation was found between data of the torque of implant removals and BMD of the femur and lumbar vertebrae.

## DISCUSSION

Analysis of literature[19-22] reveals that osteoporosis may be a risk factor for placement of implants in the mandible and the maxilla, since the disease may

affect the jaws as well as other parts of the body. Therefore when bone metabolism is impaired, repair around the implant may be negatively affected.[23,24] Bone mass loss associated to ovarian hormone deficiency during menopause is the most common type of osteoporosis.[2] However the mechanism of this deficiency which results in the bone loss is not fully understood.

The objective of this study was to evaluate, by analysis of the removal torque of implants, the influence of induced osteoporosis and treatment with estrogen and alendronate on bone tissue around implants that are already osseointegrated.

Bone densitometry of the femur and lumbar vertebrae was made to evaluate the systemic deterioration in the animals. DXA shows that animals submitted to OVX developed a systemic loss of bone mass, with the femoral and lumbar vertebral BMD of the rats of group OVX statistically lower ($p < 0.05$) in relation to the other groups, for all the regions evaluated. Da Paz et al.,[25] when evaluating long bones, obtained a similar loss of bone mass after six weeks of estrogen deficiency while Garcia-Moreno et al.[26] also presented similar results however only six months after OVX. Results of this study also agree with Kuroda et al.,[27] showing that the protocol of ovariectomy to induce estrogen deficiency brings about bone structure alterations resulting in lower BMD in long bones.

According to Da Paz et al.[25] it may also be seen, as well as in this study, that systemic administration of estrogen inhibited bone reabsorption due to OVX, in the femur as well as in the lumbar vertebrae.

The ALE group presented the highest values and was statistically different from all other groups for all regions evaluated, which agrees with Andersson et al.[16] who verified that alendronate prevents bone mass loss induced by ovariectomy.

In 1991, Johansson & Albrektsson[28] demonstrated the existence of strong positive correlation between the degree of bone/implant contact and the force necessary for removal of implants. Since then, removal torque of implants has been a biomechanical method widely used to determine the extent of bone/implant contact.

Analysis of the results of this study, related to the test of the removal torque, showed that ovariectomy has a negative influence on the biomechanical characteristics of the bone tissue around implants with established osseointegration, characterized by the need of a smaller torque for implant removal. This results agrees with Ozawa et al.,[29] in which the amount of resistance to the biomechanical test was 50% smaller for the group of ovariectomized rats in relation to the control group.

The EST group did not present any statistically significant difference in relation to the CTLE and SHAM groups. Results of this study concur with Nociti et al.,[14] who by means of histometric analysis evaluated the bone area among the threads of the implant in the regions of the cortical and medullar bone, showing that the animals that received estrogen replacement therapy presented higher values with regard to untreated animals ($p<0.05$). These results show that administration of estrogen immediately after ovariectomy

prevents negative effects of estrogen deficiency on the repair of the bone tissue around osseointegrated implants.

Alendronate is a bisphosphonate that has been widely used in the treatment of osteoporosis because bone loss is inhibited.[30] In this study it was observed that administration of alendronate significantly increased the torque needed to remove the implants, presenting values significantly higher ($p<0.05$) for the ALE group, in relation to the other groups. This is similar to Narai, Nagata[31] who evaluated implant removal torque in ovariectomized rats, with and without administration of alendronate. Higher values were found for the group that received systemic administration of the drug. This increase of torque necessary for removal of the implants probably was due to the fact that alendronate promoted an increase of bone mass due to lesser bone remodelling with subsequent increase of trabecular volume and of the number of bone bars.[25]

Although authors[32-34] have reported some cases that show association of the occurrence of osteonecrosis of the jaw and therapy with bisphosphonates, it should be stressed that these involved utilization of other types of bisphosphonates as well as with higher doses as used for treatment of cancer metastasis or as associated with an infectious process.

The absence of correlation between values of torque removal and densitometry of the femur and lumbar vertebrae may be explained by the fact that the densitometer measures mineral bone density while removal torque indirectly evaluates the bone/implant contact and the quantity of bone formed between the thread forms[35,36], thereby measuring osseointegration or the

attachment of the implant[37]. It should be emphasized, however, that results found in the evaluation of BMD and removal torque were similar.

Results of this study lead to the conclusion that ovariectomy negatively affects the biomechanical characteristics of the bone tissue around implants with established osseointegration. Therapy instituted for treatment and prevention of osteoporosis has proven to be efficient in maintaining biomechanical characteristics, with alendronate presenting increased retention of the implants. Further studies with loading of implants should be considered prior to any suggestion that alendronate therapy would be beneficial for implant survival in osteoporotic bone.

**ACKNOWLEDGEMENTS**

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**Table 1.** Distribution of animals in groups.

| | | | | |
|---|---|---|---|---|
| 58 animals | Group CTLE | Animals sacrificed for confirmation of osseointegration | | N=10 |
| | Group SHAM | Sham surgery | _ | N=12 |
| | Group OVX | Ovariectomy | _ | N=12 |
| | Group EST | | 17β-estradiol treatment | N=12 |
| | Group ALE | | Alendronate treatment | N=12 |

**Figure 1.** Experimental study protocol.

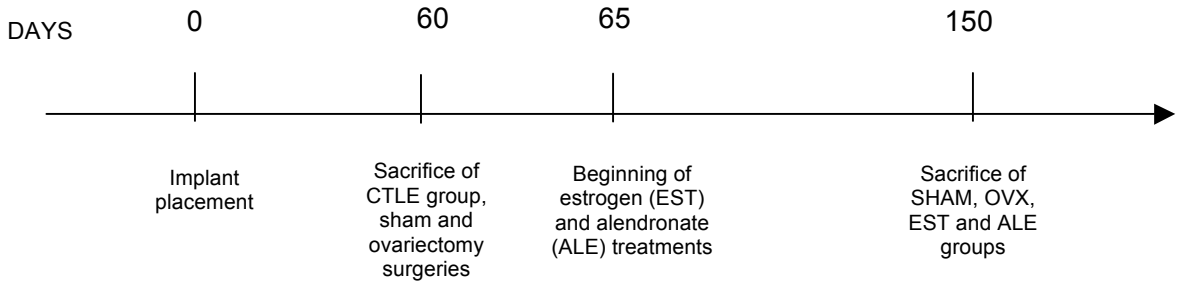


**Figure 2.** Femur bone (A) and lumbar vertebrae (B) regions evaluated by densitometry.

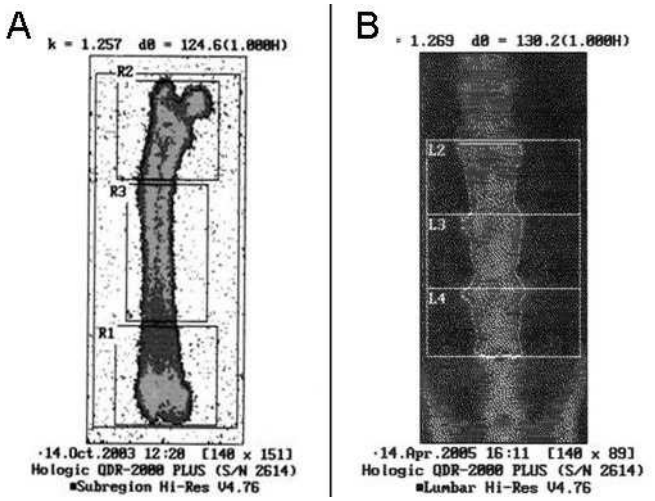


**Figure 3.** Comparison between the groups for the bone density (g/cm$^2$) of femur. *$P \leq 0.05$ for OVX group in comparison to the other groups. § $P \leq 0.05$ for ALE group in comparison to the other groups.

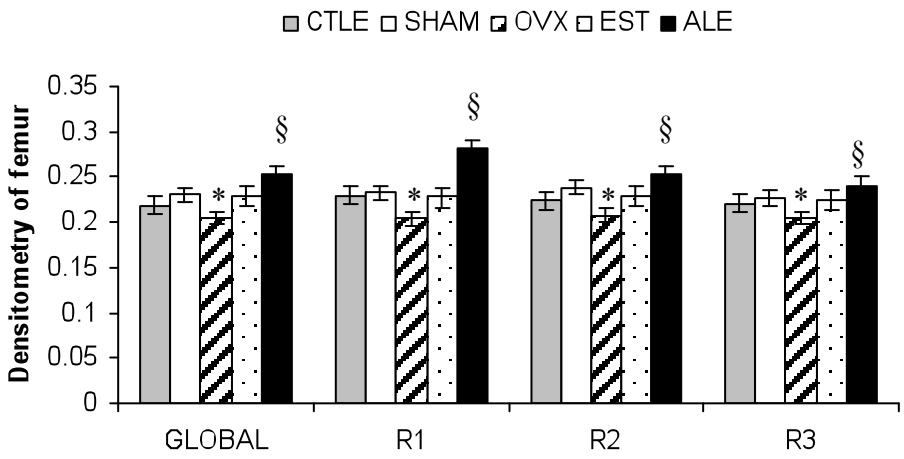


**Figure 4.** Comparison between the groups for the bone density (g/cm$^2$) of lumbar vertebrae. *$P \leq 0.05$ for OVX group in comparison to the other groups. § $P \leq 0.05$ for ALE group in comparison to the other groups.

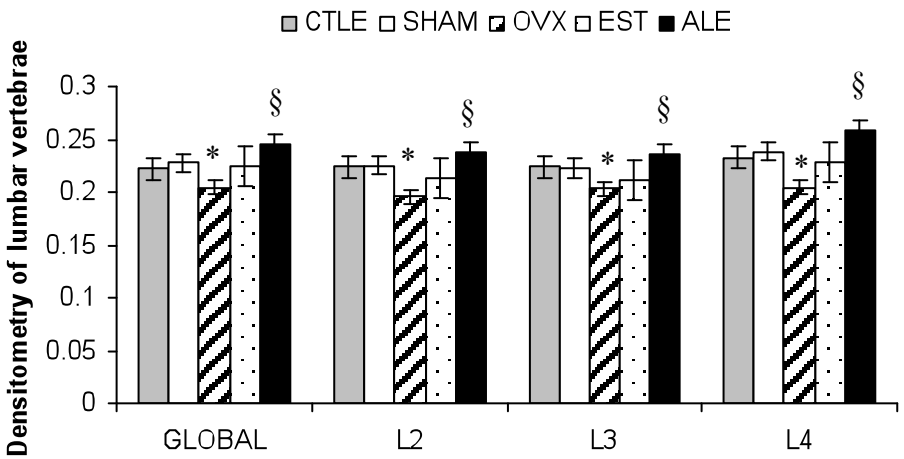
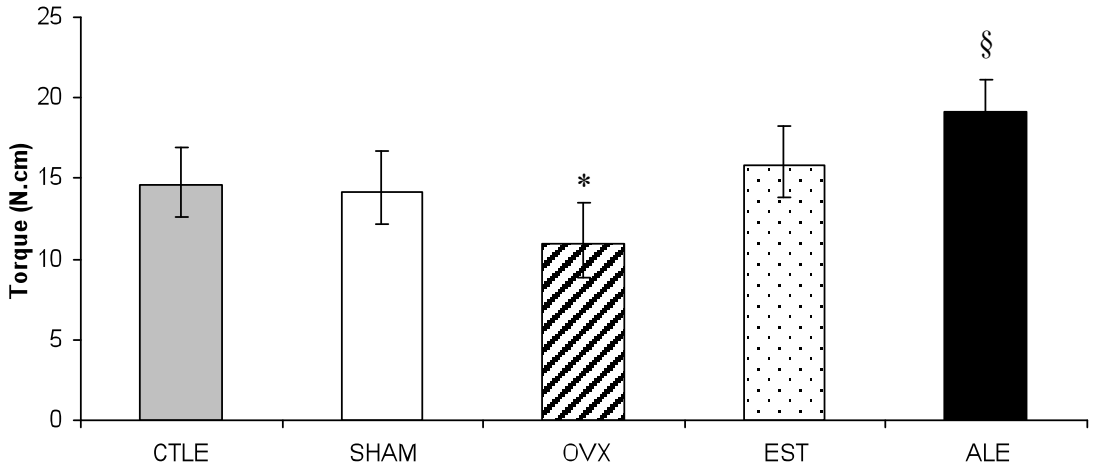


**Figure 5.** Average group values for removal torque of implants. *$P \leq 0.05$ for OVX group in comparison to the other groups. § $P \leq 0.05$ for ALE group in comparison to the other groups.



# Capítulo 2

# The effect of oestrogen and alendronate therapies on postmenopausal bone loss around osseointegrated titanium implants.*

Gabriela Giro[1], Paulo G. Coelho[2], Rosa Maria Rodrigues Pereira[3], Vanda Jorgetti[4], Elcio Marcantonio Junior[1], Silvana Regina Perez Orrico[1].

[1]Department of Oral Diagnosis and Surgery, Araraquara Dental School, UNESP – Univ Estadual Paulista, Araraquara, SP, Brazil.

[2]Department of Biomaterials and Biomimetics, New York University, New York, NY, USA

[3]Bone Metabolism Laboratory of Rheumatology Division, Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brazil

[4]Bone Metabolism Laboratory of Nephrology Division, Faculdade de Medicina da Universidade de São Paulo, Sao Paulo, SP, Brazil

**Running title:** Oestrogen/alendronate effects on osseointegrated implants

**Key words:** dental implants, ovariectomy, osteoporosis, alendronate, oestrogen replacement therapy, rats.

Author responsible for correspondence:

Gabriela Giro. Rua Humaitá, 1680, 14801-903, Araraquara, SP, Brazil.

Phone #: 55 (16) 3301-6377; Fax: 55 (16) 3301-6369

e-mail: gabi.giro@gmail.com

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**ABSTRACT**

**Objectives** This study evaluated the influence of bone mass loss and its therapies on bone tissue around osseointegrated implants.

**Methods** Implants were placed in 66 female rats tibiae. The animals were assigned to 5 groups: control (CTL), sham, ovariectomy (OVX), oestrogen (EST) and alendronate (ALE). While CTL was sacrificed 60 days after implant placement, other groups were subjected to ovariectomy or sham surgery according to group and euthanized after 90 days. Blood and urine samples were collected at sacrifice day for osteocalcin and deoxypyridinoline quantification. Densitometry of femur and lumbar vertebrae were performed in order to evaluate rats' skeletal impairment. Non-decalcified sections were referred to fluorescent and light microscopy for analyses of mineral apposition rate (MAR), eroded and osteoclastic surfaces, bone to implant contact (BIC), and bone area fraction occupancy (BAFO).

**Results** OVX showed significantly lower values for all parameters analysed compared to other groups. ALE reduced osteocalcin and deoxypyridinoline concentrations, MAR, osteoclastic and eroded surfaces, and no difference was in BIC and BAFO relative to SHAM. EST and CTL showed similar results to SHAM for measurements.

**Conclusions** Oestrogen deficiency exerted a negative influence on bone tissue around implants, while oestrogen replacement therapy and alendronate were effective against its effects. Although alendronate therapy maintained the amount of bone tissue around implants, studies evaluating bone turnover kinetics are warranted.

## INTRODUCTION

Postmenopausal osteoporosis is a common bone weakening systemic disease, characterized by a gradual loss of bone mass through bone microarchitectural deterioration induced by the lack of ovarian function (Consensus Development Conference on Osteoporosis 1993). Oestrogen deficiency increases bone turnover, resulting in a high bone remodelling rate in which the amount of bone resorption exceeds bone formation.

During the postmenopausal osteoporotic process, 30 to 50% of the trabecular bone and 25 to 35% of the cortical bone mass are lost in women (Morse et al. 1994). It is estimated that over 200 million people suffer from this disease worldwide and that more than 40 percent of postmenopausal women experience a fractured bone sometime throughout their lives (Cooper et al. 1992, Johnell & Kanis 2006). Besides that, it was estimated that osteoporosis and low bone mass would be a major public health threat for almost 44 million U.S. women and men aged 50 and older nowadays (Nelson et al. 2002).

Osteoporosis may also be a potential risk factor for dental implant therapy success. Considering that the peri-implant bone metabolism may be compromised in osteoporosis patients (Yamaguchi et al. 2003), this condition might affect the high predictability rates expected for dental implants (van Steenberghe et al. 2003).

Under the prerrogative that dental implant therapy has been a routine procedure for rehabilitation of edentulous patients over the last 10 years,

millions of women over 45 years will present implants in function during the development of osteoporosis.

Available reports in the literature have mainly targeted the influence of the estrogen deficiency on the healing of dental implant placed over the postmenopausal period (Duarte et al. 2005, Tresguerres et al. 2002, Duarte et al. 2003, Lugero et al. 2000, Vidigal et al. 2009, Viera-Negron et al. 2008, Qi et al. 2004, Mori et al. 1997, Cho et al. 2004, Du et al. 2009).

Thus, while previous studies has been performed concerning the effect of osteoporosis in dental implants that have already osseointegrated (Giro et al. 2007, Giro et al. 2008, Pan et al. 2000), the body of literature available is substantially smaller and further research in the topic may yield in better treatment protocol guidelines for affected patients. In addition, other studies are necessary since alteration in bone remodelling pathway/kinetics caused by drugs used for osteoporosis treatment, such as bisphosphonates, may also affect the bone-implant interface of previously osseointegrated surfaces.

Thus, the present investigation aimed to evaluate the influence of oestrogen deficiency induced after the osseointegration of the implants along with effectiveness of therapies for prevention of bone loss on bone parameters around implants.

## MATERIALS AND METHODS

### Animals

This study included 66 female Wistar (*Rattus norvegicus albinus*) rats approximately 30-day-old, weighing from 70 to 90g. The animals were fed a

standard laboratory diet and water *ad libitum*. Animal facility was lighting exposure, temperature and humidity controlled.

The study protocol was approved by the Ethics in Animal Research Committee of the Dental School of Araraquara (UNESP, Brazil) in compliance with the applicable ethical guidelines and regulations of the International Guiding Principles for Biomedical Research Involving Animals (Geneva, 1985).

**Study Protocol**

The animals were anaesthetized with a ketamine chloride (Ketamina Agener®; Agener União Ltda, São Paulo, SP, Brazil; 0.08 mL/100 g body weight) and xylazine 2% (Rompum®; Bayer S.A. São Paulo, SP, Brazil; 0.04 mL/100 g body weight) solution. Then, a 10mm long incision was made on the mesial aspect of the left proximal tibiae metaphysis. After careful dissection, the tibia was exposed and a bicortical osteotomy was prepared by using sequential drills to final diameter of 2 mm under sterile saline solution irrigation. An implant (AS Technology, São José dos Campos, SP, Brazil) with a sandblasted and acid etched surface (4.0 mm in length and 2.2 mm in diameter) was placed. Soft tissues were sutured and the animals received prophylactic intramuscular antibiotic injection (Pentabiótico Pequeno Porte®; Fort Dodge, Campinas, SP, Brazil; 1 mL/kg body weight). After a healing period of 60 days, 10 animals were sacrificed in order to confirm the osseointegration status of the implants (CTL group). The remaining animals were randomly divided into four groups: sham operated group (SHAM, n=14), ovariectomy

group (OVX n=14), ovariectomy + oestrogen replacement therapy group (EST n=14), and ovariectomy + alendronate group (ALE n=14).

The SHAM group was subjected to fictitious surgery and the OVX, EST, and ALE groups were subjected to ovariectomy. The animals in the EST group then received daily subcutaneous injections of 17 β-estradiol (20 μg/Kg) (Sigma Chemical Co. St. Louis, MO, USA), initiated 5 days after ovariectomy. The animals in the ALE group received subcutaneous injections of alendronate (50 μg/Kg) every other day, also initiated 5 days post-ovariectomy. Ninety days after ovariectomy (or sham surgery), the animals were euthanized by anaesthesia overdose. A chart comprising the experimental design is presented in Figure 1.

On the 2nd, 3rd, 11th, and 12th days prior to sacrifice, the animals received oxytetracycline (Sigma Aldrich, Saint Louis, CA, USA) administered by intraperitoneal injection at a dose of 20 mg/kg for bone labeling.

**Bone mineral density (BMD) evaluation**

The femur and lumbar vertebral BMD were analyzed by Dual-energy X-ray Absorptiometry (DXA) in order to assess the systemic bone mass of the animals in different groups. A densitometer (QDR 2000 Hologic, Bedford, MA, USA) operating in the high-resolution mode using specific software for small animals manufacturer was used. The technique was standardized for each bone evaluated. The DXA accuracy for determining bone mineral density was evaluated by measuring the coefficient of variation expressed as a percentage of the mean after five consecutive measurements of the same specimen.

Such approach validated the method as coefficient of variations of 0.6% for the femur and 1.2% for the lumbar vertebrae were obtained.

**Assessment of bone turnover biochemical markers**

For bone turnover biochemical markers, blood samples were collected from the rat caudal artery at the euthanization day. Serum osteocalcin (OCN) was measured using a rat osteocalcin enzyme-linked immunosorbent assay (ELISA) kit (Biomedical Technologies Inc., Stoughton, MA, USA). A urine sample comprising the experiment last 24h of the experimental period for each group was collected from each animal in metabolic cages. Urinary deoxypyridinoline (DPD) was also measured by ELISA (Metra Biosystems, Palo Alto, CA, USA). Deoxypyridinoline results were corrected for urinary concentration of creatinine (DPyr/creatinine; nM/mM).

**Bone histomorphometry**

For hard tissue histology, the proximal tibiae were retrieved by sharp dissection and the implants in bone were fixed in 4% neutral buffered formalin. Non-decalcified ground sections were prepared, according to methods described by Donath & Breuner (1982). The specimens were dehydrated using increasing concentrations of ethanol, and were then embedded in glycolmethacrylate-based resin (Technovit 7200 VLC, Heraeus Kulzer, Wehrheim, Germany). The blocks were cut to 100µm thickness sections aiming the center of the implant diameter along its long axis using a cutting-grinding unit (EXAKT Apparatebau GmbH & Co., Norderstedt,

Germany). A single section was obtained from each implant. The sections were reduced to a final thickness of 30µm by grinding and polishing using a microgrinding unit (EXAKT Apparatebau GmbH & Co., Norderstedt, Germany). The sections were stained with 1% toluidine blue (Sigma Aldrich, St. Louis, MO, USA).

Bone to implant contact (BIC) and bone area fraction occupancy (BAFO) measurements were performed by using the National Institutes of Health image analyzer software (ImageJ 1.41o, National Institutes of Health, USA). BIC was calculated as the percentage of the length of direct bone contact to the implant surface to total implant surface, while BAFO was calculated as the percentage of the area of the bone filling the area of the implant screw.

The static and structural parameters of bone formation and resorption were measured along the screws of the implant using a semi-automatic method (Osteometrics, Inc., Atlanta, GA, USA). The kinetic bone parameter was obtained by measuring the distance between bone labels by fluorescent light microscopy (Nikon, Tokyo, Japan).

**Statistical analysis**

Statistical analyses were performed using SPSS 16.0 (SPSS, Chicago, IL, USA). Kolmogorov - Smirnov test was used for testing data normality. For parametric analyses, one-way analysis of variance (ANOVA) and Tukey's post-hoc test were performed. For non-parametric analyses, Kruskal Wallis followed by Dunn's test as post-hoc tests were conducted. For both, significance level was set at 5% ($p<0.05$)

## RESULTS

### Bone Densitometry

The results for femoral and lumbar vertebral BMD showed significant differences for ALE and OVX groups when compared to the SHAM group (Table 1). The lowest BMD values were observed in the OVX group, while the highest values were observed in the ALE group. No significant differences were observed between EST, CTL, and SHAM groups (Table 1).

### Bone Turnover

Similar trends were observed for the OCN and DPD analyses. Figures 2a and 2b depict a significant increase in OCN and urinary excretion of DPD for the OVX group along with a slight decrease for the ALE group relative to the SHAM group. Both OCN and DPD levels for the EST group were comparable to the SHAM operated group.

### Histometric Analysis

The CTL group histological sections showed that uneventful healing of bone around implants led to their osseointegration at 60 days after placement. Histometry showed that the OVX group presented the lowest (mean ± standard deviation) BIC (58.04 ± 4.79) and BAFO (59.08 ± 4.81) values, while EST and ALE groups presented BIC and BAFO values that were not significantly different than the SHAM group (Figure 3).

Figure 4A shows that the kinetic parameter of bone formation presented decreased mineral apposition rate (MAR) for both OVX and ALE groups. An increase in eroded and osteoclastic surface values were observed for the OVX group, while the lowest values were observed for the ALE group (Figure 4B and 4C, respectively). There were no significant difference among CTL, SHAM and EST groups for any of the referred parameters.

Figure 5 shows that EST and ALE were effective in maintaining the bone quantity around the implants, while BIC and BAFO in OVX were evidently decreased. At the higher magnification ALE appeared to present denser bone lamellae than the other groups as a result of the quiescence of bone remodeling.

**DISCUSSION**

Osteoporosis is an osteometabolic disorder characterized by progressive bone resorption leading to a decrease in bone biomechanical competence (Nasu et al. 1998). In order to simulate skeletal conditions similar to postmenopausal osteoporosis, laboratory in vivo models have been primarily obtained by ovariectomy (Kalu 1991, Devlin & Ferguson 1990). The present investigation utilized ovariectomized rats to investigate the influence of oestrogen deficiency as well and its treatments with alendronate and oestrogen on the bone tissue around osseointegrated implants.

The evaluation of the BMD was used in order to assess the overall animal impairment, as DXA is the gold standard method used for the osteoporosis diagnosis. Bone mineral density data are presented in Table 1. The results

obtained showed that the ovariectomy surgery resulted in systemic osteopenia in the OVX group confirming that the animal model was suitable for the proposed experiment. In addition, the BMD results showed that the dosages and treatments utilized in the present study were effective in preventing the systemic loss of bone mass (no significant differences between EST, CTL, and SHAM; significantly higher BMD for ALE).

In agreement with the BMD results, the OVX and ALE groups presented significantly decreased and increased degrees of BIC and BAFO relative to other groups, respectively. These results demonstrated that oestrogen suppression did have a negative influence in the bone around implants while its subsequent treatment with a bisphosphonate overcame this scenario.

As found in previous investigation (da Paz et al. 2001), oestrogen suppression increased bone turnover. The observed increase of serum concentration of OCN led to bone resorption rates that exceeded bone formation rates, which was further supported by the increased DPD excretion, and reduced MAR during the experiment. These observations are in accordance with previous results that showed lower radiographic bone density in the tibiae along with decreased torque needed to displace the implant in ovariectomized animals (Giro et al. 2007, Giro et al. 2008, Sakakura et al. 2006). Such studies also showed a negative impact on bone healing and bone quality, loss of bone mass and a deficient bone formation around implants in ovariectomized animals. Likewise, Pan et al. (2000) found that the volume around implants and BIC were decreased in cortical and even more pronounced in cancellous bone areas compared with the sham operated animals.

Regarding the drug therapies considered in the present experiment, oestrogen replacement was considered the first choice treatment approach for prevention and management of cases of postmenopausal osteoporosis until the beginning of the past decade when the effect of this therapy by increasing the risk for coronary heart disease, stroke, and invasive breast cancer was shown (Rossouw et al. 2002). Alendronate was then widely used in the treatment of osteoporosis since its efficacy and safety were extensively investigated (Hosking et al. 1998, McClung et al. 1998, Peter & Rodan 1999, Adachi 1998, Ott 2005).

The findings of the present study demonstrated that the systemic osteopenia treatment with alendronate and oestrogen may yield preservation and/or maintenance of bone around osseointegrated implants. Regardless of the parameter assessed, the EST group did not present significant differences from CTL and SHAM groups, as previously reported in the literature (Duarte et al. 2003, Sims et al. 1996, Omi & Ezawa 1995).

On the other hand, the administration of alendronate resulted in BIC and BAFO levels comparable to the CTL, SHAM, and EST groups.

Relative to the SHAM group, decreased bone turnover markers and diminished osteoclastic and eroded surface percentages were observed for the ALE group, indicating that the medication altered the osteoclastic activity. In tandem, bone formation was also compromised, as a considerable decrease in both serum OCN concentration and MAR were observed for the ALE group. Such observations can possibly result in the alteration in bone

morphology around the implant in the ALE group compared to the SHAM group, due to inactiveness on the remodeling process.

Bisphosphonate suppressed remodeling has been related as one of the factors that plays a role in the pathophysiology of bisphosphonate related osteonecrosis of the jaws (BRONJ) (Allen & Burr 2009). The suppression of bone remodeling by treatment with bisphosphonate allows microdamage to accumulate and such accumulation should keep increasing with time as long as bone remodeling has being suppressed (Mashiba et al. 2005). The inability to repair physiologic microfractures that occur daily in the oral cavity would result in the occurrence of the BRONJ (Migliorati et al. 2005).

Since in the last years the reports regarding the association of nitrogen containing bisphosphonates with the occurrence of BRONJ have increased substantially (Dimitrakopoulos et al. 2006, Farrugia et al. 2006, Sarathy et al. 2005, Ruggiero 2008, Mawardi et al. 2009, Merigo et al. 2006, Marx 2008), our results indicate the need for further investigation concerning the alteration of early bone formation and remodelling due to bisphosphonate administration.

Within the limitations of this study, it may be concluded that oestrogen deficiency negatively affected amount of bone around implants. Both systemic therapies considered in this study were effective in preventing bone mass loss. Although alendronate yielded potentially beneficial results, further research considering bone turnover and its biomechanical influence in the implant-bone system is recommended prior to any suggestion that

alendronate therapy would be beneficial for implant survival in osteoporotic bone.

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**TABLE 1.** BMD mean ± SD of femur and lumbar vertebra (L2-L4).

| Groups | Femur | Lumbar vertebrae |
|---|---|---|
| **CTL** | 0.219 ± 0.019[a] | 0.221 ± 0.009[a] |
| **SHAM** | 0.226 ± 0.016[a] | 0.227 ± 0.011[a] |
| **OVX** | 0.204 ± 0.009[b] | 0.204 ± 0.013[b] |
| **EST** | 0.229 ± 0.014[a] | 0.224 ± 0.018[a] |
| **ALE** | 0.252 ± 0.012[c] | 0.244 ± 0.014[c] |

* Different letters indicate groups with distinct characteristics.

**Figure 1.** Study design.

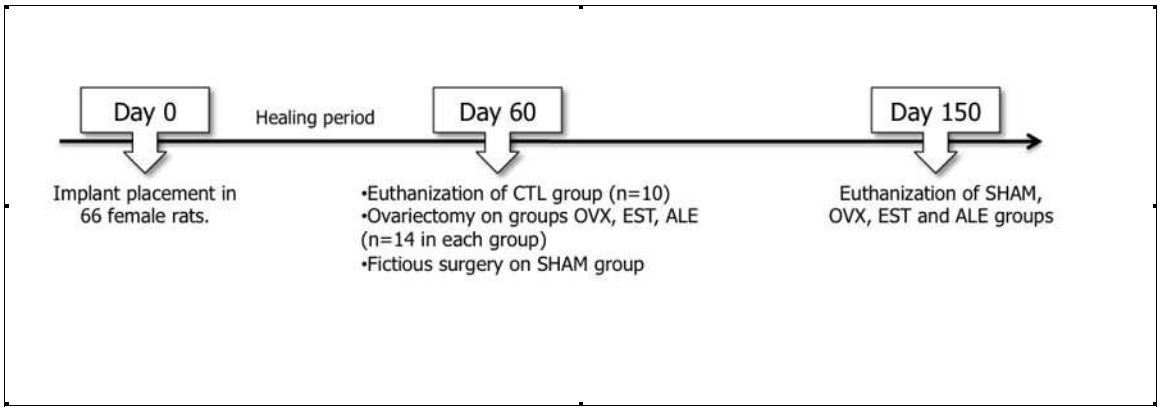


**Figure 2.** Concentration (mean ± 95% CI) of (a) serum OCN and (b) urinary DPD for the different experimental groups. The number of asterisks denote significant differences between groups ($p < 0.0001$ for both OCN and DPD).

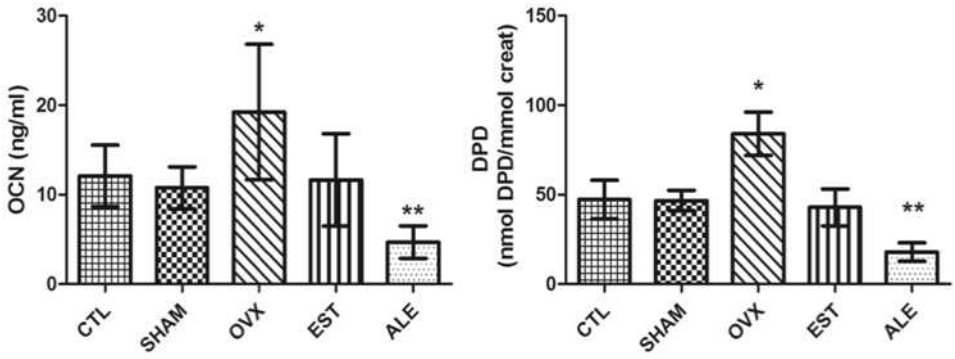


**Figure 3.** Histometric measurements around the implants: (a) BIC and (b) BAFO (mean ± 95% CI). *p<0.001 in relation to the other groups of the study.

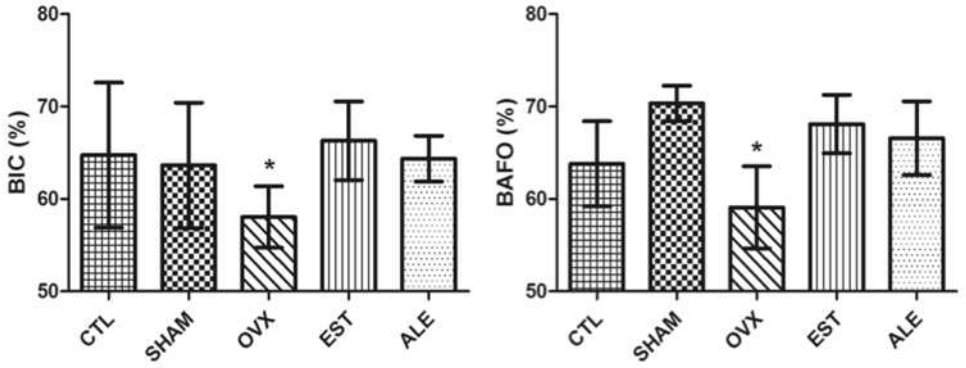


**Figure 4.** Statistical summary for (a) MAR as a kinetic parameter, percentages of (b) eroded surface and (c) osteoclastic surfaces for the different experimental groups. The number of asterisks denote significant differences between groups ((mean ± 95% CI; p<0.0001).

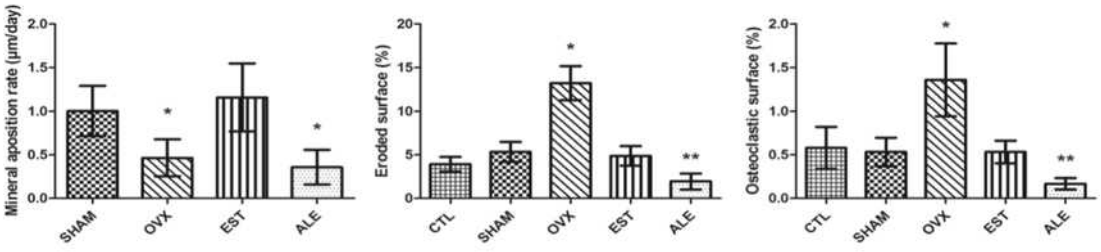
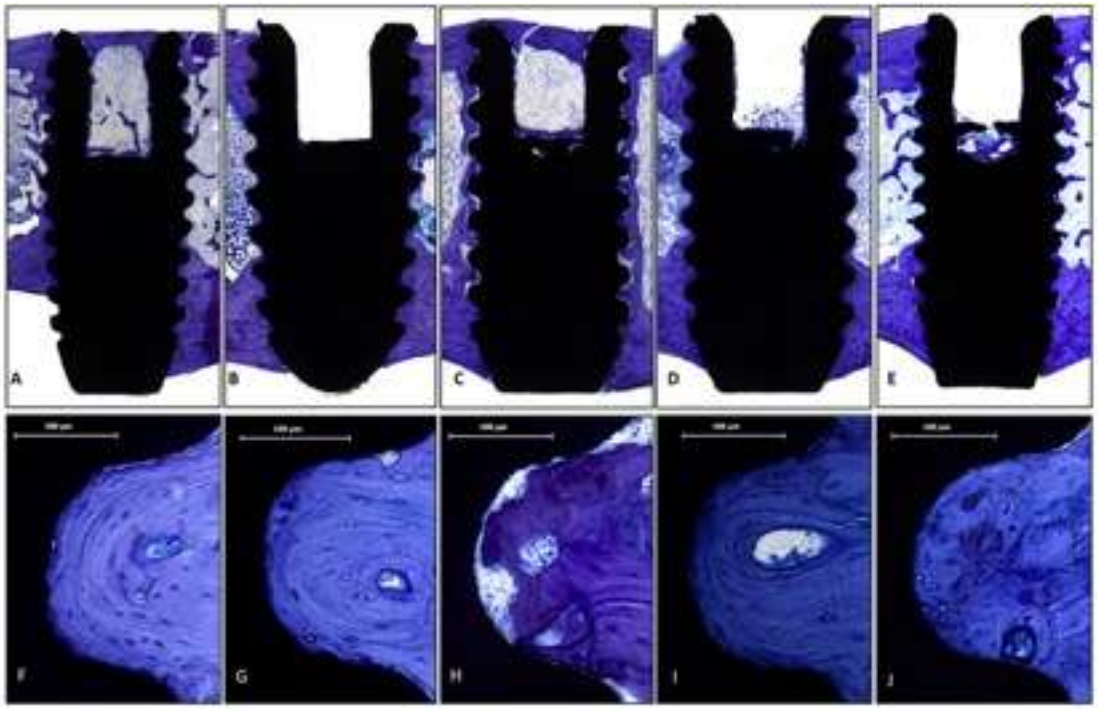


**Figure 5.** Histologic sections of the proximal tibiae metaphyses with implants (toluidine blue staining, original magnification ×100 and x400). Photomicrographs were taken from (a,f) CTL; (b,g) SHAM; (c,h) OVX; (d,i) EST and (e,j) ALE.



# Capítulo 3

# The influence of estrogen deficiency on bone around osseointegrated dental implants. An experimental study in the rat jaw model.

Gabriela Giro[1,4], Paulo G. Coelho[2], Roberto Sales e Pessoa[1], Rosa Maria Rodrigues Pereira[3], Toshihisa Kawai[4], Silvana Regina Perez Orrico[1].

[1]Department of Oral Diagnosis and Surgery, Araraquara Dental School, UNESP – Univ Estadual Paulista, Araraquara, SP, Brazil.

[2]Department of Biomaterials and Biomimetics, New York University, NY, USA.

[3]Bone Metabolism Laboratory of Rheumatology Division, Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brazil

[4]Department of Immunology, The Forsyth Institute, Boston, MA, USA

**Running title:** Estrogen deficiency on jaw bone around implants

**Key words:** dental implants, ovariectomy, osteoporosis, estrogen deficiency, rats.

Author responsible for correspondence:

Gabriela Giro. Rua Humaitá, 1680, 14801-903, Araraquara, SP, Brazil.

Phone #: 55 (16) 3301-6377; Fax: 55 (16) 3301-6369

e-mail: gabi.giro@gmail.com

**ABSTRACT**

**Objective:** The aim of this study was to evaluate the influence of estrogen deficiency on bone around osseointegrated dental implants in a rat jaw model.

**Methods**: This study utilized 16 female rats, which had the first molars bilaterally extracted and allowed to heal for 30 days prior to implant placement. After time was allowed for implant osseointegration, the animals were randomly submitted to sham surgery (SHAM) or ovariectomy (OVX). The animals were euthanized 90 days post-ovariectomy. Bone to implant contact, bone area fraction occupancy between implant threads, mineral density, turnover markers and TRAP positive cells were assessed for the different groups.

**Results:** The results showed that OVX group presented a decrease of systemic bone density, alterations on bone turnover markers, and an increased amount of TRAP positive cells relative to the SHAM group. No difference was relative to bone to implant contact and bone area fraction occupancy was observed between groups.

**Conclusion:** The findings of this study demonstrate that estrogen deficiency may not be considered as a risk factor for osseointegrated implants failure in jaw bone.

## INTRODUCTION

Osteoporosis represents a major public health threat and annually results in thousands of hip, wrist and vertebral fractures (Gosfield and Bonner, 2000; Smith and Shoukri, 2000). Estrogen is known to play an important role in regulating bone homeostasis and preventing postmenopausal bone loss. The lack of estrogen causes an imbalance in bone remodeling, so the rate of bone resorption exceeds that of bone formation (Luvizuto et al., 2009). Thus, in vivo laboratory models that allow evaluation of this systemic disease have been developed so the fundamental mechanism of this condition and related treatments can be better evaluated.

Ovariectomy is established as the most prevalent and pertinent (Kalu et al., 1989; Kalu, 1991; Wronski et al., 1988) protocol to induce estrogen deficiency related osteopenia, bringing about hormonal deficiency similar to that of menopause (Frost and Jee, 1992). In rats, estrogen deprivation appears to mimic the anatomical and kinetic patterns of loss of bone mass observed in humans (Frost and Jee, 1992; Gnudi et al., 1993). Thus, several authors have used ovariectomy in order to study the estrogen deficiency induced bone loss as a potential risk factor during the placement and maintenance of dental implants, as good quality of bone tissue have been related to their success (Duarte et al., 2003; Pan et al., 2000; Qi et al., 2004).

Previous animal studies have shown that estrogen deficiency result in bone turnover rates, bone-to-implant contact (BIC) the bone/implant interface biomechanical competence, as well as bone density on the cancellous bone

(Cho et al., 2004; Duarte et al., 2003; Giro et al., 2007; Giro et al., 2008; Pan et al., 2000; Qi et al., 2004; Viera-Negron et al., 2008).

Despite these evidences, from a clinical perspective, the literature findings in the topic are sparse and contradictory as case reports, case-controls, and retrospective clinical studies suggest that osteoporosis should not be considered a risk factor for implant therapy (Becker et al., 2000; Friberg et al., 2001; Shibli et al., 2008).

Thus, the present study evaluated the influence of estrogen deficiency on bone around osseointegrated dental implants in a rat jaw model.

## MATERIALS & METHODS

### Animals

Sixteen female Wistar rats weighing from 70 to 90g were used in this study. The animals were kept in the animal facility with controlled temperature, humidity, and light exposure. They were fed in a standard laboratory diet and water *ad libitum*. The study protocol was approved by the Committee of Ethics for Animal Experimentation of Araraquara Dental School, UNESP (Protocol# 02/2007).

The rats included in the study had both maxillary first molars extracted. For that purpose, the animals were anesthetized receiving an intramuscular injection of ketamine hydrochloride (Agener União Ltda, São Paulo, SP, Brazil) at a concentration of 0.08ml/100g and xylazine hydrochloride (Rompum®, Bayer S.A., São Paulo, SP, Brazil) at 0.04 ml/100g of body weight.

Oral cavity antisepsis was performed with chlorhexidine 0.12% prior to surgical procedures. Then, teeth were extracted using elevators. After a healing period of 30 days, two implants were placed in the healed sockets. For the placement of the implants, the rats were anesthetized and oral cavity antisepsis was performed, as described above. The transmucosal grade 5 screw shaped titanium implants (1.4 mm in diameter by 2.7 mm in length; SIN Sistema de Implante, São Paulo, SP, Brazil) were inserted in the osteotomy prepared bilaterally at the edentulous sites. Sixty days after implant placement the animals were randomly divided into a control (SHAM) and an ovariectomized (OVX) group. A fictitious surgery was carried out for SHAM while OVX had the ovaries removed, as previoulsy decribed (Orrico et al., 2005), in order to induce the estrogen deficiency. The euthanization of the animals were performed by deepening anesthesia on 180$^{th}$ day after teeth extractions.

**Bone densitometry**

Femur and lumbar vertebral bone mineral density (BMD) evaluations were performed by Dual energy X-ray Absorptionmetry (DXA) in order to assess the systemic impairment of the animals due to ovariectomy. DXA was achieved using a densitometer (QDR Discovery, Hologic, Bedford, MA, USA) operating in the high-resolution mode and using specific software for small animals supplied by the equipment's manufacturer.

**Histologic preparation of the specimens**

Two implants were inserted in each animal, one of the implants was prepared for hard tissue histology and the other was subjected to decalcification and embedded in paraffin. After euthanization, the maxillary implants were fixed in 4% buffered formaline saline for 48h. Non-decalcified ground sections were prepared after dehydration by a series of ethanol solutions and embedded in methacrylate-based resin (Technovit 9100, Heraeus Kulzer, Wehrheim, Hesse, Germany). The blocks were cut into 100μm thickness sections aiming the center of the implant diameter along its long axis using a precision diamond saw (Isomet 2000, Buehler Ltd., Lake Bluff, IL, USA). The sections were reduced to a final thickness of approximately 30μm by grinding and polishing (Metaserv 3000, Buehler Ltd., Lake Bluff, IL, USA) with a sequence of SiC abrasive papers (400, 600, 800, 1200 and 2400) (Buehler Ltd., Lake Bluff, IL, USA). The sections were stained in 1% toluidine blue (Sigma-Aldrich Co, St. Louis, MO, USA) and referred to light microscopy evaluation.

Measurements of the percentages of bone to implant contact (BIC) and the bone area fraction occupancy (BAFO) were performed at 100x magnification (Leica DM1200M, Leica Microsystems, Wetzlar, Hesse, Germany) by using the National Institutes of Health image analyzer software (ImageJ 1.41o, National Institutes of Health, USA).

The decalcified processed specimens were treated for 28 days in 10% ethylenediamine tetraacetic acid (EDTA) solution containing 0.1M Tris base (pH 6.9), dehydrated, diafanized and embedded in paraffin. Sections of 5μm were obtained and prepared for hematoxylin eosin staining, histochemically

reacted for tartrate-resistant acid phosphatase (TRAP) to demonstrate the presence of osteoclasts, and also reacted to immunolabeling of osteocalcin (OCN) and alkaline phosphatase (ALP) by using ABC system (DAB Substrate Kit, Vector Laboratories Inc. Burlingame, CA, USA).

**Measurement of biochemical markers of bone turnover**

Blood samples were collected from the rat caudal artery at the euthanization day. Serum concentrations of osteocalcin (OCN – Biomedical Technologies Inc, Stoughton, MA, USA), the cross-linked C-terminal telopeptides (CTX), osteoprotegerin (OPG - Peprotech, Rocky Hill, NJ, USA) and the receptor activator of nuclear factor-κB ligand (RANKL - Peprotech, Rocky Hill, NJ, USA) were measured by Enzyme-Linked Immunosorbent Assay (ELISA) following the manufacturer's instructions. Tartrate-resistant acid phosphate (TRAP) and alkaline phosphatase (ALP) activities were also measured in serum by biochemical assay using the α-naphthyl- phosphate (Sigma Aldrich, Saint Louis, MO, EUA) as a substrate.

**Data Analysis**

After using D'Agostino test for evaluation of data normality, both analysis of Mann Whitney and Student's t-test were used for comparison of the groups. Statistical significance was set to 95%.

## RESULTS

BMD evaluation revealed the osteopenia establishment by estrogen deficiency as OVX presented significantly lower BMD values than SHAM (p=0.0048) (Figure 2). Statistically significant differences in TRAP (p=0.0247) and ALP activity (p=0.0411) was detected between groups (Figure 3a and 3b, respectively). While the OVX group showed increased TRAP activity, its ALP activity was decreased in comparison to SHAM. These enzymes ratio also presented significantly higher values for OVX (p=0.0087) (Figure. 3c). Although both RANKL and OPG did not present statistically significant difference (Figure 3d and 3e), the ratio between these parameters was slightly increased for the OVX group relative to SHAM (p= 0.0863).

The OVX group also presented increased OCN (Fig 3g; p=0.0317) and CTX (Fig. 3h; p=0.0083) serum concentrations.

Regarding the TRAP staining of decalcified sections, at the OVX group multinucleated $TRAP^{+}$ cell was observed to be more frequently found than in sections of SHAM group on bone tissue in proximity of the implant threads. (Figure 4).

Evaluation of the bone tissue around the implants showed no significant differences in BIC and BAFO, between groups (p=0.9254 and p=0.7445, respectively) (Figure 5). From a morphologic standpoint, HE and toluidine blue staining of histologic sections showed similar results for both groups (Figure 6).

OCN and ALP immunoreactivity presented agreement with their serum concentration assessment, where both bone formation markers were strongy

expressed for OVX group. At the implantation site, OCN was expressed at the osteocytes and distributed all along the bone matrix lamellae, while ALP was detected mainly in endosteal cells for both groups (Figure 7).

## DISCUSSION

From a public health standpoint, the effects of osteoporosis account for a large number of medical and medical-related procedures per year (Gosfield and Bonner, 2000; Smith and Shoukri, 2000). Concerning potential effects of osteoporosis on dental implant therapy, the lack of relationship between laboratory in vivo and clinical studies have prevented a clear definition of practice guidelines for affected patients whether these have developed the condition prior or after implant placement.

Confirming previous reports, the results of the present study showed that ovariectomy was an efficient method to establish the estrogen deficiency related bone loss (Elovic et al., 1995; Gnudi et al., 1993; Omi and Ezawa, 1995). The systemic bone density was decreased relative to the SHAM group, as well as serum parameters of bone remodeling were altered. These results are in agreement with Rico et al. (Rico and Villa, 1993), who demonstrated that serum TRAP concentration was increased after menopause and that such observation was inversely related to bone mass. Also in agreement with previous studies, our results also revealed an increase of OCN and CTX along with a decrease in ALP serum for the OVX group (da Paz et al., 2001; French et al., 2008; Garcia-Perez et al., 2006; Sims et al., 1996).

The loss of bone mass due to estrogen deficiency during the healing period of dental implants or its implication on implants that are already osseointegrated has been previously addressed. These investigations have shown that estrogen deficiency alters bone density around implants, decreases the overall biomechanical competence of the bone-implant system, as well as decreases the degree of osseointegration (Cho et al., 2004; Duarte et al., 2003; Giro et al., 2007; Ozawa et al., 2002; Sakakura et al., 2006).

In a similar animal model but at a different implantation site (tibiae), Giro et al. had shown significant bone mass loss along the implant threads, and decreased BIC and BAFO values in ovariectomized animals relative to the SHAM group. On the contrary, such observations did not occur in the present study when jaw bone was considered. Our results support that although estrogen suppression resulted in a systemic impairment of the animals, the morphologic and morphometric parameters were relatively unaffected. Such observation could be explained by the fact that bone remodeling rate may differ on axial and appendicular skeleton (Riggs et al., 1986). Another plausible explanation is related to the fact that adult rats might have an activation of osteonal remodeling due to ovariectomy (Frost and Jee, 1992) and different turnover is expected for different bones in the skeletal system. Moreover a special attention should be given to the mechanical loading of the implants in this study. The physiological masticatory forces would stimulate periosteal mineralization (Inman et al., 1999) and, consequently, enabling the dynamics of bone turnover to reach equilibrium, thereby attenuating the loss in bone incurred by estrogen deficiency.

The results of this study are in directly agreement with a human retrieval study which did not observe differences in bone morphology between osteoporotic and non osteoporotic subjects (Shibli et al., 2008). Other authors have also shown that systemic osteoporosis was not considered an important indicator of dental implant outcome under masticatory function (Amorim et al., 2006; August et al., 2001; Friberg et al., 2001).

Within the limitations of the experimental model, the results obtained in the present study further suggests that estrogen deficiency is not a risk factor for the maintenance of jaw bone around osseointegrated implants.

## ACKNOWLEDGMENTS

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**Figure 1.** Time line for the events of the study.

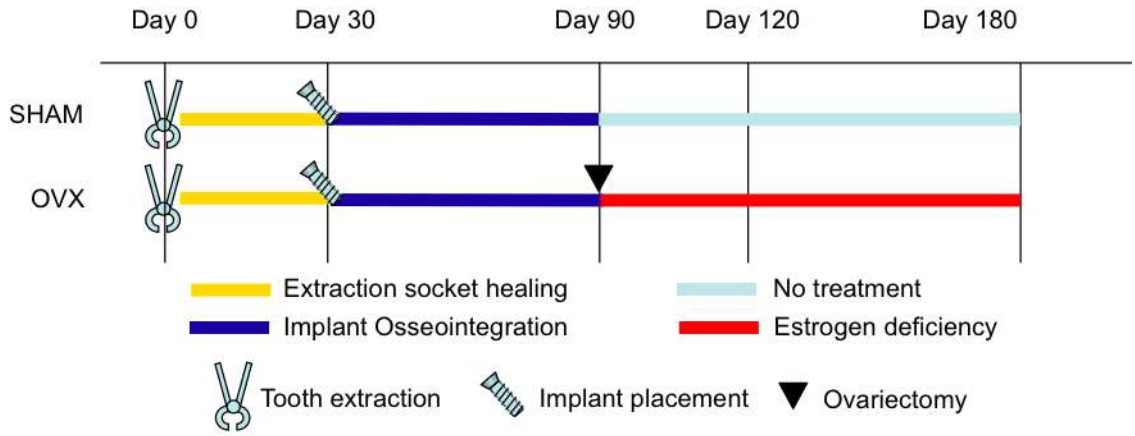


**Figure 2.** Graphical representation of the femur and lumbar vertebral BMD ($g/cm^2$).

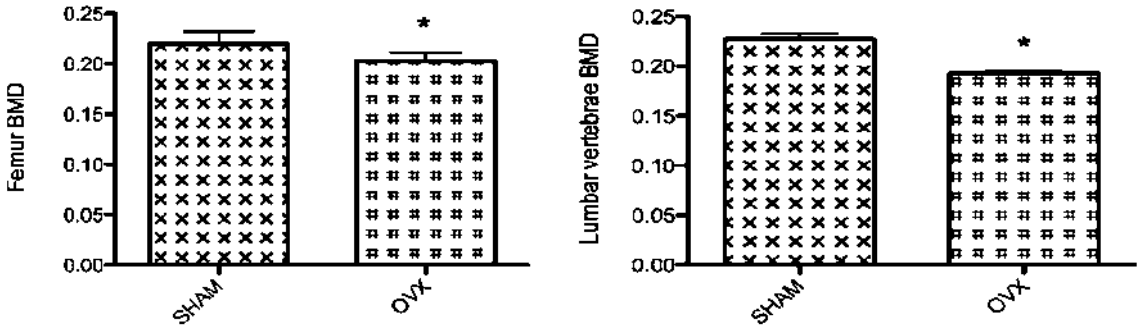


**Figure 3.** Comparison of (a) TRAP and (b) ALP activity, (d) RANKL, (e) OPG, (g) OCN and (h) CTX serum concentrations between the groups. Ratio between TRAP/ALP and RANK/OPG are represented in Figure (c) and (f), respectively.

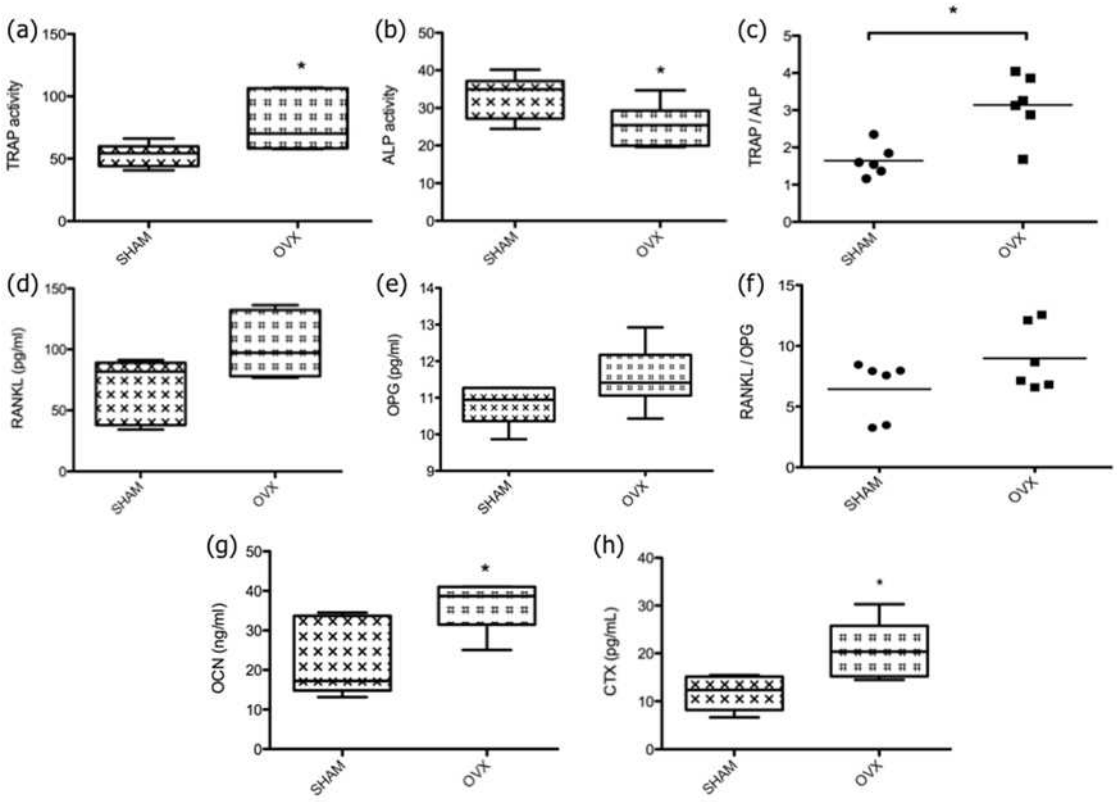


**Figure 4.** TRAP staining representing SHAM and OVX group are indicated in Figures (a) and (b), respectively. 200x magnification. (c) demonstrate the presence of osteoclast in OVX group in a higher magnification.

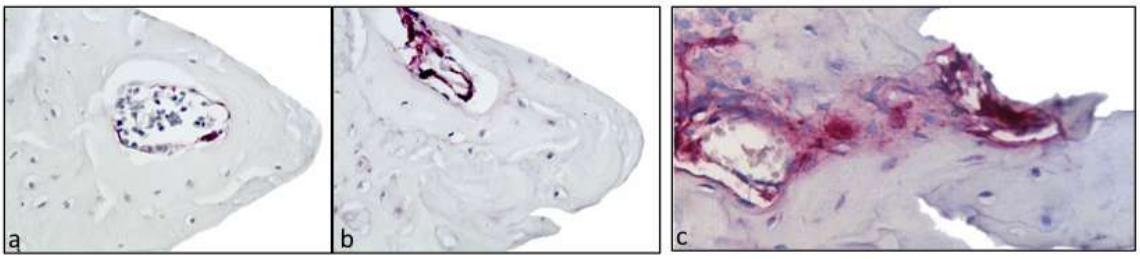


**Figure 5.** Graphical representation of histometrical parameters measured on non-decalcified section. No statistically significant difference was shown for BIC and BAFO between different groups.

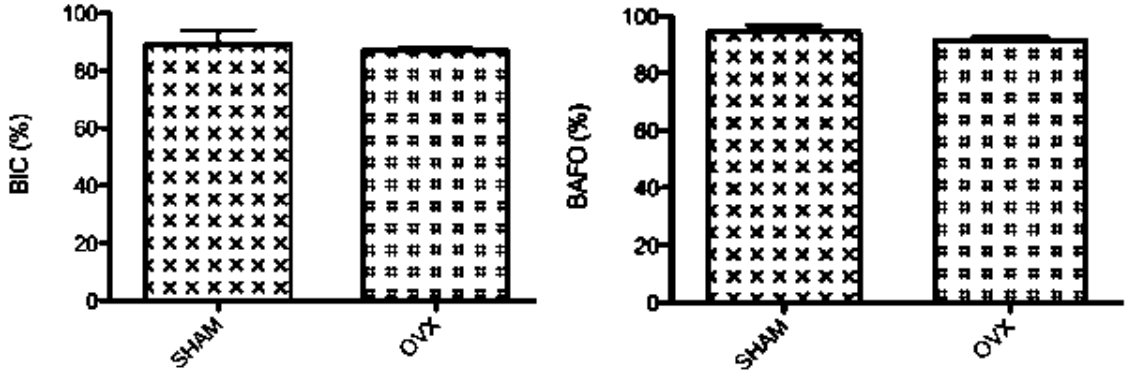


**Figure 6.** Photomicrographs of the bone/implant interface in decalcified HE stained section (a and b) and non-decalcified toluidine blue stained ones (c and d), for SHAM and OVX respectively. No difference was found in the histological morphology comparing both groups.

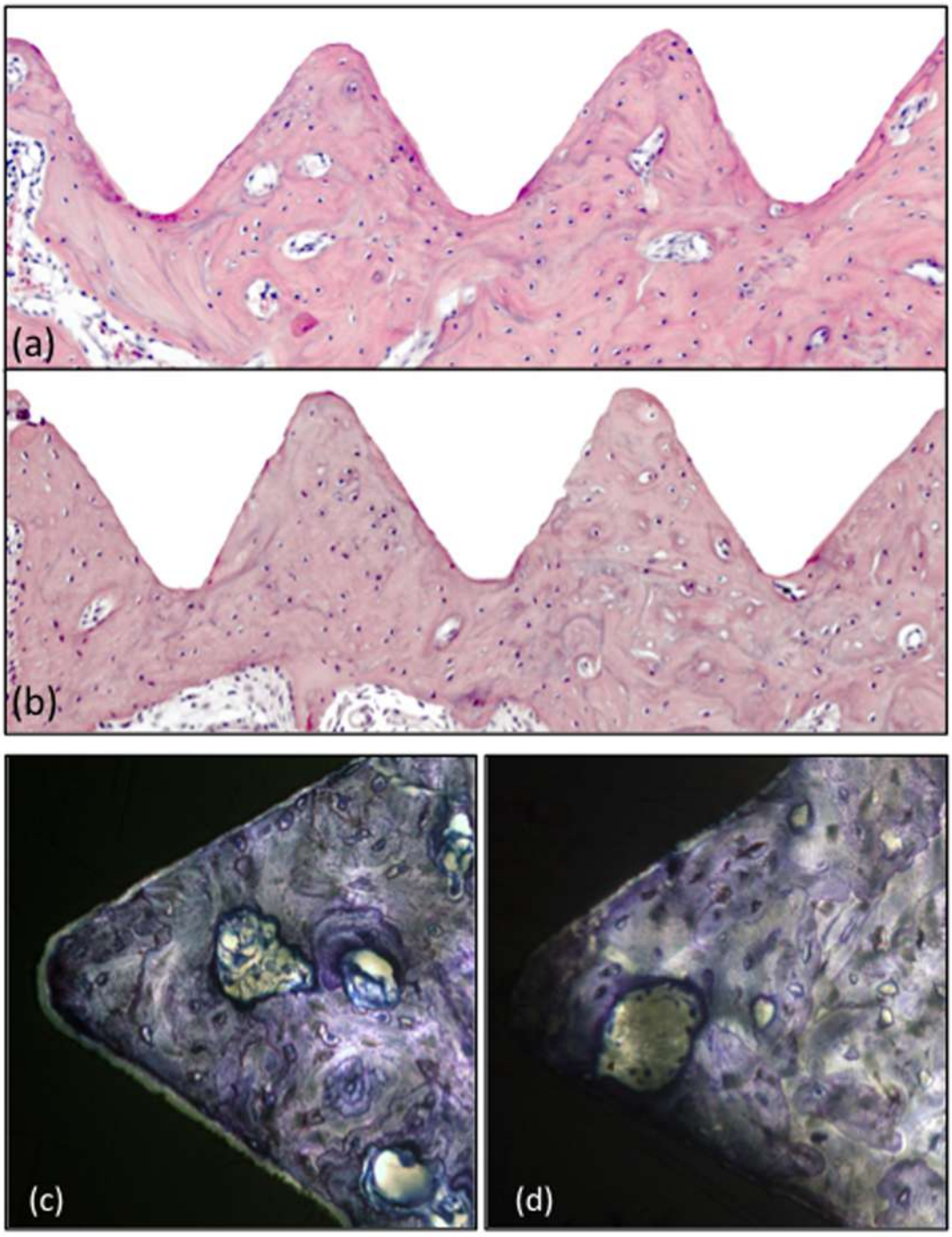
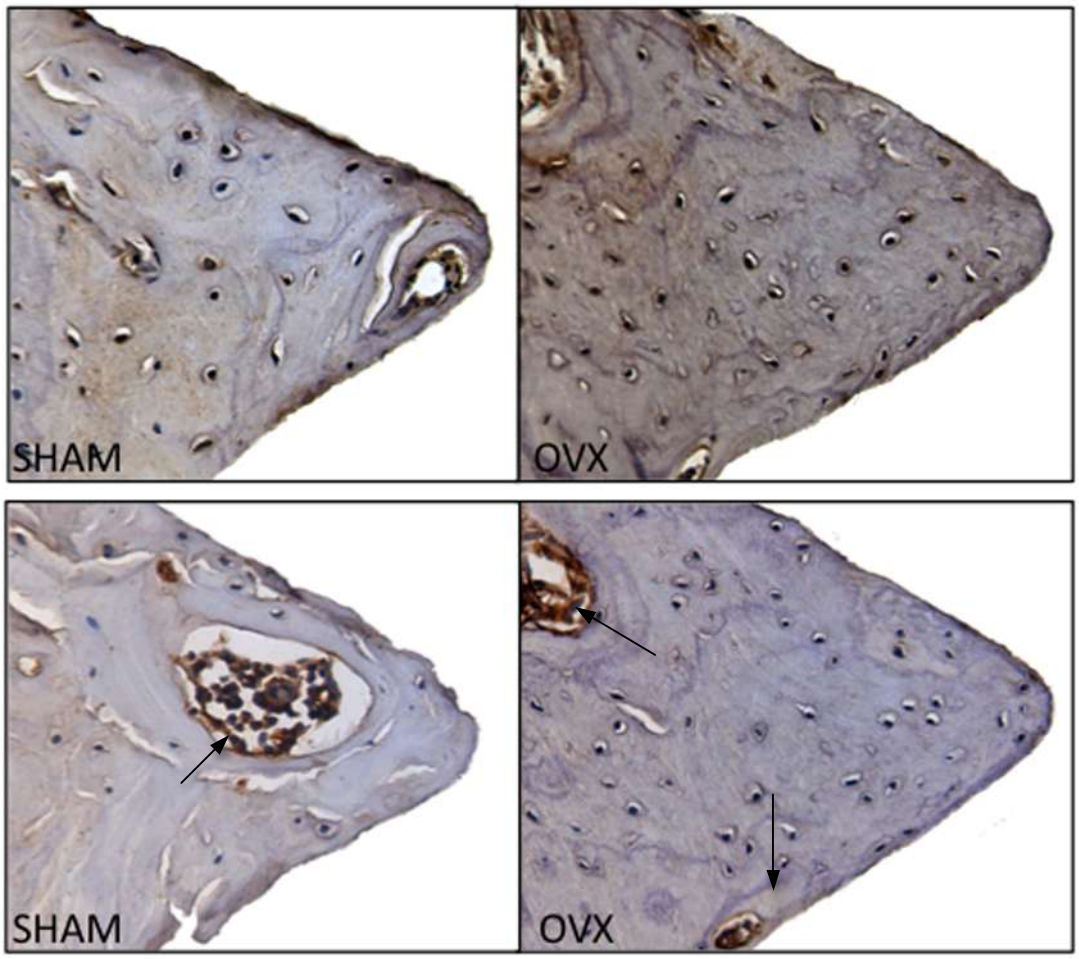


**Figure 7.** Representative sections of the expression and distribution of OCN (A and B) and ALP immunolabelling (C and D) for the different groups.



# Capítulo 4

**Systemic Bisphosphonate Inhibits Implant Integration.***

G. Giro[1,4], P.G. Coelho[2], R.S. Pessoa[1], R.M.R. Pereira[3], E. Jr Marcantonio[1], T. Kawai[4], S.R.P. Orrico[1].

[1] Department of Oral Diagnosis and Surgery, Araraquara Dental School, UNESP – Univ Estadual Paulista, Araraquara, SP, Brazil.

[2] Department of Biomaterials and Biomimetics, New York University, New York, NY, USA

[3] Bone Metabolism Laboratory of Rheumatology Division, Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brazil

[4] Department of Immunology, The Forsyth Institute, Boston, MA, USA

**Running head:** Bisphosphonate inhibits osseointegration

**Key words:** dental implants, alendronate, bisphosphonate, rats.

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**Number of cited references:** 29

Corresponding Author:

Gabriela Giro. Rua Humaitá, 1680, 14801-903, Araraquara, SP, Brazil.

Phone #: 55 (16) 3301-6377; Fax: 55 (16) 3301-6369

e-mail: gabi.giro@gmail.com.

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**ABSTRACT**

Bisphosphonates are widely used in osteoporosis treatment and have been accounted for bone remodeling suppression, potentially affecting the osseointegration of dental implants. This investigation tested the hypothesis that significant differences in biomechanical/histometric parameters would be observed for implants placed before and after the systemic administration of alendronate in a rat model. The following experimental groups were evaluated: animals that received systemic alendronate prior to implant placement, animals that received the treatment after implant osseointegration was established, and a control group (implant placement only). The implants were subjected to biomechanical testing and histologic/metric evaluation. Significant lower biomechanical and histometric measurable parameters were observed for the group that received alendronate prior to implant placement relative to the control group, resulting in partial hypothesis validation.

## INTRODUCTION

Bisphosphonates are currently the first-line class of drugs used for treating osteoporosis and other diseases characterized by increased bone resorption. Alendronate is a nitrogen containing bisphosphonate utilized in large scale for the treatment of osteoporosis. This bisphosphonate has high mineral affinity and binds to eroded bone surfaces (Sato et al., 1991), suppressing osteoclastic activity while accelerating apoptotic activity leading to increased bone mineral density and decreased bone turnover (Chesnut and Rosen, 2001; Ruggiero and Drew, 2007; Watts, 2003). Based on this mechanism of action, several reports have confirmed the clinical effectiveness of alendronate in reducing the incidence of fractures of the spine and hip in postmenopausal women (Abelson et al., 2009; Black et al., 2006; Iwamoto et al., 2008). It has also been shown that alendronate can significantly delay the progression of bone loss in experimentally induced periodontal disease using non-human primate models (Brunsvold et al., 1992; Weinreb et al., 1994), and can also promote a better biomechanical fixation of osseointegrated implants (Giro et al., 2007; Giro et al., 2008; Narai and Nagahata, 2003). While bisphosphonates have been associated with improvements in bone density and mechanical performance, they have often been associated to dental treatment complications such as osteonecrosis of the jaws after tooth extraction and other treatment modalities involving bone surgery (American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws, 2007; Ruggiero and Drew, 2007). To account for this, it has been suggested that the inhibition of bone

resorption in the jaw may drastically change initial bone healing kinetics and thereby bone morphology and mechanical properties (Mashiba et al., 2000). Under these conditions, it is plausible that such complications might arise from alterations in the physiologic remodeling and/or wound healing of the jaw bones (Ruggiero and Drew, 2007).

Specific to dental implants, current evidence providing surgical planning in patients that have been exposed to such drugs for different periods of time is sparse and contradictory (Hikita et al., 2009). Therefore, it is unclear whether the administration of bisphosphonates, either before or after implant placement, could influence the process of implant osseointegration in fundamentally different ways.

Thus, this study tested the hypothesis that significant differences in biomechanical/histometric parameters would be observed for implants placed before and after the systemic administration of alendronate in a rat model.

## MATERIALS & METHODS

### Animals

This study included 27 female Wistar rats approximately 30-days-old, weighing from 70 to 90g. The animals were fed a standard laboratory diet and water *ad libitum*. The study was approved by the Committee of Ethics for Animal Experimentation of Araraquara Dental School (UNESP), Brazil (Protocol# 02/2007).

**Study Design**

All animals included in the study had both maxillary first molar extracted at Day 0. After 30 days following teeth extraction, the animals were randomly divided into 3 groups (n=9 for each group): 1) the control group (CTL), in which implants were placed 30 days following extraction and received no systemic drug administration, 2) a group which received systemic alendronate administration for 90 days prior to implant placement (ALE prior to osseointegration), and 3) a group which implants were placed 30 days following extraction and received systemic alendronate at 60 days following implant placement (ALE after osseointegration) (Figure 1). Regardless of experimental group, two implants were bilaterally placed at the molar region (for histological assessment), and another one in the proximal tibia (biomechanical assessment).

At 30 days following extraction to 180 days (euthanization day), the ALE prior to osseointegration group received SC dosage of 50μg/kg of alendronate every other day. The same dosage was initiated to the ALE after osseointegration group at 60 days after implant placement and was maintained for 90 days. The animals were euthanized by anesthesia overdose and referred for decalcification and hard tissue non-decalcified sample processing.

**Surgical Protocol**

Oral cavity antisepsis was performed with chlorhexidine 0.12% prior to surgical procedures. The teeth were extracted by means of root elevators

under general anesthesia achieved with 0.08 ml/100g IM Ketamine Hydrochloride (Agener União Ltda, São Paulo, SP, Brazil) and 0.04 ml/100g Xylazine Hydrochloride (Rompum®, Bayer S.A., São Paulo, SP, Brazil).

The same anesthesia protocol was utilized for implant placement. The transmucosal, grade 5 screw shaped titanium implants (1.4 mm in diameter by 2.7 mm in length – SIN, Sistema de Implante, São Paulo, SP, Brazil) were inserted following surgical drilling (1.0 mm final diameter) in the healed extracted molar regions. For the tibial site, the dyaphisis was exposed by a 10 mm incision and dissection, and a grade 5 screw-shaped titanium implant (AS Technology, São José dos Campos, SP, Brazil) of 2.2 mm in diameter and 4 mm in length was inserted following surgical drilling (2.0 mm final diameter) under saline irrigation. Soft tissue closure was performed with standard suture techniques (Seda 4.0 Ethicon, Johnson & Johnson do Brasil Ind. Com. Prod. para Saúde Ltda, São José dos Campos, SP, Brazil).

Following all surgical procedures, the animals received a single IM injection of penicillin (2.400 UI/100g) associated with streptomycin (1mg/100g) (Pentabiótico Pequeno Porte, Fort Dodge, Campinas, SP, Brazil).

**Bone mineral density (BMD) assessment by Dual-energy X-ray Absorptiometry (DXA)**

In order to obtain information regarding the systemic condition of the animals from different groups, femur BMD was performed by DXA. This was accomplished using a densitometer (QDR 2000, Hologic, Bedford, MA, USA) in

the Hologic QDR® for Windows® XP operating system for small animals in the high-resolution mode.

**Histomorphometric analyses**

For each animal, one of the implants placed in the oral cavity was non decalcified processed for non-decalcified hard tissue histology. After euthanization, the left side maxillary implant was fixed in 4% buffered formalin for 48h. The implants in bone were dehydrated using increasing concentrations series of ethanol solutions, then embedded in methacrylate-based resin (Technovit 9100, Heraeus Kulzer, Wehrheim, Hesse, Germany). The blocks were cut into 100μm thick sections using a precision diamond saw (Isomet 2000, Buehler Ltd., Lake Bluff, IL, USA). The sections were reduced to a final thickness of approximately 30μm by means of a series of SiC abrasive papers (400, 600, 800, 1200 and 2400) (Buehler Ltd., Lake Bluff, IL, USA) in a grinding/polishing machine (Metaserv 3000, Buehler Ltd., Lake Bluff, IL, USA) under water irrigation. The sections were stained in toluidine blue 1% (Sigma-Aldrich Co, St. Louis, MO, USA) and referred to optical microscopy evaluation.

Measurements of the percentages of bone to implant contact (BIC) and the bone area fraction occupancy (BAFO) between implant thread regions (Leonard et al., 2009) were performed at 100x magnification (Leica DM1200M, Leica Microsystems, Wetzlar, Hesse, Germany) by using the National Institutes of Health image analyzer software (ImageJ 1.41o, National Institutes of Health, USA). BIC was calculated as a length percentage of the

direct bone contact to the implant surface to total implant surface, while BAFO was calculated as a percentage of the area between threads filled with bone.

The remaining implant was decalcified for 28 days in 10% ethylenediamine tetraacetic acid (EDTA) solution containing 0.1M Tris base (pH 6.9). After the completion of decalcification, the implant was gently removed, and the remaining decalcified tissues were embedded in paraffin. Sections of 5µm were subjected to a picrosirius red staining for collagen histochemistry. All histomorphologic observations were performed at various magnifications (Leica DM1200M, Leica Microsystems, Wetzlar, Hesse, Germany) in both transmitted and circularly polarized light mode.

**Removal torque testing**

Biomechanical testing was performed immediately after animal euthanization. The tibia containing the implant was removed and attached to a vise for stabilization to assure orientation and stability during testing. A 0.88 mm wrench was adapted to the internal connection of the implant, and a counter clockwise torque was applied until complete rupture of the bone-implant interface. The maximum torque for interface rupture was measured with a torque gauge (ATG24CN-S, Tohnichi MFG Co. LTD. Tokyo, Japan).

**Data Analysis**

Following D'Agostino normality test, both analysis of variance (ANOVA) and Tukey's post-hoc tests were used for multiple comparisons between the groups. The significance level was set at $p<0.05$.

**RESULTS**

**Bone Densitometry**

Figure 2 shows that both groups receiving systemic alendronate presented significantly higher femoral BMD compared to the control group ($p<0.0001$). A significant difference was also observed between both ALE groups ($p=0.0004$).

**BIC and BAFO analyses**

BIC and BAFO results are presented in Figures 2c and 2d, respectively. Significant differences were observed when comparing ALE prior to osseointegration group with the others groups of the study ($p<0.0001$ for BIC, and $p=0.0002$ for BAFO).

**Removal torque testing**

While the removal torque testing showed significantly lower values to the ALE prior to osseointegration group (Figure 2b), the highest torque degree was recorded for the ALE after osseointegration group, and intermediate values were observed for the control group ($p<0.0001$).

**Histomorphology**

The histomorphologic results presented in Figures 3 and 4 showed similar bone morphology at regions in proximity with the implant surface between the CTL and ALE after osseointegration groups. On the other hand, morphologic evaluation of ALE prior to osseointegration group presented an absence of the concentric configuration of collagen fibers in disorganized/diffuse bone morphology. The absence of osteocytes along with the haversian system pattern without concentric layers indicative of bone remodeling also characterized the ALE after osseointegration bone morphology.

**DISCUSSION**

Complications due to bisphosphonate related osteonecrosis of the jaws (BRONJ) have been described in the dental literature as a potential limiting factor in bone related procedures such as teeth extraction, bone grafting, and dental implant placement (Bamias et al., 2005; Bedogni et al., 2008; Dimitrakopoulos et al., 2006; Marx, 2003; Merigo et al., 2006; Schwartz et al., 2008). It has been proposed that possible mechanisms (Ruggiero and Drew, 2007) resulting in BRONJ include the utilization of systemic bisphophonates and related alterations in physiologic bone remodeling, where the inhibition of osteoclast function may inhibit normal bone turnover following mechanical loading or injury, ultimately resulting in bone necrosis. Another possible mechanism is related to the fact that bisphosphonates are preferentially deposited in bones with high turnover rates, and since the jaws present high

degrees of bone remodeling, the levels of bisphosphonate within the jaws may increase the risk of complications following hard tissue interventions (Ruggiero and Drew, 2007).

While reviews on the topic have been published to date (Grant et al., 2008; Madrid and Sanz, 2009; Mellado-Valero et al.; Serra et al., 2008; Wang et al., 2007), information regarding bone healing following implant placement in patients subjected to systemic bisphosphonate administration prior, during, or post treatment is lacking in the literature (Duarte et al., 2005; Giro et al., 2007; Narai and Nagahata, 2003; Viera-Negron et al., 2008) . Thus, we hypothesized that significant differences in histomorphometric and biomechanical parameters would be observed between implants placed before and after the administration of a bisphosphonate (alendronate) in a rat model.

The femur densitometry results confirmed the systemic impairment of the animals (Giro et al., 2007; 2008). As expected, the animals included in the groups receiving systemic alendronate showed higher BMD values. This increase of the BMD values demonstrates the effectiveness of the animal model and posology utilized in the present and previous studies for the ALE experimental groups (Andersson et al., 2002; da Paz et al., 2001; Giro et al., 2007; Giro et al., 2008).

The biomechanical testing results showed that higher torque values were observed for the ALE after osseointegration group, and are in agreement with a previous report which evaluated implant removal torque in ovariectomized rats with and without administration of systemic alendronate (Narai and

Nagahata, 2003). The increase in torque levels necessary for removal of the implants of ALE after osseointegration group was possibly due to an increase in bone density as lower degrees of bone remodeling after osseointegration occurred (Kuroda et al., 2003). However, in contrast to the findings of Chacon et al. (2006) and Viera-Negron et al. (2008), systemic administration of alendronate prior to implant placement significantly decreased the amount of torque necessary for implant removal and resulted in less organized bone morphology relative to the other groups. While our biomechanical results are in direct contrast with previous work, the morphologic differences between groups along with the BIC and BAFO results obtained support the torque testing findings, as higher degrees of BIC and BAFO and bone organization for the CTL and ALE after osseointegration groups possibly led to higher biomechanical fixation. It should be noted that differences in administration periods and dosages may have accounted for the differences between studies.

Even though the majority of osteonecrosis reports are related to intravenous bisphosphonate administration for the treatment of cancer metastatic lesions, the present study points that further investigation regarding the administration of bisphosphonates prior, during, and post implant surgery are warranted in order to determine their effects on osseointegration, especially as systemic kinetics may substantially differ between bisphosphonate drugs. Since significant differences were observed in both biomechanical and histomorphometric results between the ALE prior to osseointegration and the control group but not for the ALE after osseonintegration group, the

postulated hypothesis was partially accepted.

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**Figure 1.** Study design showing the experimental groups and time frames for drug administration and implant placement.

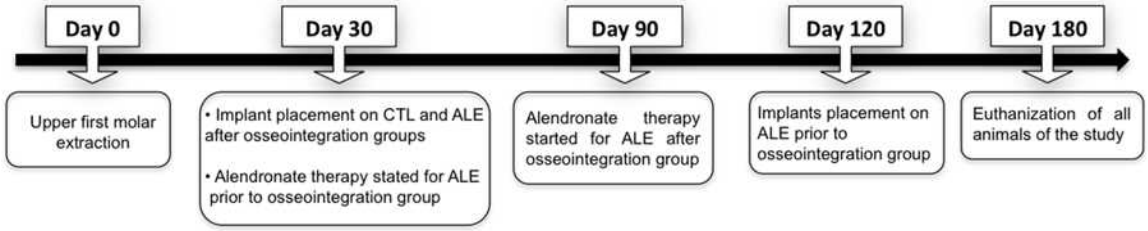


**Figure 2.** Graphic representation of the means and SD for BMD (a), removal torque (b) and BIC (c) and BAFO (d) values. *, Φ p<0.001 in relation to the other groups of the study.

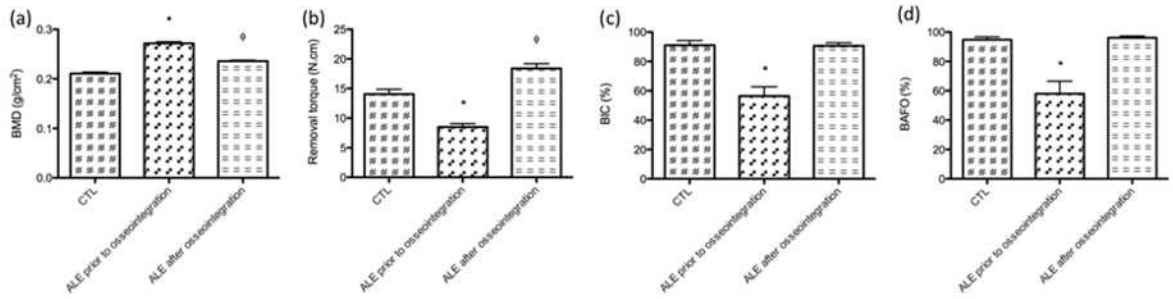


**Figure 3.** Representative histological section for each group of the study. 1% toluidine blue staining, 100x and 200x magnification.

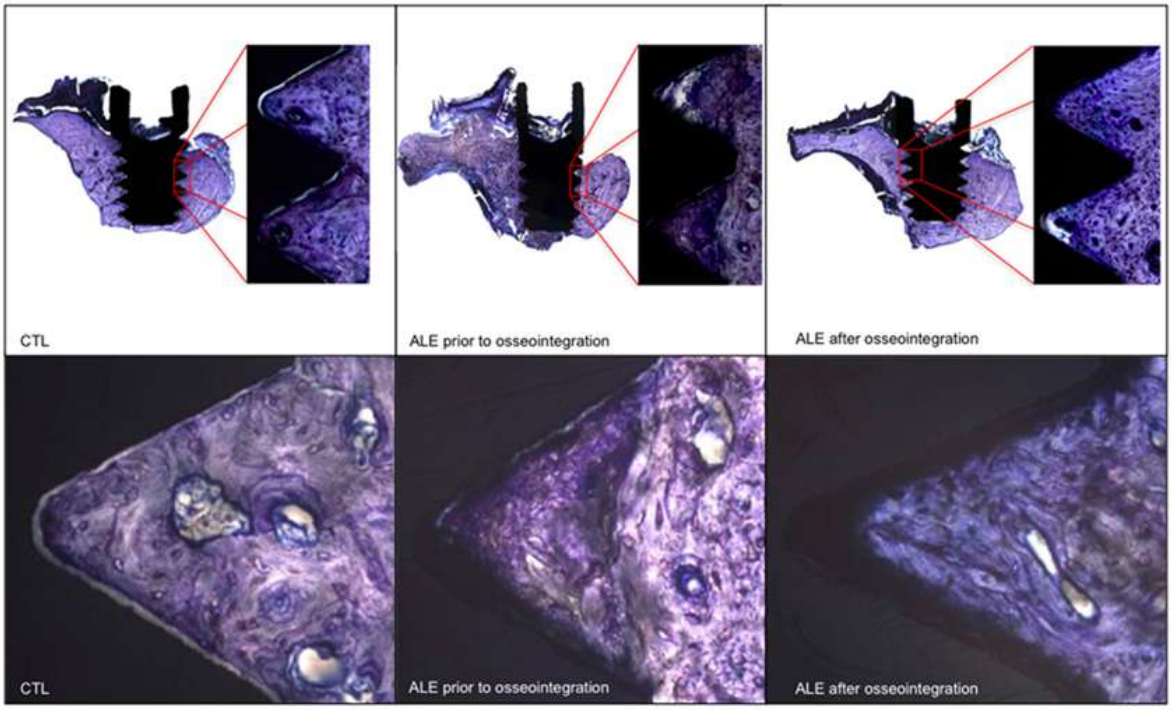
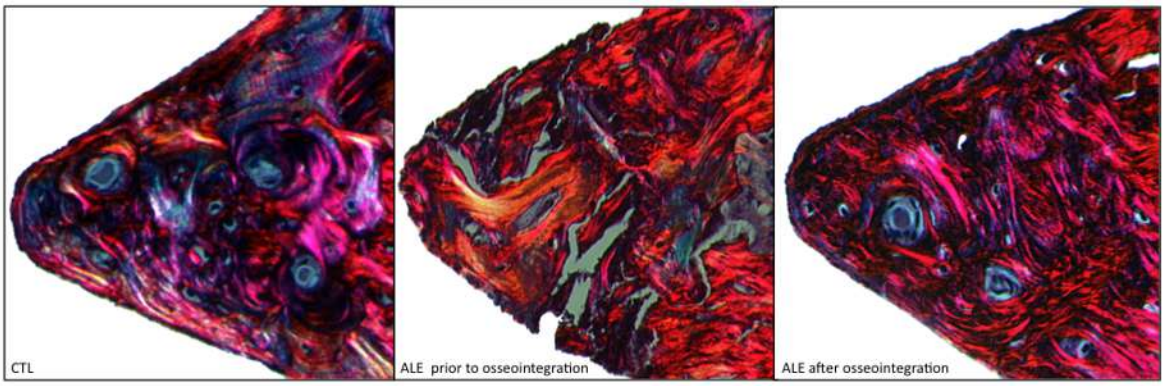


**Figure 4.** Photomicrograph showing bone organization and collagen fibers orientation. Picrosirius red staining, 200x magnification.



# Capítulo 5

# Alterations in implant osseointegration on animals under alendronate therapy.

Gabriela Giro (CORRESPONDING AUTHOR)

Department of Oral Diagnosis and Surgery, Araraquara Dental School, UNESP – Univ Estadual Paulista. Rua Humaitá 1680. Araraquara, SP, 14801-903, Brazil. Phone #: 55 16 3301-6377. email: gabi.giro@gmail.com

Paulo G. Coelho

Department of Biomaterials & Biomimetics, New York University. Arnold and Marie Schwartz Hall of Dental Sciences. 345 East 24th Street (Rm 804S). New York, NY 10010. email:pc92@nyu.edu

Lukasz Witek

Department of Biomaterials & Biomimetics, New York University. Arnold and Marie Schwartz Hall of Dental Sciences. 345 East 24th Street (Rm 804S). New York, NY 10010. email:pc92@nyu.edu

Roberto Sales e Pessoa

Department of Oral Diagnosis and Surgery, Araraquara Dental School, UNESP – Univ Estadual Paulista. Rua Humaitá 1680. Araraquara, SP, 14801-903, Brazil. email: rspessoa@uol.com.br

Rosa Maria Rodrigues Pereira

Bone Metabolism Laboratory of Rheumatology Division, Faculdade de Medicina da Universidade de São Paulo. Av. Dr. Arnaldo, 455, 3o andar, Reumatologia, São Paulo, SP 0124-6903, Brazil. email: rosamariarp@yahoo.com.br

Toshihisa Kawai

Department of Immunology, The Forsyth Institute. 140 The Fenway, Boston, MA, USA. 02115. email: tkawai@forsyth.org

Silvana Regina Perez Orrico

Department of Oral Diagnosis and Surgery, Araraquara Dental School, UNESP – Univ Estadual Paulista. Rua Humaitá 1680. Araraquara, SP, 14801-903, Brazil. email: s-orrico@foar.unesp.br

**ABSTRACT**

The influence of alendronate administration during healing and after osseointegration was evaluated on bone tissue surrounding endosseous implants in a rat model. The study included 56 female Wistar rats, randomly divided into 7 groups. All animals had the upper first molars extracted. After 30 days, sham surgery (SHAM), ovariectomy (OVX), estrogen replacement therapy (EST), and the groups that received alendronate therapy after implant integration (ALE 50μg after osseointegration and ALE 1mg after osseointegration) received 2 implants in order to replace the missing teeth. Sixty days after implant placement these animals were submitted to the respective treatment. Two groups (ALE 50 μg prior to osseointegration and ALE 1mg prior to osseointegration) started receiving alendronate 90 days prior to implant placement (implants kept in place for 60 days). Histomorphometric analysis showed a decrease of bone to implant contact and bone area fraction occupancy between implant threads for animals that received alendronate treatment previously to the implant placement. Alendronate administration also increased the amount of empty osteocyte lacunae and apoptotic cells in proximity with the implant surface. It was concluded that alendronate treatment negatively affected the bone tissue healing and establishment of osseointegration of dental implants.

**Key words:** dental implants, ovariectomy, osteoporosis, alendronate, bisphosphonate, rats.

## 1. INTRODUCTION

Understanding bisphosphonate related osteonecrosis of the jaws (BRONJ) has received substantial attention by the dental and medical research communities since its pathophysiology mechanism is not completely understood. At the present stage, there is no consensus regarding how and why this condition exclusively affects the oral cavity [1].

Although the majority of cases of BRONJ are related to intravenous administration of bisphosphonates for treatment of bone metastasis, oral nitrogen-containing bisphosphonates such as alendronate have also been associated to BRONJ [2-4]. Although the occurrence of BRONJ in patients under alendronate for osteoporosis treatment are rare when compared to the intravenous administration, the suppression of bone remodeling caused by this drug may be a complicating factor in therapies where bone healing and/or modeling/remodeling is required for success. For instance, the bisphosphonate induced remodeling suppression could preclude the successful osseointegration (intimate contact between bone and implant at the optical microscope level, allowing implant load bearing capability) of dental implants and/or affect in long-term stability as the remodeling process plays an important role bone healing and in the microdamaged bone substitution.

Regarding the fact that oral bisphosphonates are widely prescribed drugs on the market (27 million prescriptions for oral bisphosphonates were written last year only in the United States) [5], assessment regarding the relationship between these drugs administration, its cellular and tissue level interference,

and related clinical scenarios is desirable for better determining treatment guidelines.

The present study aimed to evaluate the influence of alendronate administration on bone tissue surrounding endosseous implants during healing and after osseointegration in a rat model.

## 2. MATERIALS & METHODS

### 2.1. Animals

The experimental protocol received the approval from the Committee of Ethics for Animal Experimentation of Araraquara Dental School, UNESP (Protocol# 02/2007). This study included 56 female Wistar rats approximately 30 days old weighing from 70 to 90g. The animals were fed a standard laboratory diet and received tap water *ad libitum*. The animal facility had controlled environmental temperature, humidity, and light exposure.

### 2.2. Study design

All rats included in the study had both maxillary first molar extracted at Day 0. On the 30$^{th}$ day following teeth extraction, the animals were randomly divided into 7 groups, as described in Table 1. At this time, groups SHAM, OVX, EST, ALE 50μg after osseointegration and ALE 1mg after osseointegration had two implants bilaterally placed at the molar region, while ALE 50μg prior to osseointegration and ALE 1mg prior to osseointegration were submitted to ovariectomy and started alendronate therapy five days post-operatively. The therapy consisted in sub-cutaneous (SC) injections of

50µg/kg every other day and 1mg/kg once a week of alendronate for ALE 50µg prior to osseointegration and ALE 1mg prior to osseointegration groups, respectively.

At day 90, the animals that received the implants were then submitted to sham surgery or ovariectomy (Table 1). The estrogen replacement therapy was started in the 5$^{th}$ day post-ovariectomy for EST group with daily SC injections of 20µg/Kg of 17 β-estradiol (Sigma-Aldrich Inc., Saint Louis, MO, USA). At this time frame, ALE 50µg after osseointegration and ALE 1mg after osseointegration groups started the alendronate therapy as previously described (Table 1). These treatments were maintained for 90 days.

Finally, Groups ALE 50µg prior to osseointegration and ALE 1mg prior to osseointegration were subjected to implant placement 90 days after ovariectomy. The animals were euthanized by anesthesia overdose 180 days after teeth extraction and referred for decalcified and hard tissue nondecalcified sample processing.

## 2.3. Teeth extraction

Oral cavity antisepsis was done with chlorhexidine 0.12% prior to each surgical procedure. Teeth are extracted using a tooth extractor under general anesthesia by an intramuscular (IM) injection of ketamine hydrochloride (Agener União Ltda, São Paulo, SP, Brazil) at a concentration of 0.08ml/100g and xylazine hydrochloride (Rompum®, Bayer S.A., São Paulo, SP, Brazil) at 0.04 ml/100g of body weight. The animals received a single intramuscular injection of penicillin associated with streptomycin (Pentabiótico Pequeno

Porte, Fort Dodge, Campinas, SP, Brazil) after all surgical procedures. No special diet was provided after the surgery.

### 2.4. Surgery for implant placement

The implants used in this study were sandblasted and acid etched surface screw shaped transmucosal grade 5 titanium implants (SIN, Sistema de Implante, São Paulo, SP, Brazil) measuring 1.4 mm in diameter by 2.7 mm in length. For the placement of the implants, the same anesthesia protocol was used as previously described. They were inserted in the prepared cavities in the extraction area following surgical drilling of 1.0 mm as final diameter, under sterile saline solution irrigation. Postoperative antibiotic therapy was ensured by IM injection of penicillin associated with streptomycin. The clinical sequence of the implant placement is presented in Figure 1.

### 2.5. Ovariectomy and sham surgery

Ovariectomy was performed under general anesthesia and bilateral body shaving. For that purpose, bilateral dorsal skin incision was performed, the muscle layers dissected, and the ovaries removed. The SHAM group underwent similar procedures to those of the OVX group, with the difference that the ovaries were exposed and replaced in their original position in order to simulate surgical stress.

## 2.6. Bone mineral density (BMD) assessment by Dual-energy X-ray Absorptiometry (DXA)

Femur BMD was performed by DXA in order to confirm the systemic condition of the animals. This assessment required a densitometer (QDR 2000, Hologic, Bedford, MA, USA) operating in the high-resolution mode and using specific software for small animals supplied by the equipment's manufacturer.

## 2.7. Histological preparation

Specimen preparation for hard tissue histology was performed as previously described [6]. The left side maxillary implant was fixed in 4% buffered formalin saline for 48h. An increasing concentration series of ethanol solutions were used for dehydration prior to the embodiment in methacrylate-based resin (Technovit 9100, Heraeus Kulzer, Wehrheim, Hesse, Germany). The 100μm thickness sections obtained using a precision diamond saw (Isomet 2000, Buehler Ltd., Lake Bluff, IL, USA) were reduced to approximately 30μm by grinding and polishing with a series of abrasive papers (400, 600, 800, 1200 and 2400) (Buehler Ltd., Lake Bluff, IL, USA) under water irrigation. The sections were stained within 1% (toluidine blue Sigma-Aldrich Co, St. Louis, MO, USA) and referred to optical microscopy and backscattered electron (BSE) mode for morphologic evaluation. The BSE imaging was performed under 10 Pa and 20 KeV in an environmental scanning electron microscope (EVO 50, Zeiss, Wetzlar, Germany).

For decalcified specimen processing, the right side implant was decalcified for 28 days in 10% ethylenediamine tetraacetic acid (EDTA) solution containing

0.1M tris base (pH 6.9) after fixation and embedded in paraffin. Sections of 5μm were obtained and stained with hematoxylin-eosin (HE), Masson-Trichrome, and Picrosirius Red polarization method for bone tissue analyses. The sections were also TUNEL stained as subsequently described.

### 2.8. Histomorphometry

Measurements of the percentages of bone to implant contact (BIC) and the bone area fraction occupancy (BAFO) were performed in non-decalcified sections at 100x magnification (Leica DM1200M, Leica Microsystems, Wetzlar, Hesse, Germany) by using the National Institutes of Health (NIH) image analyzer software (ImageJ 1.41o, National Institutes of Health, USA). BIC was calculated as a length percentage of the direct bone contact to the implant surface to total implant surface, while BAFO was calculated as a percentage of the area between screw threads filled with bone.

NIH software was used for the quantification of the number of lacunae-containing osteocytes. One 5μm thick section from each biopsy was scored for the number of osteocyte lacunae containing live cells (presence of the hematoxylin stained nuclei) and empty osteocyte lacunae in a squared area with length of 500μm each side (shown in Figure 6A) along the 2$^{nd}$ and 3$^{rd}$ threads in both sides of the implant. The mean of the percentage of lacunae-containing cells by the total lacunae amount in the related area was calculated. HE stained sections were also evaluated for the presence of a necrotic bone regions around the implant.

### 2.9. TUNEL assay

Apoptotic cells were detected using an *in situ* apoptosis detection kit (ApopTag® Plus, Chemicon International Inc., Temecula, CA, USA). Apoptotic cells were labeled by modifying DNA fragments utilizing terminal deoxynucleotidyl transferase (TdT) nick end-labeling (TUNEL). After dewaxing, the sections were washed in PBS for 5 minutes and were then incubated with proteinase K in a concentration of 20 μg/mL (Trevigen Inc., Gaithersburg, MD, USA) for 15 minutes at room temperature (RT). Endogenous peroxidase was subsequently blocked by incubating the sections in 3% hydrogen peroxide in PBS for 5 minutes at RT. After incubation with TdT enzyme at 37ºC for 1h, the sections were reacted with anti-digoxigenin conjugate at RT for 30 min. The apoptotic reaction was visible after incubation with a peroxidase substrate. The sections were faintly counterstained with 0.5% methyl green.

### 2.10. Data Analysis

Following D'Agostino normality test, analysis of variance (ANOVA) and Tukey's post-hoc tests were used for multiple comparisons between the groups. The significance level was set at 5%.

## 3. RESULTS

### 3.1. BMD assessment

Densitometry of femur and lumbar vertebrae showed similar results. The OVX group presented a decrease of BMD while the proposed treatments inhibited

the bone mass loss caused by ovariectomy. No significant difference was found between SHAM and EST groups for any of the evaluated areas. Groups receiving alendronate treatment showed higher femoral BMD. ALE 50μg prior to osseointegration and ALE 1mg prior to osseointegration showed the highest levels of BMD with statistically significant difference in relation to the other groups of the study ($p<0.001$), including ALE 50μg after osseointegration and ALE 1mg after osseointegration groups although these showed significantly higher values compared to SHAM, OVX and EST groups (Figure 2).

### 3.2. Histomorphometry

BIC and BAFO data were similar for SHAM, OVX, EST, ALE 50μg after osseointegration, and ALE 1mg after osseointegration (Figure 3). On the other hand, ALE 50μg prior to osseointegration and ALE 1mg prior to osseointegration groups showed significantly lower BIC and BAFO values compared to other groups ($p<0.0001$ for both BIC and BAFO).

Histomorphologic evaluation at the optical and BSE modes showed that the bone tissue formed around implants showed substantial variation depending on group evaluated (Figures 4 and 5).

No difference in bone morphology was observed between SHAM (Figure 4A), EST and OVX (Figures 4 and 5). Alendronate administered after the osseointegration of the implants also presented the complete filling of the threads (Figures 4 and 5).

Regardless the dosage, animals that received the ALE treatment previous to implant placement showed poor bone matrix deposition along and between threads and/or a deposition of an unspecific matrix without mineralized bone matrix characteristics (Figures 4 and 5). For both groups that received ALE prior to implant placement, the osteotomy line was visible even after 2 months of healing.

Quantification of osteocyte presence in the lacunae (Figure 6) showed that groups that received the bisphosphonate therapy prior to the placement of the implant presented significantly lower values compared to all other groups. ALE 50μg after osseointegration and ALE 1mg after osseointegration groups showed a reduction of 20% on live cell percentage with the presence of a focal loss of viable bone matrix in the area adjacent to the threads (as could be seen at Figure 6), whereas in SHAM, OVX and EST the empty osteocytes lacunae were regularly distributed at regions in proximity and away from the implant. ALE 50μg prior to osseointegration and ALE 1mg prior to osseointegration presented a 60% decrease in the number of live cells compared to the SHAM (Figure 6). For these groups, an extensive area of necrotic bone matrix with a high concentration of empty osteocytes lacunae along the implant threads were observed (Figure 6). No significant differences in the number of osteocyte filled lacunae were observed between SHAM, OVX, and EST.

The deposition of an amorphous organic matrix was noted in most threads of the implants from groups ALE 50μg prior to osseointegration and ALE 1mg prior to osseointegration (Figures 6c and 7b). In several instances, empty

medullar spaces with complete absence of live cells in the whole maxilla revealed the necrotic bone degeneration (Figure 7b, 7b').

The TUNEL staining assay results showed that the SHAM, EST, and OVX groups presented a reduced number of apoptotic cells (no significant differences between groups). On the other hand, experimental groups that received alendronate presented large quantities of labeled apoptotic cells. Lower amounts of TUNEL labeled cells were observed for the ALE 50μg and ALE 1mg prior to implant placement groups compared to ALE 50μg and ALE 1mg after osseointegration groups (Figure 8).

The picrosirius-polarization light microscopy evaluation showed the lack of bone matrix organization for all groups receiving alendronate therapy (Figure 9). While SHAM, OVX, and EST presented thick collagen fiber arrangement with concentric lamellar-like spatial arrangement of the haversian system, no well defined osteonic structures were observed for the ALE groups prior and after osseointegration (Figures 9E and 9F).

## 4. DISCUSSION

Despite the substantial increase of the BMD for groups submitted to the systemic alendronate treatment, the overall results of this study showed a negative effect of alendronate therapy on bone tissue around dental implants. In contrast with Viera-Negron [7], who reported the enhancement of implant osseointegration following alendronate administration prior to implant placement, even with dosages an order of magnitude higher than the one utilized in the present study.

From a histomorphometric perspective, our results showed that the administration of systemic alendronate prior to implant placement resulted in significant reductions in the BIC and BAFO parameters, suggesting that the biomechanical support for these implant groups may be decreased compared to other groups. In addition, the bone morphology observed for the ALE 50μg prior to osseointegration and ALE 1mg prior to osseointegration groups presented particular characteristics such as the absence of live cells, poor organization of collagen fibers, and an amorphous mineralized matrix. Such morphology substantially differed from the bone morphology observed for the ALE after osseointegration, SHAM, OVX, and EST group, which did not present significant differences in histometric parameters between them. However, higher numbers of empty osteocyte lacunae and higher amounts of apoptotic cells were observed at the tissue adjacent to the implant threads of the ALE after osseointegration groups, even though such observation may not necessarily implicate in detrimental clinical performance.

Under normal physiologic conditions, loss of osteocytes and the associated changes to tissue can likely be held in check by bone remodeling. However with the suppression of remodeling, non-viable osteocytes would be expected to accumulate, resulting in a dose or potency-dependent accumulation of non-viable bone [1]. An alternative hypothesis for the accumulation of non-viable osteocytes is that bisphosphonates become embedded in the skeleton and therefore accumulate over time, which in turn could affect cell viability [8].

Another mechanism that could be involved in the presence of necrotic area could be related to the fact that tissue blood flow is directly proportional to its metabolic activity. The suppression of bone turnover, with consequently lower metabolic demand would possibly result in vascular remodeling with blood vessels becoming smaller and thus less likely to accommodate the demands for skeletal perfusion known to exist after bone injury. Such inability to raise blood flow in these circumstances could compromise tissue viability and could play a role in BRONJ [1].

When considering all histometric and morphologic results obtained around implants for both ALE prior to osseointegration groups, where significantly lower measurable parameters such as BIC and BAFO were observed along with significant deviations from normal bone healing around implants, previous research supports that such deviation likely occurred after one of the first steps in the implant healing process [9]. Our results showed that osteoid matrix was deposited throughout the space between osteotomy and implant as a response to surgical instrumentation and implantation, and that the subsequent steps did not follow the normal bone healing around implant pathway [9], as subsequent organization and mineralization through remodeling did not occur.

## 5. CONCLUSIONS

From a clinical perspective, the literature concerning guidelines for implant placement in patients under bisphosphonate therapy is sparse and contradictory. While several authors contraindicate implant placement in

patients under intravenous bisphosphonate therapy as such treatment is often considered elective [10, 11], indication of dental implants therapy in patients under oral bisphosphonates for osteoporosis is supported by several reports that did not associate the occurrence of BRONJ to oral bisphosphonate therapies [12-14]. Thus, further basic and clinical investigations concerning administration route, dosage, and specific drugs should be warranted for better understanding bisphosphonate effects on short- and long-term implant therapy success.

## 6. ACKNOWLEDGMENTS

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## 7. DISCLOSURE STATEMENT

The authors have related no conflict of interests regarding this study.

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**Chart 1.** Sequence of events for each groups of the study.

| | Day 0 | Day 30 | Day 35 | Day 90 | Day 95 | Day 120 | Day 180 |
|---|---|---|---|---|---|---|---|
| **SHAM** | Teeth extraction | Placement of the implants | - | Fictious surgery | - | - | Euthanization |
| **OVX** | Teeth extraction | Placement of the implants | - | Ovariectomy | - | - | Euthanization |
| **EST** | Teeth extraction | Placement of the implants | - | Ovariectomy | 17β-estradiol therapy (daily) | - | Euthanization |
| **ALE 50µg after osseointegration** | Teeth extraction | Placement of the implants | | Ovariectomy | Alendronate therapy (50µg/kg every other day) | | Euthanization |
| **ALE 1mg after osseointegration** | Teeth extraction | Placement of the implants | | Ovariectomy | Alendronate therapy (1mg/kg once a week) | | Euthanization |
| **ALE 50µg prior to osseointegration** | Teeth extraction | Ovariectomy | Alendronate therapy (50µg/kg every other day) | - | - | Placement of the implants | Euthanization |
| **ALE 1mg prior to osseointegration** | Teeth extraction | Ovariectomy | Alendronate therapy (1mg/kg once a week) | - | - | Placement of the implants | Euthanization |

**Figure 1.** Illustration of the surgical procedures: (A) Teeth that will be extracted; (B) Healed extraction site; (C) Osteotomy; (D) Implant being inserted at the osteotomy; (E) Both implants placed at the first molar region; (F) Radiograph confirming the implant placed in the maxillary bone.

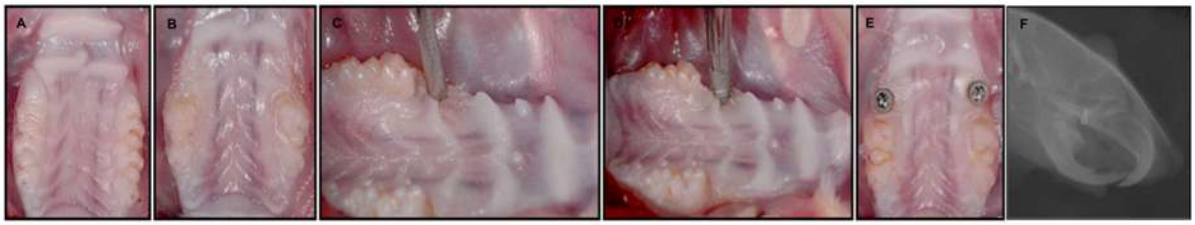


**Figure 2.** Systemic impairment and/or the effectiveness of the drug administration were assessed by femur and lumbar vertebrae global densitometry.

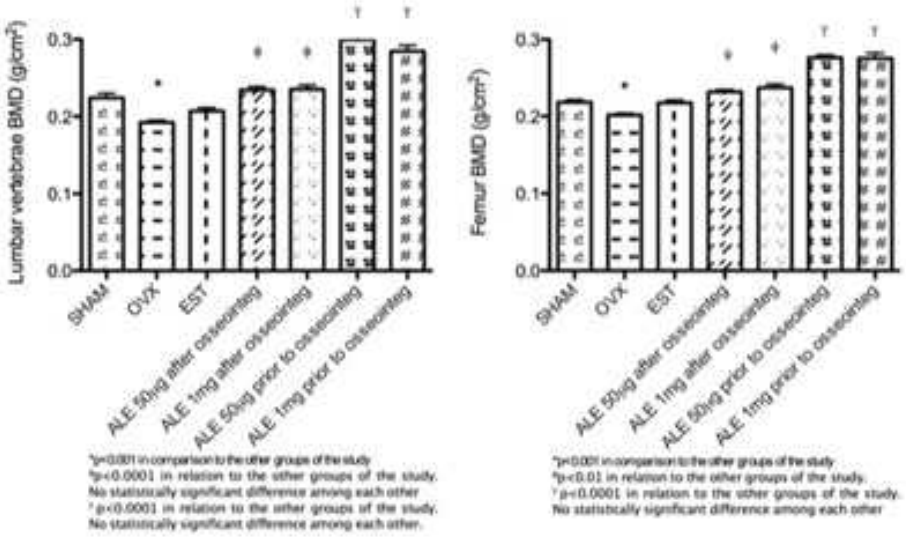


**Figure 3.** Comparison between the groups of the study for bone contact to the implant surface (BIC) and the bone area filling the threads of the implants (BAFO). Results were expressed in percentage.

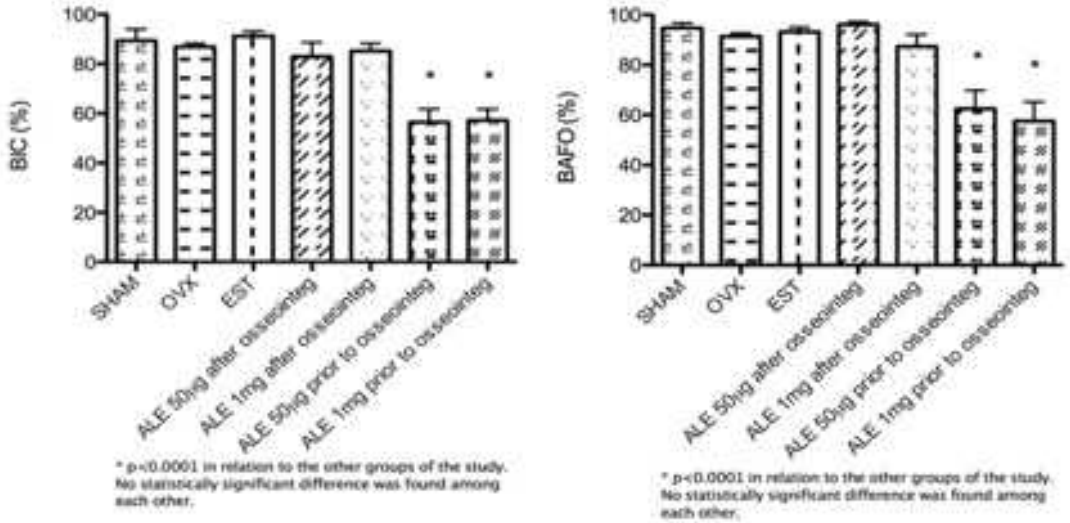


**Figure 4.** Photomicrographs of nondecalcified bone sections around the implants. (A) represents SHAM; (B) OVX; (C) ALE 50μg after osseointegration; (D) ALE 1mg after osseointegration; (E and F) are representing ALE 50μg prior to osseointegration and (G and H) ALE 1mg prior to osseointegration.

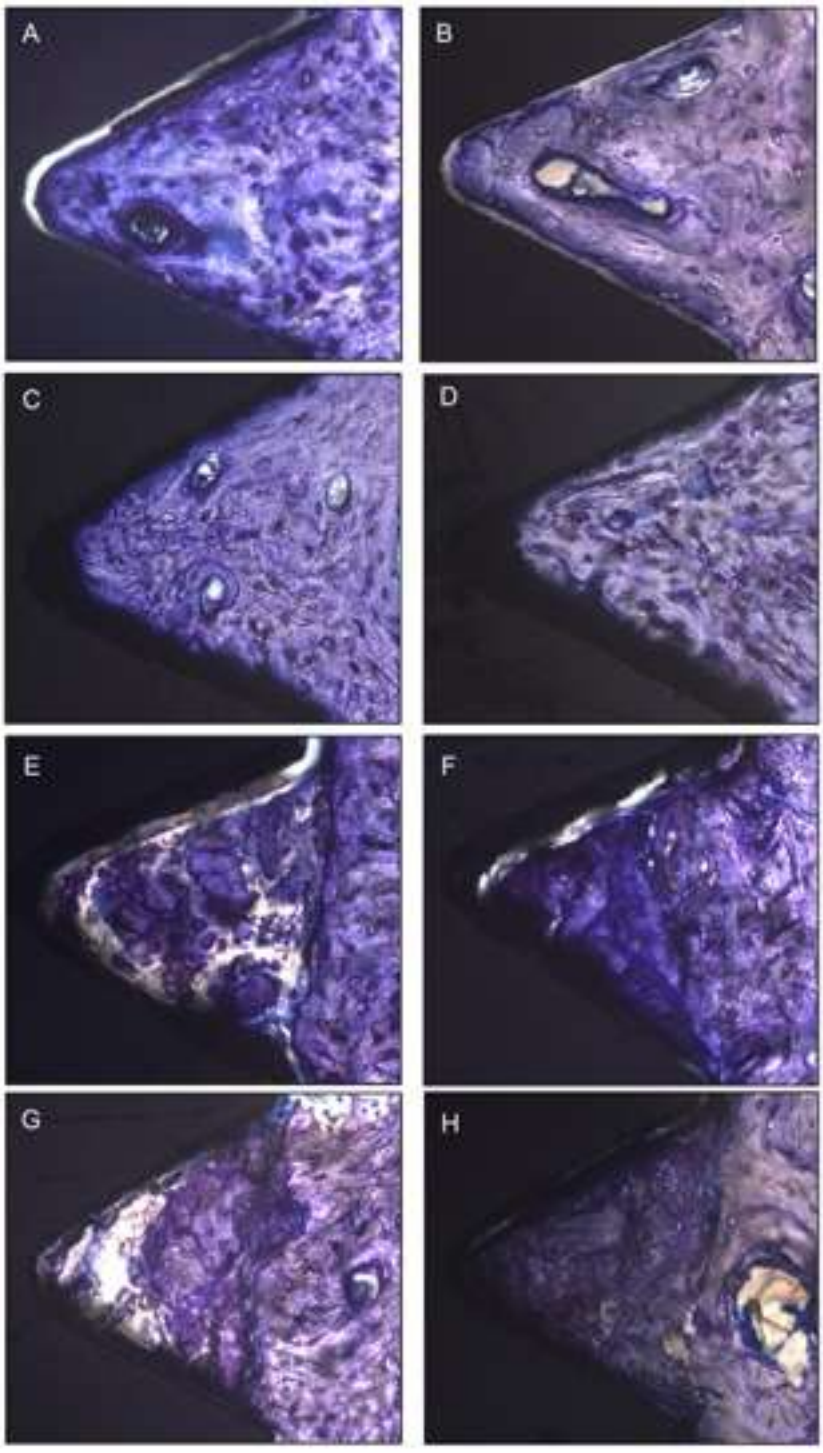


**Figure 5.** Backscattered scanning electron micrographs showing the mineralization pattern for all groups of the study.

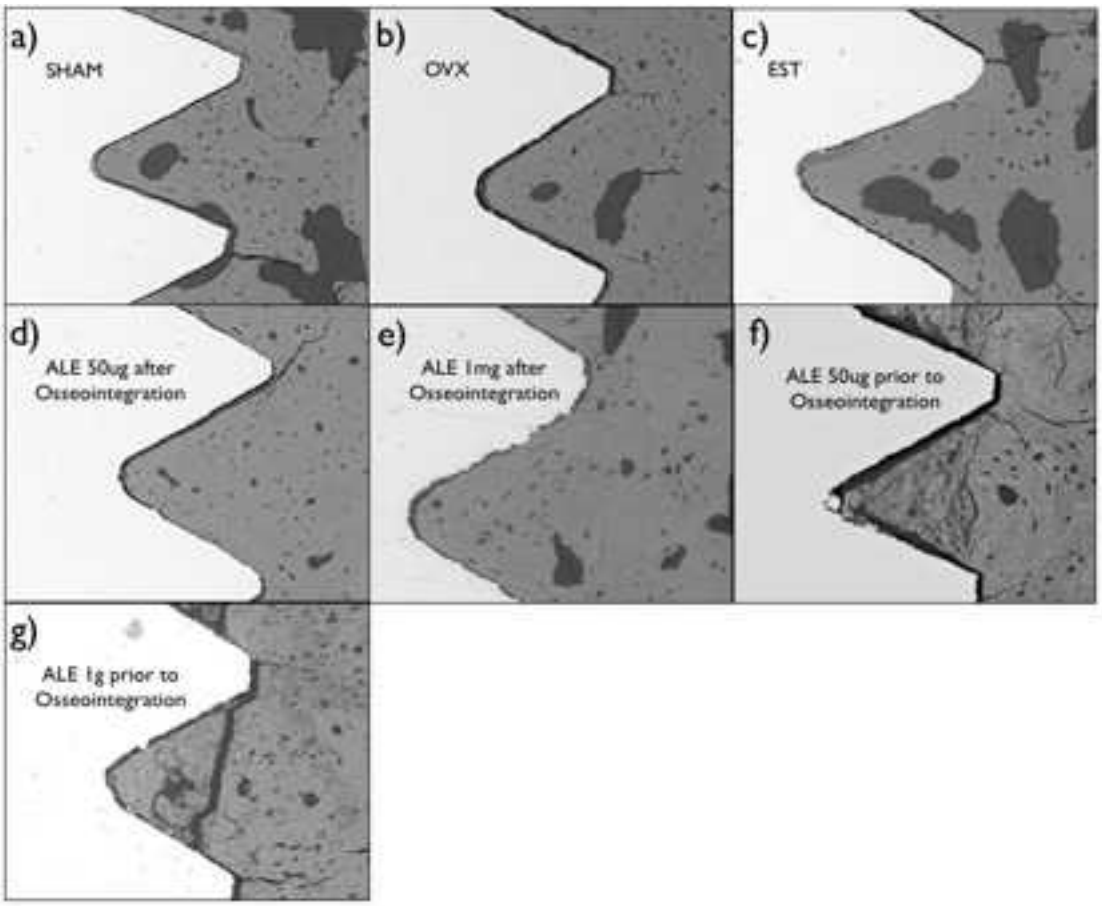


**Figure 6.** (A) Graphical representation of the incidence of areas with accumulated nonviable osteocytes. Osteocyte lacunae number in bone around the threads of the implants was counted (at magnification x100) in a square area of 500 μm each side (shown in Figure 4D), in both sides of the implant. Results are expressed as (B) the total amount of osteocyte lacunae at the referred areas, including the empty ones and those containing live cells and (C) the mean percentage values of live osteocytes (± SD) evaluated from all groups. (D) Representative photomicrograph of SHAM, EST and OVX, since there is no difference on bone morphology between the groups. The black square represents the area used for osteocytes counting at the implant site; Yellow squares represent the empty lacunae accumulation on bone around implants for (E) ALE 50μg and 1mg after osseointegration and (F) ALE 50μg and 1 mg prior to osseointegration.

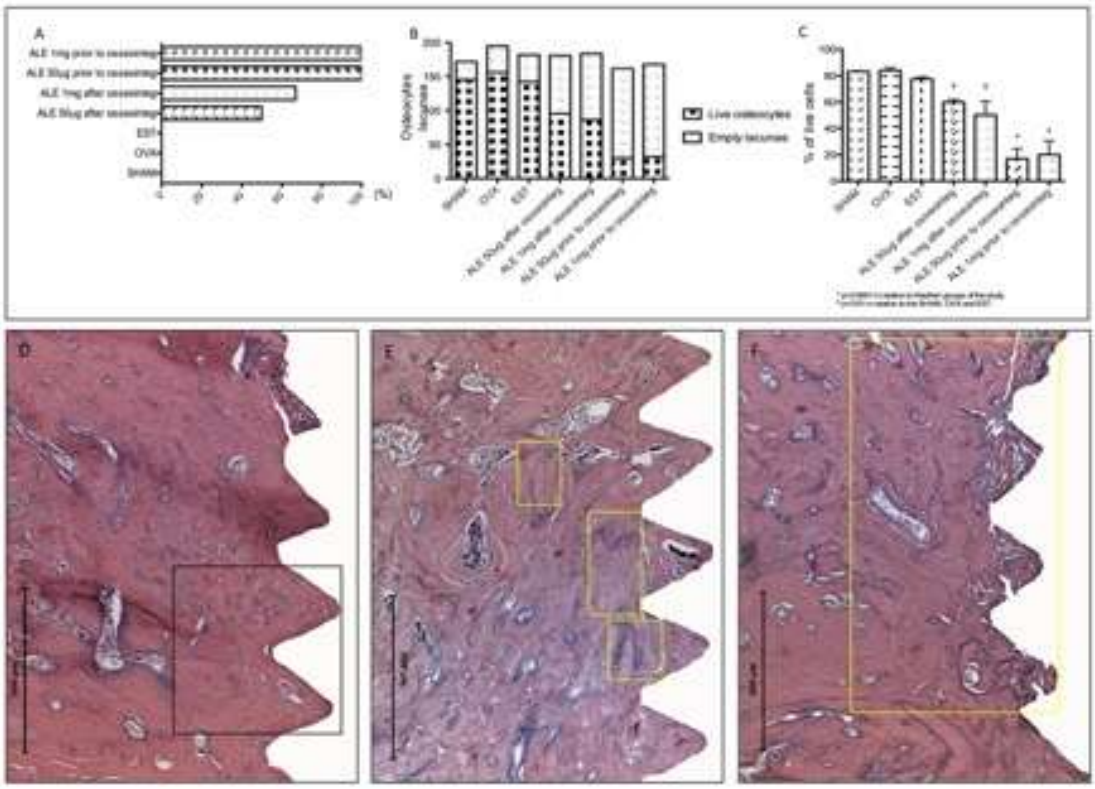


**Figure 7.** HE and Masson's trichrome staining for SHAM (A and B, respectively) and ALE 1mg prior to osseointegration (B and B') showing the compromised bone tissue around the implant due to implant placement in rats under alendronate treatment. A and A' represents a normal bone tissue with viable osteocytes, medullar spaces filled with fibroblasts and the reversal lines regarding bone remodeling. Moreover, (B) represents a necrotic bone tissue full of dead osteocytes, empty medullar spaces and with an amorphous matrix secreted at the threads of the implants. (B') confirms the absence of collagen fibers especially on the top thread of the implant with a lack of adhesion with the bone tissue. Light microscopy, x200 magnification.

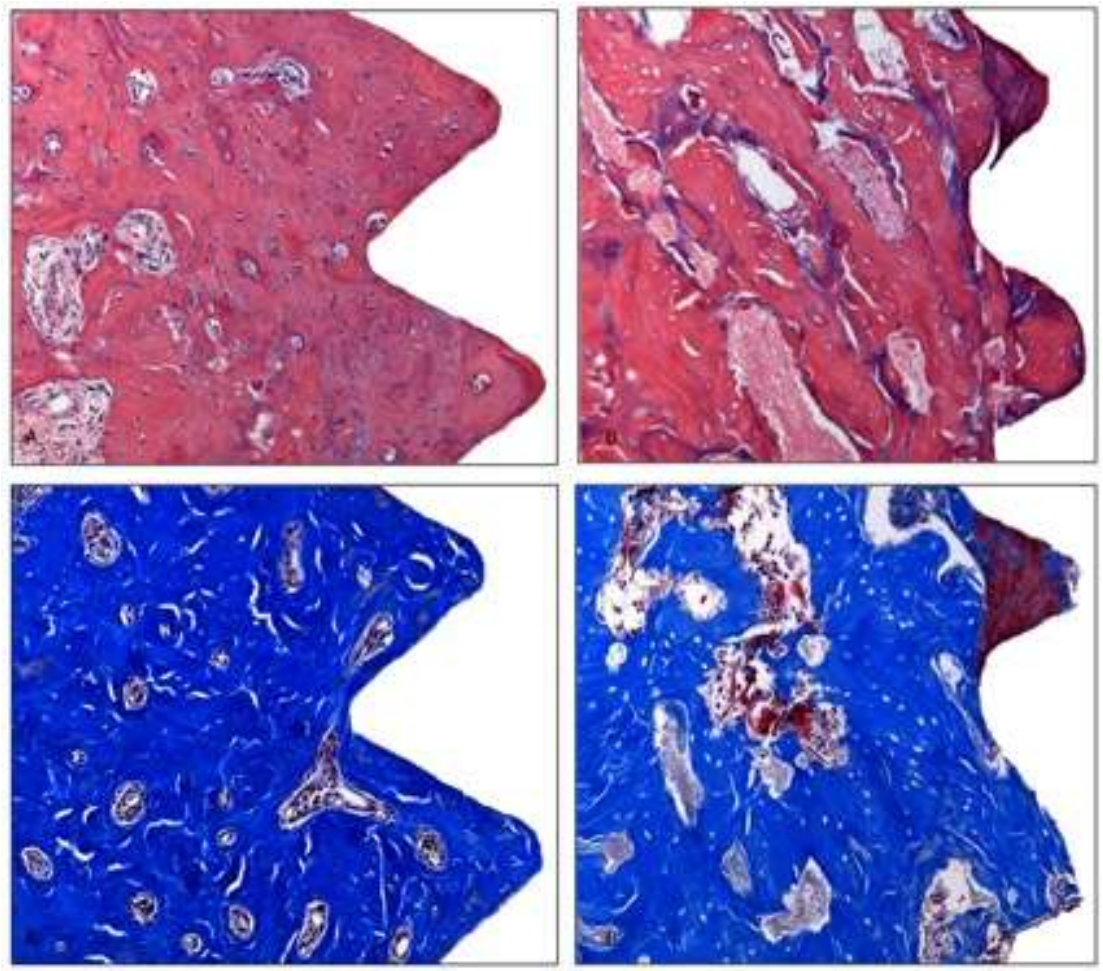


**Figure 8.** (A), (B) and (C) HE stained photomicrographs demonstrated the organizational pattern of the bone tissue on the threads of the implants for the selected groups (SHAM, ALE 50µg after osseointegration and ALE 50µg prior to osseointegration, respectively) and the TUNEL staining pattern for the related groups (A', B', C'). 200x magnification.

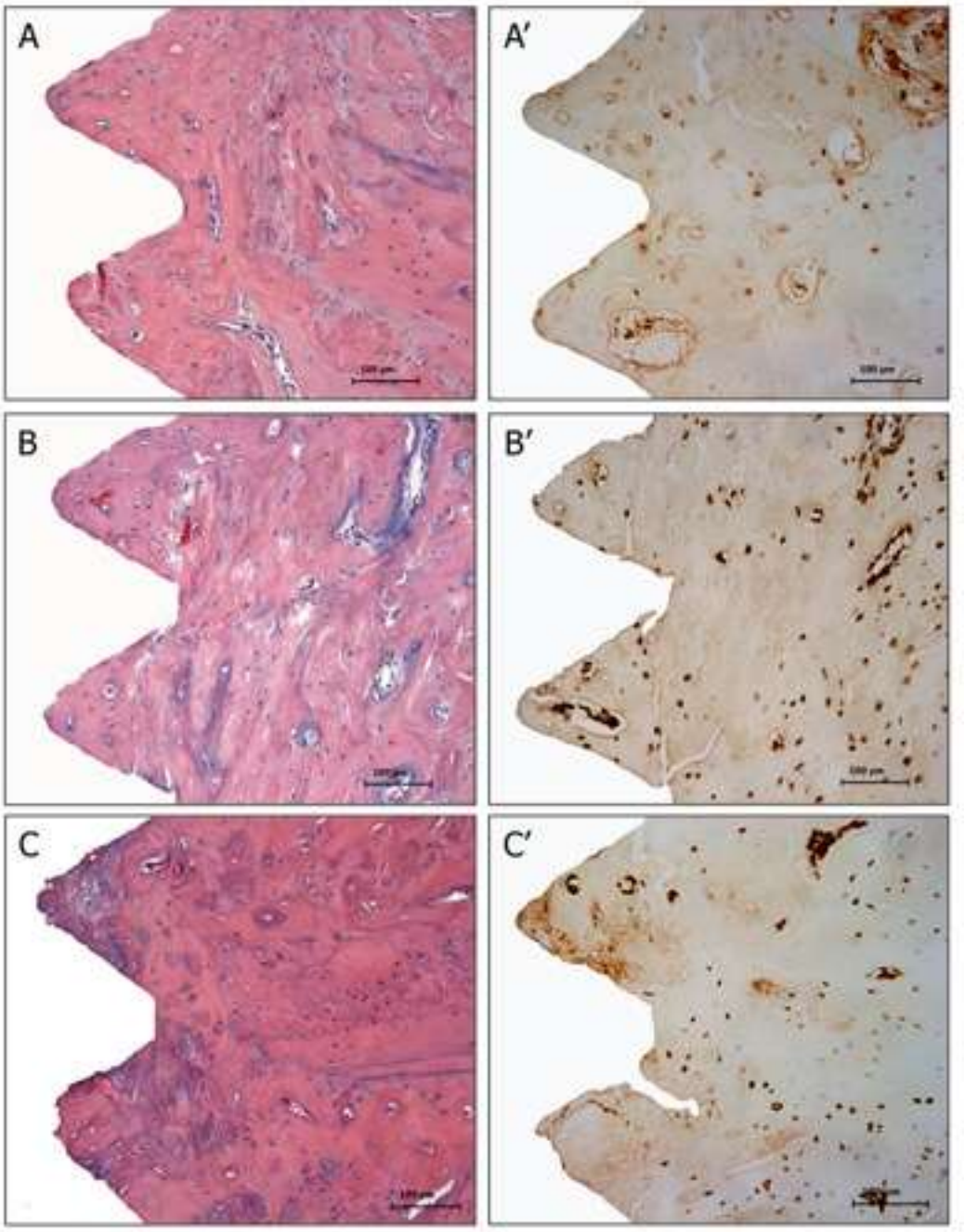
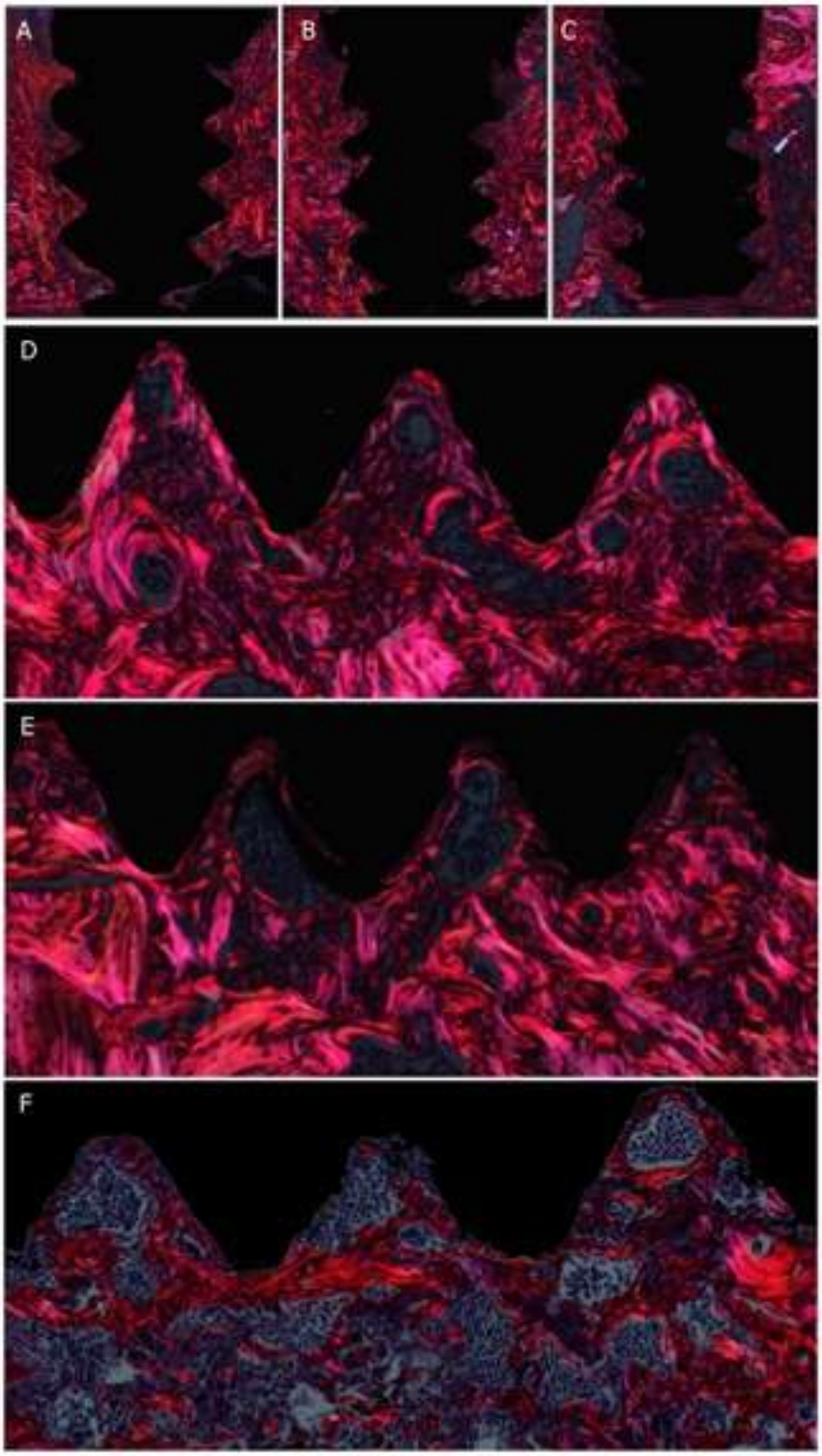


**Figure 9.** Series of images illustrating bone sections stained with Picrosirius red examined by circularly polarized light showing in (A) and (D) SHAM, (B) and (E) ALE 1mg after osseointegration and (C) and (F) for ALE 1mg prior to osseointegration.



# Capítulo 6

**Possible pathogenic association of osteopontin with the inhibition of implant osseointegration on a rat model.**

Gabriela Giro[1,2], Roberto Sales e Pessoa[1], Toshihisa Kawai[2], Silvana Regina Perez Orrico[1].

[1] Department of Oral Diagnosis and Surgery, Araraquara Dental School, UNESP – Univ Estadual Paulista, Araraquara, SP, Brazil.

[2] Department of Immunology, The Forsyth Institute, Boston, MA, USA

**Key words:** osteonecrosis, dental implant, osteopontin, inflammation, T cells

Corresponding Author:

Gabriela Giro. Rua Humaitá, 1680, 14801-903, Araraquara, SP, Brazil.

Phone #: 55 (16) 3301-6377; Fax: 55 (16) 3301-6369

e-mail: gabi.giro@gmail.com

**ABSTRACT**

Bisphosphonate administration has been reported to cause osteonecrosis of the jaws as a complication of the treatment. Therefore, it is necessary to understand the possible pathological mechanism(s) underlying bisphosphonate-mediated osteonecrosis in the context of dental implant placement. To accomplish this, we first investigate the placement of implants in a rat model receiving administration of alendronate (ALE) prior to the implant placement and after its osseointegration. The expression patterns of skeletal remodeling factors and cytokine production were then evaluated in the serum and gingival tissue around implants. The ovariectomy by itself did not affect the integrity of bone tissue around implants. However, examination of the peri-implant bone tissue indicated that ovariectomized rats receiving ALE prior to implant placement showed diminished osseointegration by the up-regulation of immune inflammatory responses accompanied by tissue necrosis and cellular infiltration. The levels of RANKL and TNF-$\alpha$ were elevated in ovariectomized rats receiving ALE either prior to, or after, implant placement. However, elevated levels of IL-6 and osteopontin were only characteristic to the peri-implant necrotic tissue of ovariectomized rats receiving ALE prior to implant placement. Irrespective of diminished expression of osteopontin in bone tissue, the increased level of osteopontin on gingival tissue appeared to be derived from the infiltrating CD5+ T cells or activated macrophages. Therefore, these results suggest that increased osteopontin-expressing cells to play a key role in inhibition of dental implant integration under bisphosphonate therapy.

## INTRODUCTION

Bisphosphonates, the drugs widely used to treat osteoporosis, have been reported to cause the complications in the oral cavity, such as osteonecrosis, in patients under its treatment due to invasive dental procedures (Madrid and Sanz, 2009). Moreover, dental implants are currently contraindicated in patients undergoing intravenous bisphosphonate treatment based on the risk of bisphosphonate-mediated osteonecrosis of the jaw (BONJ) (Dao et al., 1993). Therefore, understanding the pathological mechanism(s) underlying BONJ will help to estimate the potential risk of developing BONJ associated with the placement of dental implants in patients receiving alendronate therapy.

It has been postulated that BONJ results from the death of osteoclasts caused by the cytotoxicity of bisphosphonates and that this, in turn, results in bone necrosis. However, a recent clinical study demonstrated that massive inflammatory cell infiltration, along with hypervascularization, accompanies human BONJ lesion (Lesclous et al., 2009). The roles of such inflammatory cells in the BONJ lesion remain to be elucidated. Immune cells in the inflammatory bone lesion can produce pro-inflammatory cytokines, such as TNF-α, receptor activator of NF-kappaB ligand (RANKL) and IL-6, which can promote cell death of bone cells as well as osteolysis (Herman et al., 2008; Irwin and Myrillas, 1998).

After the placement of dental implants, bone regeneration normally occurs as a result of calcium deposition on the organic matrix of bone tissue, mostly composed of collagen (Boskey and Posner, 1984). The engagement of non-

collagenous bone matrix proteins, such as osteocalcin (OCN) and osteopontin (OPN), plays an important role in the bone remodeling process by facilitating calcium deposition and matrix maturation as well as absorbing bone mineral (Butler and Ritchie, 1995; Rodan and Noda, 1991). However, while the production of osteocalcin is a sign of bone formation, osteopontin facilitates osteoclast-mediated bone resorption because OPN deposited on bone allows the adhesion of osteoclasts via αVβ5 integrin (Reinholt et al., 1990; Rodan and Rodan, 1997). Interestingly, it is OPN that it is also known to function as a cytokine (Buback et al., 2009; O'Regan et al., 2000). OPN has been classified as a pro-inflammatory, or Th1-type, cytokine, and it is therefore thought to exacerbate inflammation in several chronic inflammatory diseases (Buback et al., 2009; O'Regan et al., 2000). While OPN is not expressed in circulating lymphocytes, it is one of the most copious proteins produced by macrophages and T cells and is a potent chemo-attractant stimuli for macrophage recruitment (Scatena et al., 2007). Therefore, OPN appears to regulate the infiltration of macrophages and T cells into the inflammatory lesion (Crawford et al., 1998; Steinman, 2009; Weber and Cantor, 1996). While OPN expression by osteoblasts during implant osseointegration has been reported (Colnot et al., 2007), it is unknown whether lymphocytes infiltrating to the peri-implant tissue express OPN as a pro-inflammatory cytokine. If this can be confirmed, it would mean that, in addition to TNF-α and RANKL, OPN can be included as a pathogenic factor that would compromise implant integration.

Considering the facts, as enumerated above, we undertook a study to validate

our hypothesis, in a dental implant animal model in ovariectomized rats that had been subjected to alendronate administration prior to the placement of the implants. The results showed the inhibition of the adequate bone formation around these implants and the induction of bone necrosis in most of the cases (Giro et al., 2010). In order to elucidate these findings we evaluated the characteristics of both skeletal remodeling and inflammatory responses in the peri-implant tissue.

## MATERIALS AND METHODS

### Study Design

This study utilized 56 30-day-old female Wistar rats weighing approximately 100g. Study protocol, animal care and treatment of the animals were conducted in compliance with the guidelines established by the Committee of Ethics for Animal Experimentation of Araraquara Dental School (UNESP), Brazil (Approval # 02/2007). The timeline of the protocol used in this study is shown in Figure 1.

The animals were anesthetized on Day 0 of the study by an intramuscular (i.m.) injection of ketamine/xylazine. After oral antisepsis, all rats had both right and left maxillary first molars extracted. The rats received an IM injection of penicillin and streptomycin post-operatively.

After 30 days (= Day 30) from the tooth extraction, the animals were randomly divided into 7 groups (n=8/group). The SHAM group received only two implants (left and right maxilla) placed at Day 30 (control group) and underwent a sham operation without ovariectomy. The OVX group received

two implants placed at Day 30 and, after a 60-day healing period, rats underwent ovariectomy (Day 90). The EST group received the same treatments as the OVX group, but additional estrogen replacement therapy was administered via subcutaneous (SC) injection of 17β-estradiol (20µg/kg) started at 5 days post-ovariectomy (=Day 95-180). The ALE 50µg after osseointegration group received two implants placed at Day 30 and, after a 60-day healing period (Day-90), rats underwent ovariectomy followed by alendronate therapy (50µg/kg every other day) started 5 days post-ovariectomy (Day 95-180). 5) The ALE 1mg after osseointegration group received the same treatment as group ALE 50µg after osseointegration, but with a higher dose of alendronate (1 mg/kg once a week; Day 95-180). The ALE 50µg prior to osseointegration group was ovariectomized after healing of tooth extraction site (Day 30) followed by administration of alendronate (50µg/kg every other day; Day 35-180). After implant placement (Day 120), rats were kept receiving the drug treatment until the end of experiment (Day 180). The ALE 1mg prior to osseointegration group received the same treatment as the last cited groups, but with a higher dose of alendronate (1 mg/kg once a week; Day 35-180).

**Implant placement**

The rats were anesthetized as described above. Transmucosal grade 5 implants, 1.4 mm in diameter by 2.7 mm in length, were placed in the maxilla in order to replace the missing teeth. The osteotomy was prepared under saline solution irrigation. The animals also received a single intramuscular

injection of penicillin/streptomycin post-operatively.

**Ovariectomy**

On Day 30, both ALE prior to osseointegration groups underwent ovariectomy, and the respective alendronate therapy was begun 5 days post-surgery. SHAM, OVX, EST and both ALE after osseointegration groups underwent ovariectomy or sham surgery on Day 90 of the study (60 days after implant placement). All animals were sacrificed on Day 180 of the study by deepening anesthesia.

**Blood sample collection and the measurement of serum biomarkers**

On Day 180, blood samples were collected from rat caudal artery early in the morning. Serum concentrations of osteocalcin (OCN), osteopontin (OPN), osteoprotegerin (OPG), receptor activator of nuclear factor NF-κB ligand (RANKL), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were measured by an enzyme-linked immunosorbent assay (ELISA) kit related to each parameter, following the manufacturer's instructions.

Serum samples were also reacted for tartrate resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) activity. For the TRAP activity assay, a 5µl serum aliquot was incubated in 245 µl freshly prepared buffered solution of 125mM Tartrate buffer with 2mM $MgCl_2$ and 1mg/ml of Phosphatase substrate (P-Nitrophenyl phosphate, Disodium; Sigma Aldrich, Saint Louis, MO, USA), buffered to pH 4.5. Fifty µl of 2N NaOH were used as stop

solution, and the optical density of the reaction was read immediately at 405nm.

**Preparation of Gingival Tissue Homogenates**

Gingival tissues surrounding the implants were collected and homogenized with a glass Dounce homogenizer in phosphate buffered saline (PBS) supplemented with 0.05% Tween 20 (Sigma, St. Louis, MO, USA) and protease inhibitor cocktail (Sigma Aldrich, Saint Louis, CA, USA).

The concentrations of proinflammatory (TNF-α and IL-6) and anti-inflammatory (IL-10) cytokines, as well as RANKL, OPG and OPN, in the serum or tissue homogenates were measured by ELISA kits (PeproTech, Rocky Hill, NJ, USA), following the instructions provided by the manufacturer.

**Histological preparation**

Maxillae containing the implant were fixed in 4% buffered formalin saline at 4ºC for 48 hours. The specimens were then decalcified for 28 days in 10% ethylenediamine tetra-acetic acid (EDTA) solution containing 0.1M tris base (pH 6.9) by exchanging the solution every 24 hours. After decalcification, the samples dehydrated through a series of ethanol solutions of increasing concentrations followed by 100% xylene embedded in paraffin. Serial mesio-distal sections (thickness at 5 μm) were obtained. The sections were stained with hematoxylin eosin (H/E) or subjected to other staining patterns as described below. Each stained specimen was examined by light microscopy.

**Immunohistochemistry**

Selected sections mounted on silane-coated glass slides were diafanized in xylene, rehydrated through descending ethanol concentrations, and rinsed for 20 min in PBS. After blocking the endogenous peroxidase in 3% $H_2O_2$ in PBS solution containing 1% bovine serum albumin for 30 min at room temperature (RT), the sections were rinsed three times in PBS and then reacted with the primary antibodies in a humid chamber overnight at 4ºC. The primary antibodies used for this study are described in Table 1. Isotype-matched mouse IgG or whole rabbit IgG was used for negative controls, and no immunoreactivity was confirmed.

Subsequent to the reaction with primary antibodies, sections were incubated with a biotinylated goat anti-rabbit IgG (1:200 dilution; Chemicon International Inc., Temecula, CA, USA) or biotinylated rat anti-mouse IgG (1:200 dilution; Jackson Immunoresearch Laboratories, West Grove, PA, USA) as a secondary antibody for 2 hours in a humidity chamber at RT.

For the detection of binding pattern of mouse monoclonal antibody, sections were incubated with avidin/biotin complex (ABC) with horseradish peroxidase (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA).

Color development of the peroxidase activity was performed with diaminobenzidine substrate (DAB Substrate Kit, Vector Laboratories, Burlingame, CA, USA) in the presence of $H_2O_2$, yielding a brown-staining product. Slides were counterstained with hematoxylin and mounted in a resin-based mounting medium (Permount Mounting Medium, Fisher Scientific, Pittsburg, PA, USA).

A subjective quantification was carried out on bone tissue surrounding the

implants for the assessment of the labeling intensity. The quantifications were performed at a magnification of x100 by light microscope (Leica DM1200M; Leica Microsystems, Wetzlar, Hesse, Germany).

**Fluorescent immunostaining**

Paraffin sections were blocked using BSA 2% in PBS for 45 min. The samples were incubated overnight at 4°C with the primary antibodies: anti-CD5 MAb (OX-19, Mouse IgG1, Serotec, Raleigh, NC, USA) and anti-MHC class-II (OX-6, mouse IgG1, Serotec). Isotype-matched mouse IgG1 MAb, PA20 (Kawai et al., 1998), was used for negative control. Subsequently, Flourescein Isothiocyanate (FITC)-conjugated donkey $F(ab')_2$ fragment anti-mouse IgG (minimum reaction to rat immunoglobulin, Jackson ImmunoResearch) was reacted to the primary antibodies. Mouse MAb specific to OPN (AKm2A1, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was biotinylated using Sulfo-NHS-LC-Biotin (Thermo Scientific, Rockford, IL, USA). After the washing out the unbound FITC-conjugated donkey $F(ab')_2$ anti-mouse IgG from the sections, the biotinylated anti-OPN MAb was reacted with the section at RT for 2 hours, followed by incubation with Phycoerythrin (PE)-conjugated Streptavidin (BD Biosciences, San Jose, CA, USA) at RT for 2 hours. The stained section was mounted in a Fluoromount-G medium (SouthernBiotech, Birmingham, AL, USA) and evaluated using a fluorescent microscope (FSX100, Olympus, Center Valley, PA, USA).

**Tartrate resistant acid phosphatase (TRAP) enzyme histochemistry**

Selected sections, which were reacted for TRAP activity, were incubated in a freshly prepared 125 mM Tartrate buffer solution for 2 hours at room temperature (RT). After that, sections were incubated in an acid phosphatase solution for 90 minutes at RT, washed in distilled water twice, counterstained with hematoxylin, and diafanized to mount the cover glass.

### Statistical analysis

Differences between the groups were analyzed by Kruskal Wallis and Dunn's post-hoc test.

## RESULTS

### Histological findings

As shown in our previous study, it was possible to observe that alendronate treatment prior to the placement of the implants inhibited osseointegration process with the deposition of an amorphous matrix between the some threads of the implants and the induction of bone tissue necrosis, as shown in Figure 2. This necrotic process seems to be a late tissue response, since it is possible to see the deposited matrix, mineralized in some case even in the complete necrotic tissue surrounding the implant as shown in Figure 2.

### Immunohistochemical evaluation of peri-implant bone tissue

The staining of skeletal remodeling biomarkers, including ALP, OCN and OPN, was also monitored in the peri-implant bone sections (Figure 3). Alkaline phosphatase (ALP) activity was present in osteoblasts, vascular endothelial

cells and, sometimes, lymphocytes (Hayhoe, 1983). Alkaline phosphatase (ALP) was weakly stained on bone tissue surrounding the implants. The cells present in the medullary spaces of the SHAM, OVX and EST groups presented a stronger stain for ALP than the ALE after osseointegration groups (both doses 50 μg/kg and 1 mg/kg). However, the cellular infiltrates in the necrotic/amorphous peri-implant tissue of ALE prior to osseointegration groups (both doses 50 μg/kg and 1 mg/kg) showed stronger ALP staining than the other groups. Osteocalcin (OCN), the protein involved in the up-regulation of mineralization (Mundy, 1995; Wolf, 1996), was expressed weakly in osteocytes and bone matrix along the lamellar matrix in alendronate treated groups (Figure 4 A-C) than the other groups of the study.

Regarding OPN staining pattern, SHAM, EST and OVX groups presented a staining pattern of concentric configuration at the cement lines. The ALE treated groups demonstrated a strong staining pattern of OPN at the perilacunar matrix, and some osteocytes were presented in the marrow lacunae. It is noteworthy that the OPN staining pattern on the cement lines diminished on these groups, suggesting the stagnancy of bone remodeling (illustrated at Figure 4 D-F).

**Skeletal remodeling factors in gingival tissue homogenates**

The concentrations of RANKL were higher in the OVX (Figure 5) group than the SHAM group ($p<0.001$), whereas such increased RANKL expression in the ovariectomized rats was abrogated by the administration of estrogen (EST). However, administration of ALE to the ovariectomized rats, either prior to, or

after, osseointegration, did not alter the level of RANKL in gingival tissue in relation to the OVX group. Neither OPG nor OPN showed any significant difference between the SHAM and OVX groups, in contrast to RANKL. Furthermore, administration of EST or ALE after osseointegration did not change the level of OPG or OPN in the gingival tissue of ovariectomized rats. Very interestingly, significant increase of both OPG and OPN could only be observed in peri-implant tissue homogenates from rats receiving ALE administration prior to osseointegration (Figure 5).

**Skeletal remodeling biomarkers in serum**

Figure 6 shows the skeletal remodeling biomarkers measured in the serum isolated from rats at Day 180. Compared to SHAM, the OVX group showed an increasing trend of skeletal remodeling biomarkers: TRAP, RANKL, OPN and OCN concentrations in serum which were abrogated by the administration of estrogen replacement therapy (EST group), suggesting, in turn, the sufficient efficacy of estrogen replacement therapy in the ovariectomized rats. It is noteworthy that statistical significant difference between the OVX group and the SHAM or EST groups was observed only for the concentrations of OPN ($p<0.0001$) and OCN ($p<0.001$).

Both RANKL and OPG are factors that regulate osteoclast-mediated bone resorption, and concentrations of RANKL and OPG in the ALE after and prior to osseointegration groups were significantly higher compared to the remaining groups ($p<0.001$) (Figure 6). However, the total systemic bone turnover involving osteoclastogenesis might be reduced in the groups

receiving ALE because the ratio of RANKL/OPG showed a slight decrease compared to the SHAM, EST and OVX groups not receiving ALE ($p<0.05$, data not shown). All four groups receiving ALE showed a decreased level of TRAP activity compared to the groups not receiving ALE (SHAM, OVX and EST), whereas a significant decrease was only shown in the ALE 1mg/kg prior to osseointegration group compared to the OVX group (Figure 6). ALP activity which, in general, indicates new bone formation mediated by osteoblasts, showed no difference between the groups tested.

Serum concentrations of OPN for the OVX group increased substantively compared to the SHAM and EST groups ($p<0.0001$), but no difference was found between the OVX group and the four groups receiving ALE (Figure 6). On the other hand, the OPN level on gingival tissue was elevated only for the ALE prior to osseointegration groups, suggesting that such elevated concentration is derived from local source rather than systemic origin. Moreover, significantly lower serum concentrations of OCN were found in four groups receiving ALE compared to the other three groups that did not receive alendronate (Figure 6), indicating that alendronate administration attenuated the systemic bone remodeling processes as a result of the suppression of osteoclasts-mediated bone turnover.

**TRAP staining**

The number of multinucleated $TRAP^{+}$ cells present on the surface of medullary cavities of the OVX group was higher than that of the SHAM group (Figure 7A, SHAM; and 7B, OVX). Interestingly, alendronate treated groups

also presented an increased $TRAP^+$ cell compared to the SHAM group (Figure 7C, ALE 50μg after osseointegration; and Figure 7D, ALE 50μg prior to osseointegration). However, these $TRAP^+$ cells in alendronate treated groups were generally round shaped, contained only one or two nuclei, and most of these TRAP+ cells were found in the sites of inflammatory infiltrates. These results might indicate that an increased number of osteoclast-precursors or macrophages (Hayman, 2008) were presented in the peri-implant tissue in the rats receiving alendronate treatment.

**Measurements of serum and gingival tissue level of pro-inflammatory and anti-inflammatory cytokines**

The concentrations of TNF-α, IL-6 and IL-10 in serum were increased in all four tested groups of rats receiving alendronate than the other groups (SHAM, OVX and EST). The concentrations of TNF-α in the gingival tissue showed a trend similar to that of serum TNF-α, i.e., all four tested groups of rats receiving alendronate demonstrated significantly higher TNF- α than the remaining three groups. However, the groups of rats receiving ALE prior to osseointegration (50μg and 1mg) presented significantly higher IL-6 and IL-10 concentrations in gingival tissue than both the non-drug groups (SHAM, OVX and EST) and the groups receiving ALE after osseointegration (Figure 8).

**Immunofluorescent staining of OPN-expressing cells in peri-implant tissue**

The cellular source of OPN was evaluated by double-color immunofluorescent

staining (Figure 9). CD5+ (T lymphocytes) in the inflammatory infiltrates expressed OPN in the rats receiving ALE prior to osseointegration, whereas bone tissue in the same sample expressed little or no OPN. The rats receiving ALE after implant placement showed a few CD5/OPN double-positive cells, while OPN was also expressed in bone tissue. There was no area of peri-implant tissue that contained an infiltration of dense CD5+ cells in SHAM, EST and OVX. However, CD5+ T cells presented in the bone medullary cavities expressed little or no OPN. Such CD5+ T cells are considered to be non-stimulated naïve T cells. In contrast, the CD5+ T cells are activated in the peri-implant tissue of rats that received ALE prior to osseointegration, probably because of the elevated proinflammatory cytokine IL-6 in the tissue (Figure 8), which can activate T cells. Also there were a few cells expressing MHC-class-II (monocyte linage cells; data not shown), that appear to express OPN. Taken together, these immunofluorescent staining results demonstrate that activated CD5+ T cells and monocytes/macrophages rather appeared to express OPN even at areas of necrotic bone tissue of ovariectomized rats receiving ALE prior to osseointegration.

## DISCUSSION

The results of this study demonstrated that peri-implant tissue of ovariectomized rats that had received ALE prior to osseointegration showed a deteriorated osseointegration by causing inflammatory responses accompanied by an insufficient mineralization of the deposited matrix and tissue necrosis. The ovariectomy by itself did not affect the integrity of

implant integration. OPN expressed from activated CD5+ T cells and macrophages appeared to be a key characteristic of this impairment of bone mineralization caused by ALE treatment prior to osseointegration. Although detailed molecular mechanisms underlying the induction of inflammatory response, as well as the tissue necrosis, are still to be elucidated.

The precise functions of most non-collagenous proteins composing bone are not known. The fact that they are deposited into bone at different stages of bone formation may reflect their distinctive functions. For instance, OCN is only present in calcified matrix (Groot et al., 1986), and, furthermore, it is reported that synthesis of OCN does not take place until the mineralization process occurs during the bone regeneration (Kasai et al., 1994; McKee et al., 1992). Therefore, it is plausible to observe that there is an inhibition of the mineralization process at the alendronate treated groups, since OCN expression pattern at these groups is decreased comparing to the other groups of the study. In contrast to OCN, OPN is only incorporated in the bone matrix prior to mineralization (Kasugai et al., 1992; Mark et al., 1988; McKee et al., 1992), and it is probably involved in both cell-matrix adhesion by osteoclasts and the initiation of mineralization (Roach, 1994). OPN deposited in bone matrix is a natural ligand for the αVβ5 integrin expressed on the osteoclasts, facilitating important role to generate the ruffle border during the bone resorption mediated by activated osteoclasts (Reinholt et al., 1990; Rodan and Rodan, 1997). Very interestingly, OPN had decreased expression on bone tissues of alendronate treated groups, whereas the remaining groups showed remarkable staining patterns. While OPN is not expressed in

circulating lymphocytes, it is one of the most copious proteins produced by activated macrophages and T cells, and it is also a potent chemo-attractant stimuli for macrophage recruitment (Scatena et al., 2007), which corroborates with the increased TRAP+ cells were still found in the peri-implant bone tissue of ALE-treated groups.

In addition, OPN appears to regulate the infiltration of macrophages and T cells to inflammatory lesion (Crawford et al., 1998; Steinman, 2009; Weber and Cantor, 1996). Therefore, as noted above, it is conceivable that ALP positive cellular infiltrates found in the peri-implant tissue of ALE prior to osseointegration group may be recruited by locally secreted OPN. Moreover, the increased production of OPN seems to contribute to the inhibition of bone mineralization process, once OPN in the non-mineralized phase is responsible for the regulation of the mineralization by inhibiting calcium oxalate formation (Shiraga et al., 1992).

The skeletal, or bone-specific, isoform of alkaline phosphatase is a tetrameric glycoprotein found on the surface of osteoblast cells. Although bone-specific alkaline phosphatase (BAP) has been accepted as a biochemical indicator of bone turnover, the precise function of BAP is not clearly understood. Interestingly, while there was no difference in the serum level of ALP in any of the groups of rats tested in this study, remarkable staining of ALP-positive cells was observed in the inflammatory infiltrations in the ALE prior to osseointegration groups. Both BAP and ALP are expressed by osteoblasts, but ALP is also expressed by many other cells, including hepatocytes, intestinal

epithelial cells, macrophages and neutrophils. Thus, the ALP-positive cells found in the inflammatory infiltrations of the alendronate treated groups are considered to be activated neutrophils or macrophages.

TNF-α super-family molecules, such as TNF-α and RANKL, are considered to play critical roles in inflammatory bone resorption diseases (Gravallese, 2002; Kwak et al., 2005). TNF-α is one of the most potent tissue-destructive cytokines produced from T cells and monocyte lineage cells in inflammation. Activated T cells express both membrane-bound and soluble forms of receptor activator of NF-kappaB ligand (RANKL) (Gravallese, 2002; Kawai et al., 2006; Valverde et al., 2004) that up-regulate bone resorption by the differentiation and activation of osteoclasts. In the present study, all groups of rats that received ALE demonstrated similar expression patterns of TNF-a and RANKL in the peri-implant tissue. However, similar to OPN, the concentrations of OPG in the peri-implant gingival tissue were elevated in a manner specific to ALE pre-treatment (Figure 5). Osteoprotegerin (OPG) plays a critical role in bone remodeling by neutralizing the effect of RANKL on differentiation and activation of osteoclasts (Boyle et al., 2003; Simonet et al., 1997). Therefore, the elevated amount of OPG appeared to reduce the RANKL/OPG ratio in the peri-implant tissue of ALE prior to osseointegration groups (data not shown), which may, in turn, down-regulate the bone remodeling processes. In terms of role of OPG in the context of inflammation, although it is reported that macrophages and mesenchymal cells produce osteoprotegerin (OPG), there are little or no reports demonstrating that OPG can contribute to the disuse destruction processes.

In conclusion, the present study demonstrates that 1) pre-administration of alendronate to ovariectomized rats can inhibit implant osseointegration processes, 2) such deteriorated osseointegration is followed by necrosis of peri-implant tissue and inflammatory cell infiltration, 3) increased production of osteopontin appeared to be the key characteristic of alendronate mediated inhibition of dental implant integration. Since the incidence of osteoporosis is in the increasing trend, it is expected that the use of bisphosphonates will also increase. Thus, these findings offer insight into the pathogenic mechanism(s) underlying BONJ induced in relation to dental implant placement especially in subjects under bisphosphonate treatment.

## ACKNOWLEDGMENTS

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**Table 1.** Primary antibody used for immunohistochemistry

| Primary antibody | Isotype | Dilution | Producer |
|---|---|---|---|
| Osteocalcin (OCN) | Rabbit polyclonal | 1:100 | Santa Cruz Biotech. |
| Osteopontin (OPN) | Rabbit polyclonal | 1:100 | Abcam Inc. |
| Alkaline phosphatase (ALP) | Rabbit IgG | 1:50 | Sigma Aldrich |

**Figure 1.** Experimental protocol of the study.

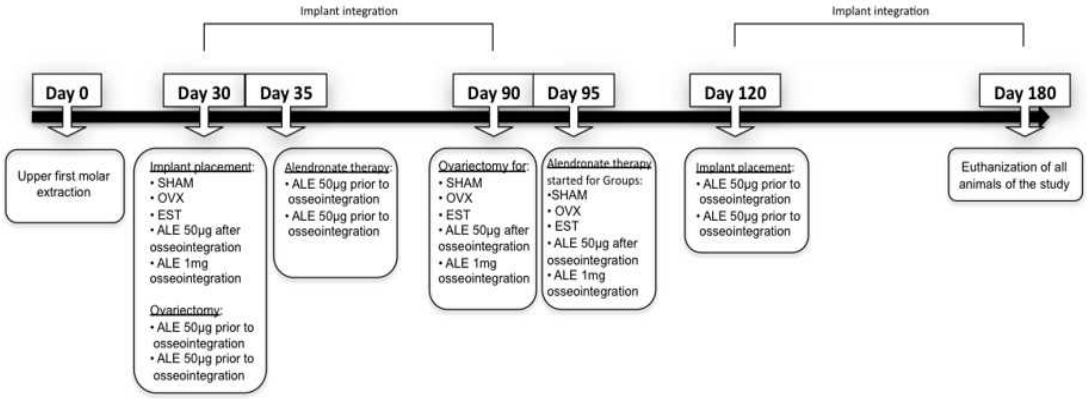


**Figure 2.** Representative photomicrographs from the necrotic area presented by the alendronate treated groups: (A, C and G) corresponding to ALE 1mg prior to osseointegration; (B and D) related to ALE 50µg prior to osseointegration; (E) from ALE 50µg after osseointegration and (F) from ALE 1mg after osseointegration. Slides were hematoxylin counterstained; (A-D) x200 magnification and (E-G) x400 magnification.

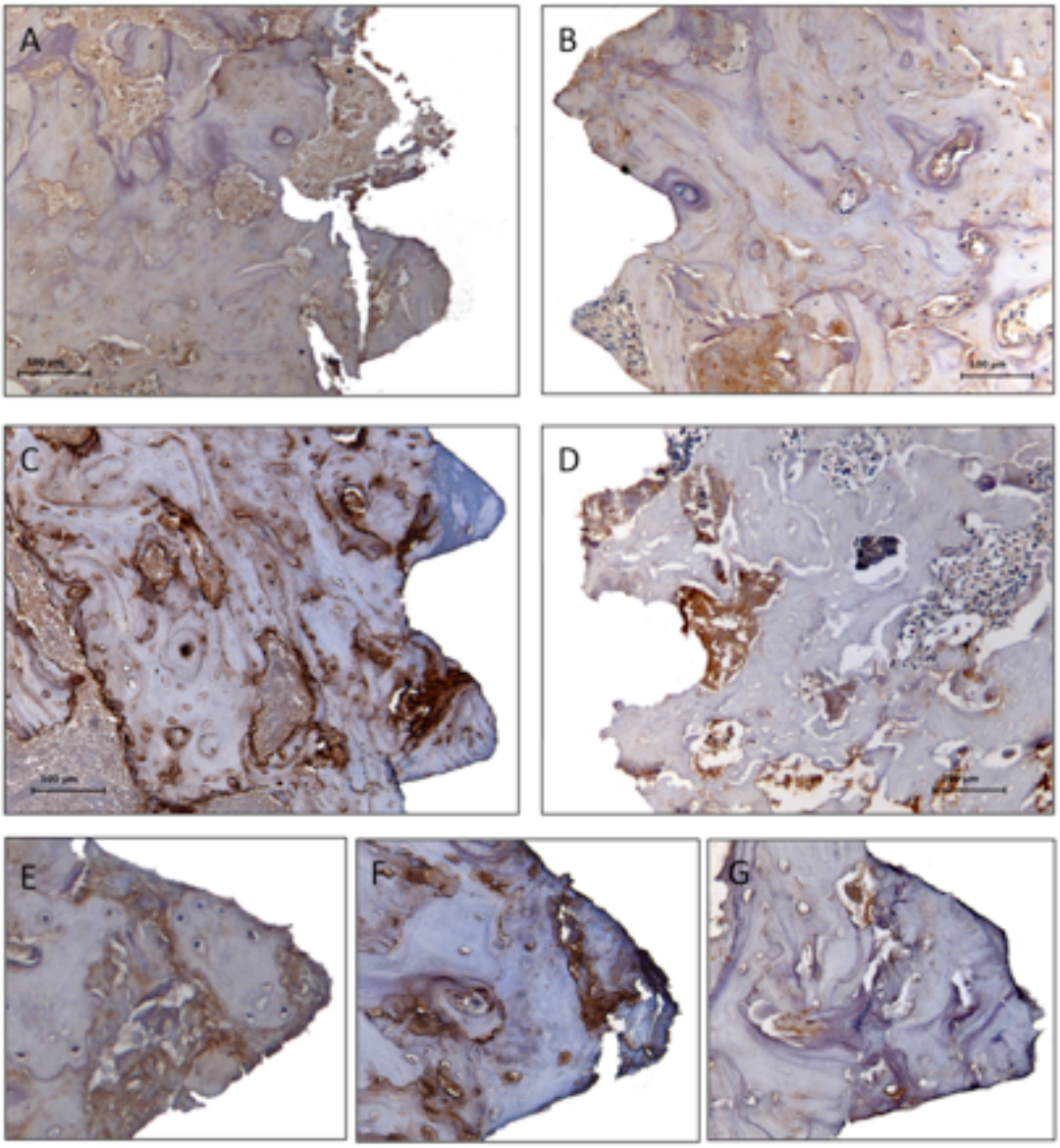


**Figure 3.** Representative photomicrographs of the immunohistochemical reaction patterns for the parameters involved on bone formation on the threads of the implants.

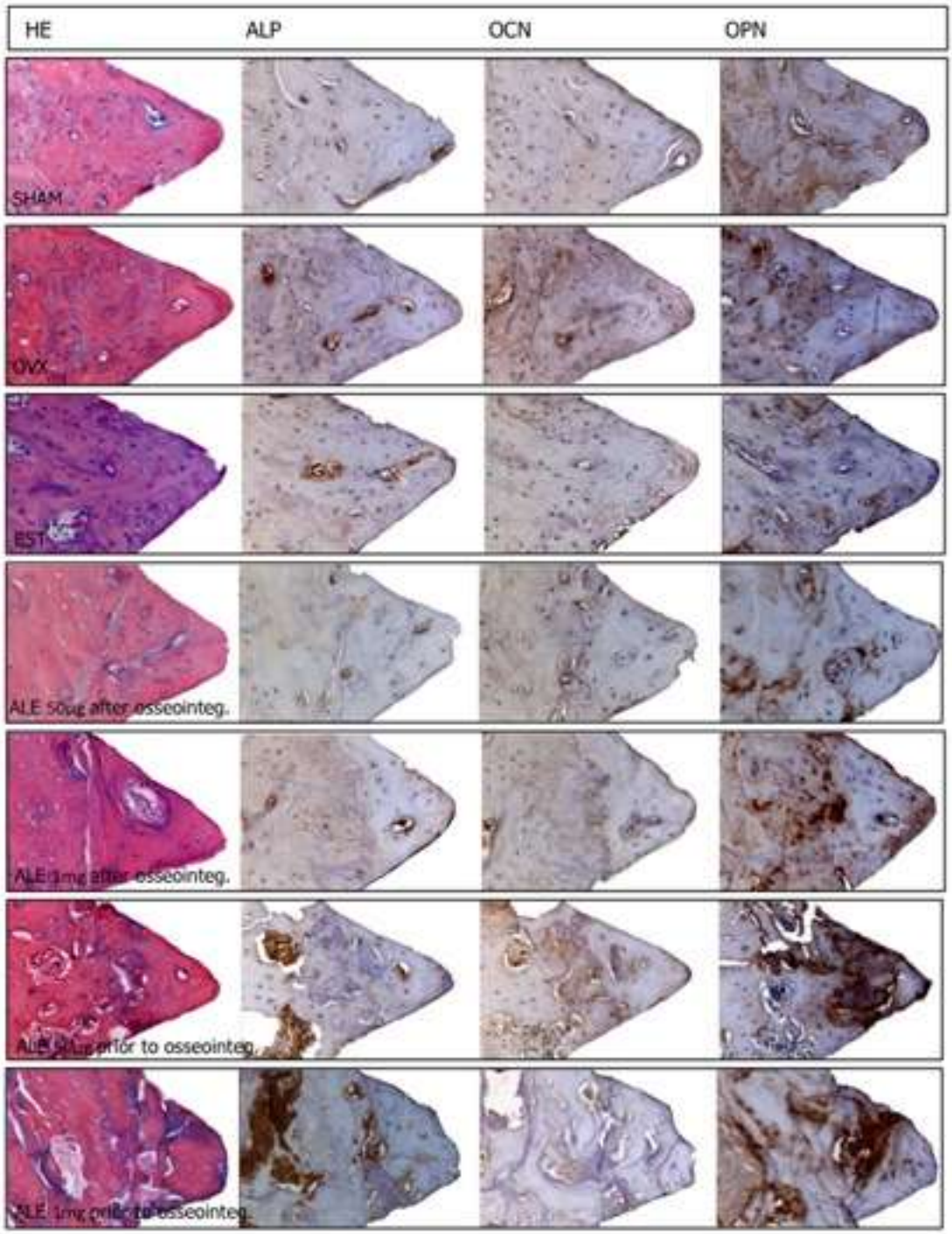


**Figure 4.** Representation for immunohistochemistry reaction against OCN for (A) SHAM, (B) ALE 1mg after osseointegration and (C) ALE 1mg prior to osseointegration and distribution of osteopontin-immunopositive cement lines representative for (D) SHAM (E) ALE after osseointegration and (F) ALE prior to osseointegration.

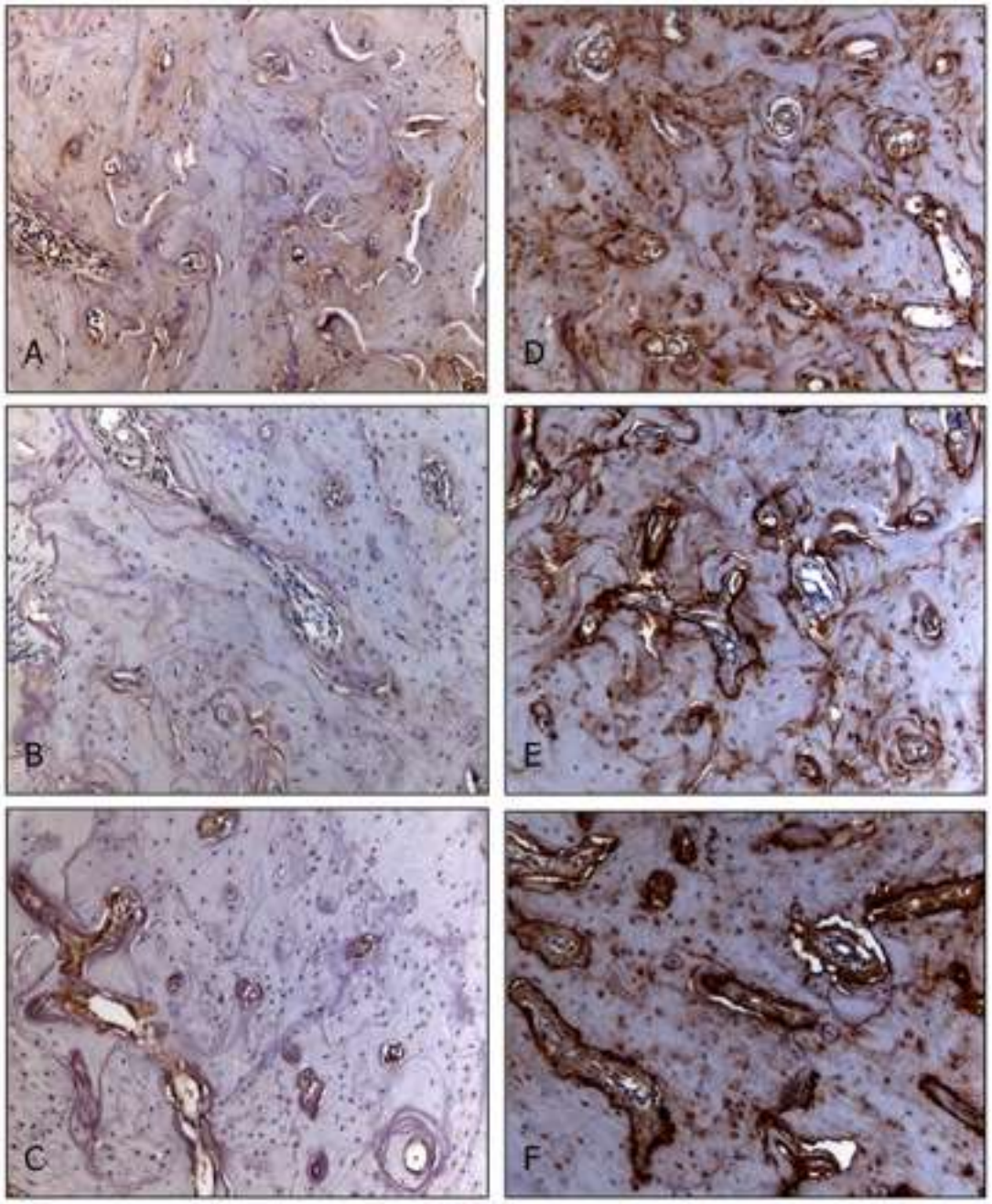


**Figure 5.** Biomarkers concentration on gingival tissue homogenates for all groups of the study. *p<0.001 in relation to the remaining groups of the study.

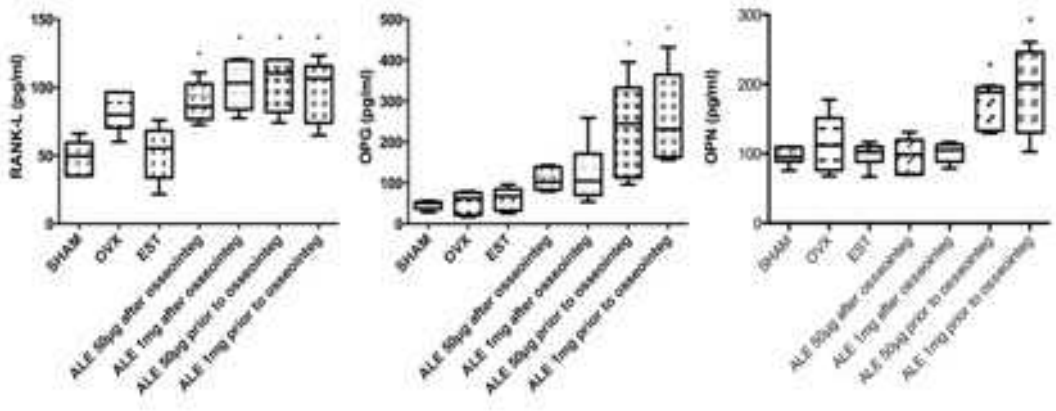


**Figure 6.** Serum concentrations for TRAP and ALP activity, RANKL, OPG, OPN and OCN for all groups of the study.

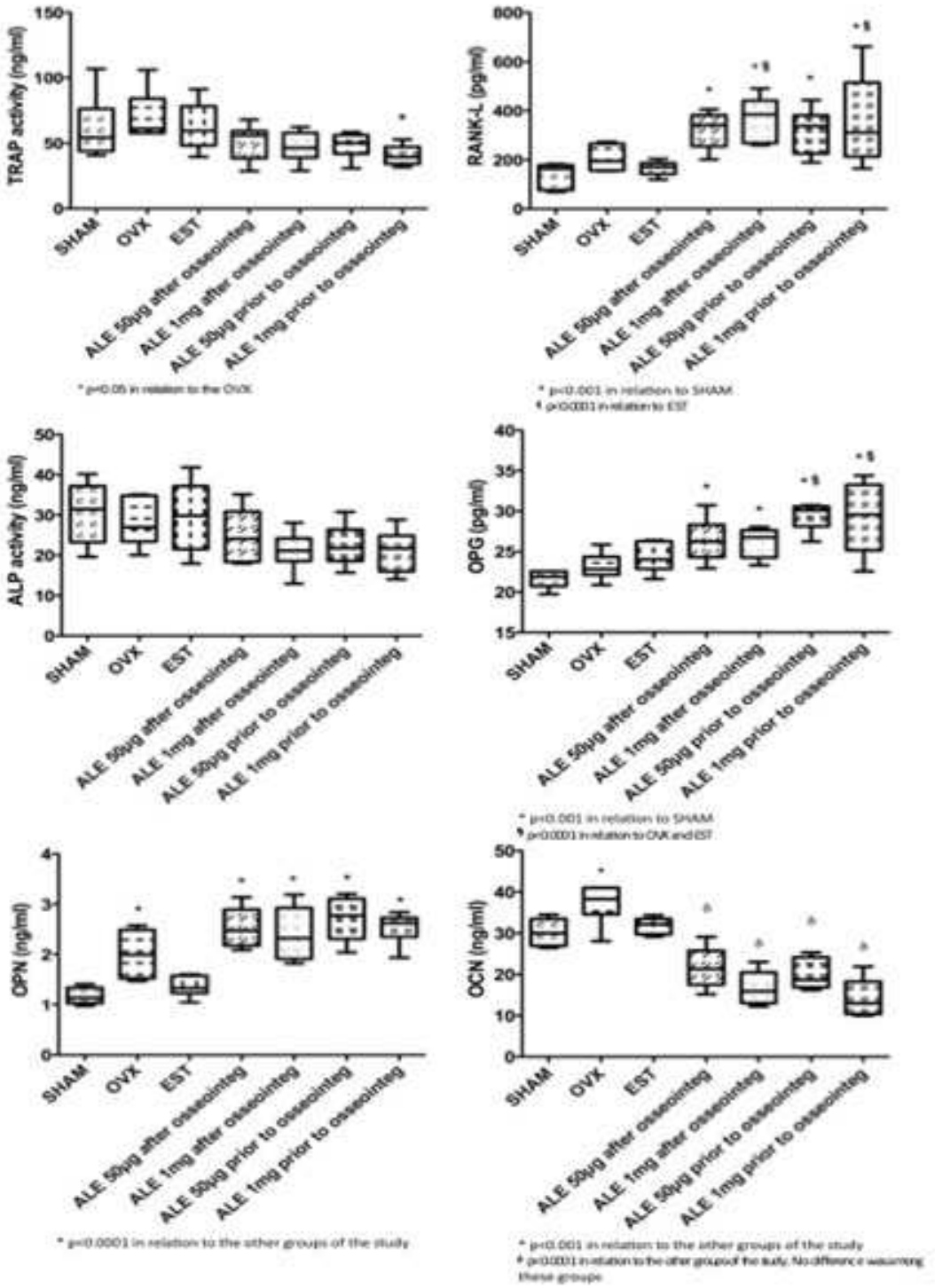


**Figure 7.** TRAP activity photomicrographs from (A) SHAM, (B) OVX, (C) ALE 50µg after osseointegration and (D) ALE 50µg prior to osseointegration. 200x magnification, hematoxylin counterstained.

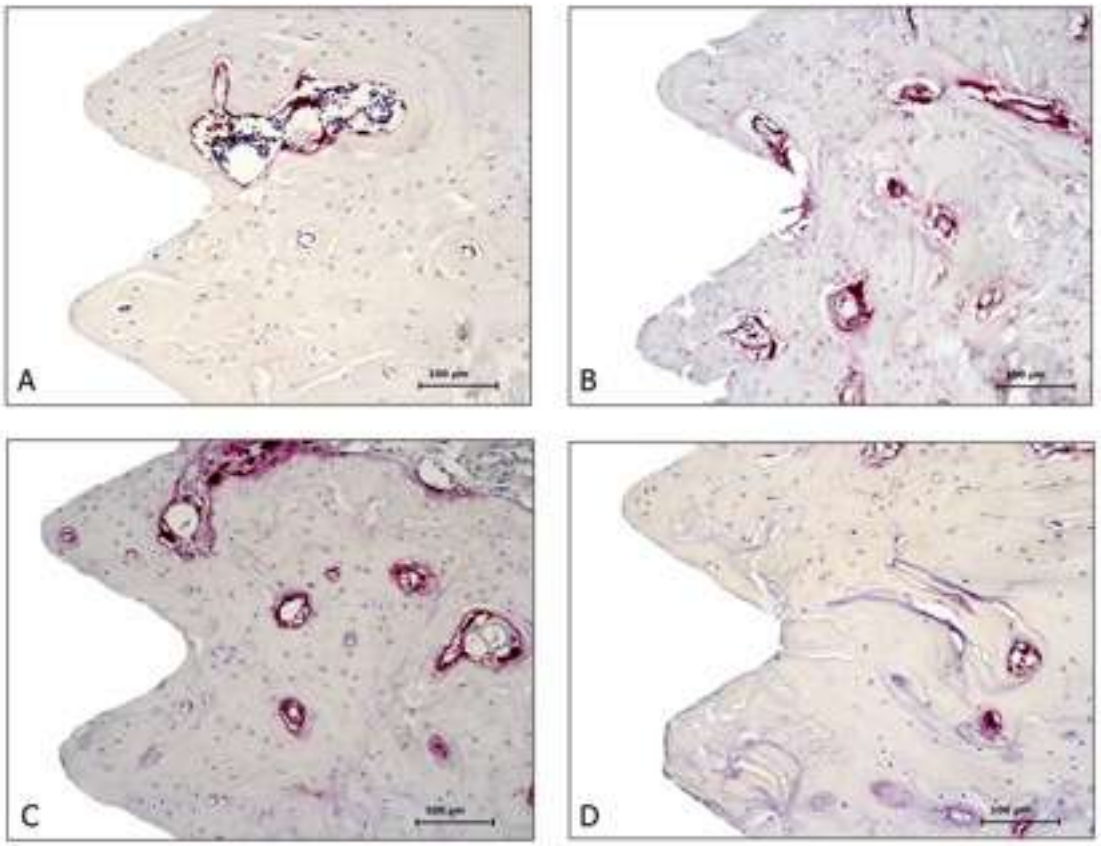


**Figure 8.** Comparison of the pro-inflammatory and anti-inflammatory cytokines on (A) serum and (B) gingival tissue between the different groups of the study.

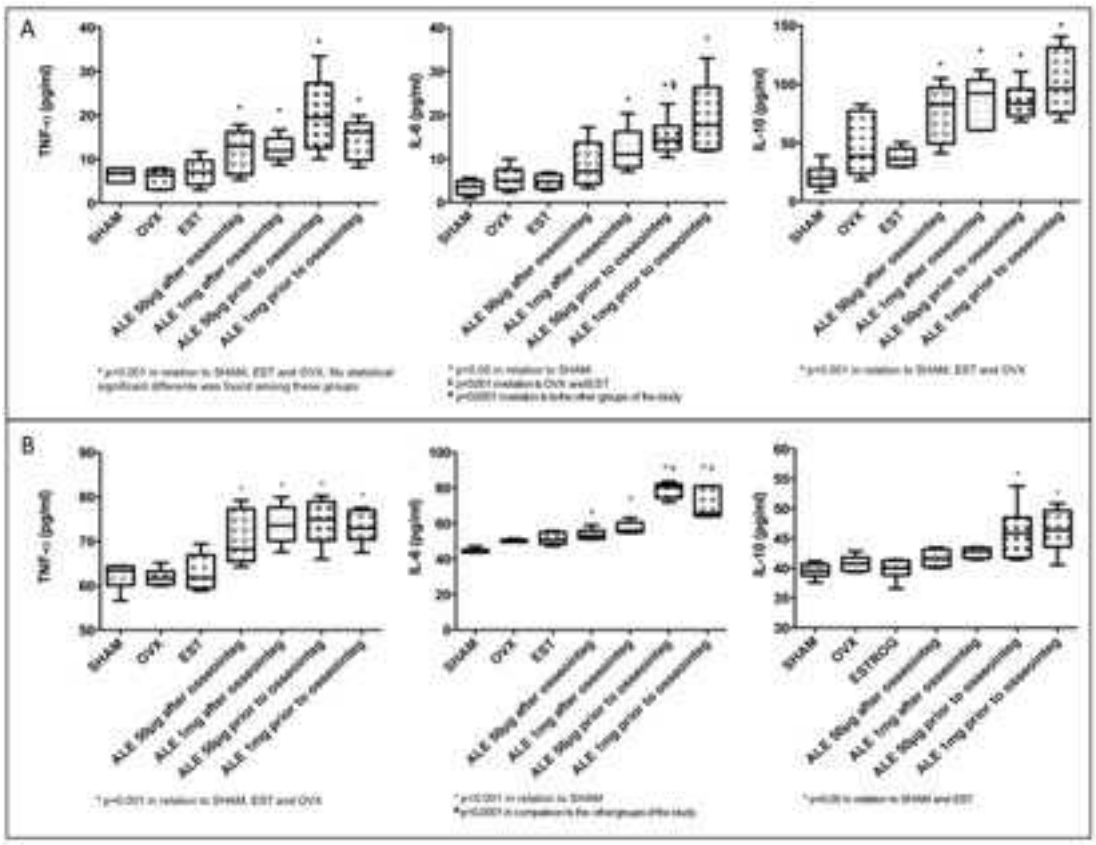
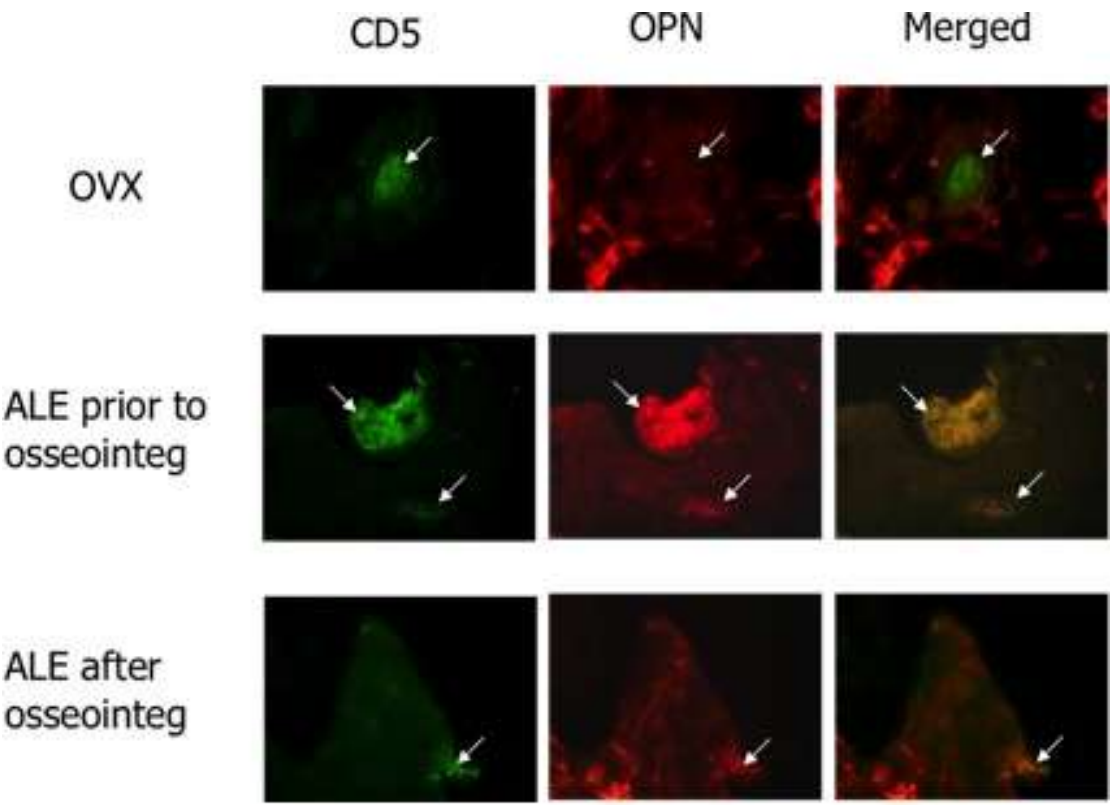
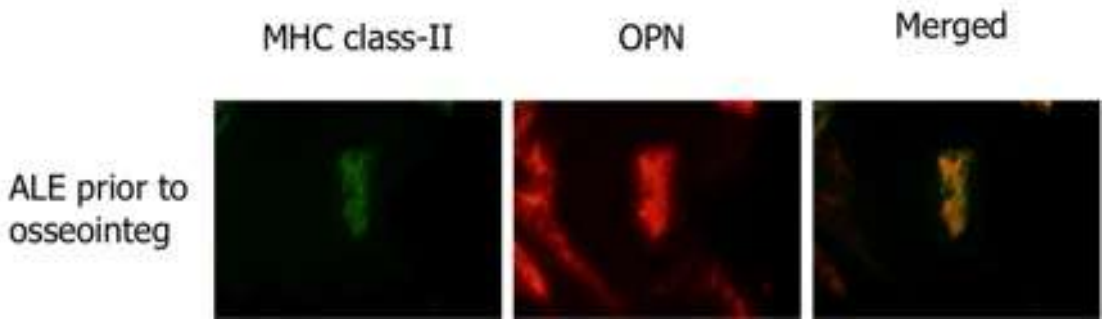


**Figure 9.** Immunofluorescent staining of OPN-expressing cells on bone tissue surrounding implants.



**Supplement results.** Double positive stained cells expressing MHC-class-II and osteopontin.



# Discussão Geral

O objetivo desse projeto, a princípio, foi avaliar os efeitos deletérios da deficiência estrogênica sobre a manutenção de implantes que se encontravam osseointegrados, previamente à indução do desafio sistêmico, uma vez que os relatos na literatura com relação a esse tópico eram escassos e bastante controversos. Alguns estudos demonstraram que a colocação de implantes em tíbias de ratas ovariectomizadas foi associada a uma diminuição do contato osso/implante e da densidade do tecido ósseo, principalmente em regiões de osso medular, demonstrando que a deficiência de estrógeno é capaz de gerar prejuízos ao tecido ósseo ao redor de implantes (Duarte et al.[13], 2003; Pan et al.[42], 2000; Yamazaki et al.[70], 1999). Porém, o presente estudo foi o primeiro a demonstrar as implicações da deficiência estrogênica e das terapias propostas sobre o tecido ósseo ao redor de implantes osseointegrados.

Utilizando-se o modelo de ovariectomia em ratas, com o intuito de mimetizar o período pós-menopausa em humanos, a deficiência estrogênica foi induzida, levando consequentemente à perda de massa óssea sistêmica evidenciada pela análise da densidade mineral óssea (BMD), exame padrão para o diagnóstico de osteoporose.

A avaliação da osseointegração foi realizada em implantes inseridos nas tíbias dos animais. Como o objetivo principal do estudo foi avaliar os efeitos da condição sistêmica sobre implantes osseointegrados, utilizamos um grupo controle da osseointegração (CTL), a fim de comprovar a completa reparação do tecido ósseo ao redor dos implantes após um período de 60 dias. Esse período foi escolhido baseado em resultados anteriores que

mostraram a ausência de diferença em relação às características biomecânicas e quantidade de osso formado, quando comparados períodos de 2 e 5 meses de osseointegração.

Em relação ao grupo OVX, foi demonstrado que a ovariectomia estabeleceu o envolvimento sistêmico esperado, confirmado pela diminuição da densidade mineral óssea sistêmica nesse animais. Em relação ao tecido ósseo ao redor dos implantes, os resultados demonstraram que o desequilíbrio sistêmico influenciou na manutenção desse tecido. As propriedades biomecânicas foram alteradas, com diminuição da força necessária para a ruptura da interface osso/implante (torque de remoção), concordando com os resultado obtidos por Ozawa et al.[41] (2002) em que a deficiência hormonal resultou em redução das propriedades biomecânicas, do percentual de contato osso/implante e do preenchimento do espaço entre as roscas por tecido ósseo. Além disso, animais ovariectomizados sem nenhum outro tratamento apresentaram menor taxa de aposição mineral, mostrando o desequilíbrio da homeostase óssea, com ambos os marcadores de formação e reabsorção óssea aumentados. Apesar de todas as evidências encontradas corroborarem outros estudos em animais (Chow et al.[10], 1992; Duarte et al.[14], 2005; Lugero et al.[26], 2000; Narai, Nagahata[37] 2003; Sims et al.[54], 1996; Wronski et al.[68], 1988), um aspecto que deve ser levado em consideração previamente à extrapolação desses resultados para a prática clinica, é o de que os implantes não foram submetidos à ação de cargas. A implicação das forças mecânicas atuando de modo protetor sobre a perda

óssea associada à privação estrogênica foi verificada tanto em relatos de casos como em estudos clínicos longitudinais, realizados em pacientes com diagnóstico de osteoporose e que possuíam implantes dentais, demonstrando a ausência de diferença quanto à quantidade e morfologia do tecido ósseo em comparação a pacientes saudáveis (Becker et al.[7], 2000; Friberg et al.[18], 2001; Shibli et al.[52], 2008).

Em relação aos tratamentos selecionados para controle dos efeitos deletérios da supressão hormonal, deve-se esclarecer que a terapia de reposição hormonal é a primeira escolha e a mais utilizada para a prevenção e tratamento da osteoporose. A seleção do alendronato, um bifosfonato nitrogenado de 3ª geração, deu-se ao fato deste ser uma droga rotineiramente empregada no tratamento da osteoporose, devido a sua capacidade de inibir a perda óssea (Rodan[45], 1998), sendo considerada umas das drogas com maior numero de prescrição nos EUA (Assael[5], 2009) e uma alternativa aos estrógenos, os quais são relacionados, entre outros efeitos colaterais, ao desenvolvimento de câncer de endométrio (Rossouw et al.[46], 2002).

Ambos os tratamentos propostos neste estudo mostraram-se eficientes na inibição da perda de massa óssea. A reposição hormonal pelo estradiol não resultou em diferença estatisticamente significante em relação ao grupo SHAM para os parâmetros estudados. Em contrapartida, o alendronato promoveu um aumento significativo da BMD nas regiões analisadas (fêmur e vértebras lombares), principalmente quando comparado ao grupo OVX, concordando com os relatos encontrados na literatura

(Andersson et al.[3], 2002; Andersson et al.[4], 2004), que demonstraram que o alendronato promove uma elevação da massa óssea, devido à diminuição da remodelação, com conseqüente aumento do volume trabecular e do número de traves ósseas (da Paz et al.[43], 2001). A utilização desse medicamento mostrou-se efetivo na prevenção dos efeitos deletérios da deficiência estrogênica com relação aos implantes, uma vez que foi mantida a quantidade de tecido ósseo presente em contato com a superfície e entre as roscas do implante, além do aumento do torque de remoção desses implantes. Esses resultados estão de acordo com aqueles encontrados por Duarte et al.[14] (2005) e Narai, Nagahata[37] (2003) que demonstraram, por meio de análise histométrica e biomecânica, que os animais tratados com alendronato possuíam maior força necessária para a remoção dos implantes, maior área de contato osso/implante, maior área de osso entre as roscas e maior densidade óssea em uma área lateral à superfície do implante.

Por outro lado, a abrupta diminuição da concentração sérica de osteocalcina e deoxipiridinolina, importantes indicadores de formação e reabsorção óssea, respectivamente, somados à diminuição da taxa de aposição mineral desses animais, alertaram para um possível efeito indesejável da supressão da remodelação óssea, característica dessa classe de medicamentos. Foi, então, levantada a hipótese de que pacientes em uso crônico dessa droga poderiam apresentar dificuldades de reparação do tecido ósseo frente à instalação de implantes, uma vez que o processo de osseointegração parte do princípio de reabsorção inicial do tecido ósseo

acometido pela fresagem e posterior neoformação, levando à estabilização desses implantes (Albrektsson, Johansson[1] 2001).

Frente aos questionamentos levantados, novos estudos foram propostos, envolvendo basicamente três idéias, com o objetivo de avaliar: a influência da carga mastigatória sobre os efeitos deletérios da deficiência estrogênica, a influência da administração do alendronato sobre a qualidade do tecido ósseo ao redor de implantes osseointegrados e a qualidade da osseointegração em animais sob terapia com alendronato.

Contrariando os achados previamente descritos, os implantes instalados na maxila de ratas, sob função, não apresentaram diferença em relação aos parâmetros histométricos avaliados (contato osso/implante e fração da área de preenchimento das roscas), apesar das ratas ovariectomizadas apresentarem diminuição da densidade óssea sistêmica e alteração dos marcadores de formação (osteocalcina, fosfatase alcalina e osteoprotegerina) e reabsorção óssea (peptídeo C-terminal, fosfatase ácida resistente ao tartarato e RANKL). Esses resultados vêm de encontro aos achados de estudos clínicos em pacientes saudáveis e osteoporóticos sugerindo que a deficiência estrogênica não é um fator de risco para a manutenção de implantes osseointegrados, desde que estes estejam em função.

Na avaliação dos animais submetidos ao tratamento com alendronato após a osseointegração dos implantes, apesar desses animais não apresentarem diferença estatisticamente significante em relação ao grupo SHAM, OVX e EST para os parâmetros histométricos avaliados, foi constatada

a escassez de linhas de reversão, indicativas de remodelamento ósseo, e maior número de lacunas sem osteócitos na área ao redor do implante, com uma tendência de acúmulo dessas lacunas sem células viáveis, enquanto os demais grupos citados apresentaram uma distribuição uniforme das mesmas. Um achado interessante em relação a esse grupo diz respeito ao aumento significativo de células mononucleares TRAP positivas, sugerindo um maior recrutamento de macrófagos para a área ao redor dos implantes e dentes adjacentes, provavelmente ativados pela presença da medicação (Toyras et al.[60], 2003), corroborando com o aumento da expressão de osteopontina, IL-6 e TNF-α, marcadores também produzidos por macrófagos.

Para os animais que receberam a administração de alendronato previamente à colocação dos implantes foram evidenciados importantes resultados, que alertam para o cuidado na instalação de implantes em pacientes sob uso crônico da droga. Os achados desses grupos caracterizaram-se pela diminuição acentuada da área de contato osso/implante e do percentual de área entre as roscas preenchida por tecido ósseo, além do acúmulo de lacunas sem osteócitos, sendo que grande parte dos animais apresentou extensas áreas de necrose ao redor dos implantes. É importante ressaltar que os quadros de necrose ocorridos neste estudo são sugestivos de complicação tardia, uma vez que apesar de observada a deposição da matriz óssea, em alguns casos esta apresentou características de matriz amorfa, sem a presença de células ou organização tecidual condizente.

Com relação às dosagens empregadas neste projeto, a posologia de 1mg/kg/semana foi escolhida por ser comparativa à utilizada em seres humanos para o tratamento da osteoporose, enquanto o esquema terapêutico de 50µg/kg em dias alternados foi escolhido por ter sido empregado anteriormente na literatura (Andersson et al.[3], 2002). Além disso, por ser uma dose quatro vezes menor do que a empregada no tratamento da osteoporose em humanos, seu uso teve também o objetivo de constatar se os efeitos colaterais inerentes a essa classe de medicamentos poderiam ser amenizados com a diminuição da dosagem empregada.

Embora possa ser levantada a hipótese que os piores resultados encontrados para os grupos ALE antes da osseointegração estejam relacionados a um maior período de tratamento com a droga e a um menor tempo de osseointegração, quando comparados aos outros grupos de administração do alendronato, deve-se lembrar que uma vez que o tecido encontra-se necrosado este perde a capacidade de reparação, não tendo portanto a possibilidade de adquirir características próximas ao tecido normal em um curto período de tempo. Isso, portanto, excluiria o questionamento sobre a avaliação do tecido ósseo ao redor dos implantes em um maior período de osseointegração. Além disso, nossos estudos iniciais demonstraram a ausência de diferença quanto às características do tecido ósseo quando comparados os períodos de reparação de dois e cinco meses de instalação dos implantes.

Quanto ao fato do maior tempo de administração da droga, deve-se ressaltar que a principal questão levantada foi o efeito da

administração da medicação por um tempo relativamente longo, com a manutenção desta durante a instalação e osseointegração dos implantes, sendo este um questionamento até agora inédito em experimentos com ratos.

# Conclusão

Baseados nas metodologias utilizadas, a análise dos resultados apresentados e discutidos nos estudos anteriormente citados, sugerem que:

⇒ A deficiência estrogênica não pode ser considerado um fator de risco para a manutenção de implantes que encontram-se osseointegrados e em função na cavidade oral;

⇒ A reposição hormonal com estradiol mostrou-se bastante eficiente no manutenção do equilíbrio do tecido ósseo, uma vez que nenhuma alteração dos parâmetros envolvidos nos estudos em questão foi observada nesses animais;

⇒ A administração do alendronato após o período de osseointegração dos implantes mostrou-se efetivo na prevenção dos efeitos deletérios da deficiência estrogênica, uma vez que foram mantidos a quantidade de tecido ósseo e aumentada a qualidade biomecânica desse tecido. Entretanto, o estudo mais aprofundado das características morfológicas do tecido ósseo mostra um ressalva em relação ao acompanhamento cauteloso dos pacientes que receberam implantes dentários e tenham que ser submetidos ao tratamento com qualquer tipo de bifosfonato;

⇒ A terapia com alendronato pode ser considerada com grande fator de risco ao insucesso da terapia com implantes, extrapolando para a o risco em

relação à ocorrência da osteonecrose dos maxilares ligadas ao tratamento com bifosfonatos, uma vez que sérias alterações foram observadas nesses animais.

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# Anexos

# UNIVERSIDADE ESTADUAL PAULISTA
## Faculdade de Odontologia
## Campus de Araraquara

## Comitê de Ética em Experimentação Animal-CEEA

# CERTIFICADO

Certicamos que o protocolo nr. *15/2004* referente à pesquisa *"Avaliação da influência da osteoporose induzida e seu tratamento com estrógeno e alendronato sobre o tecido ósseo ao redor de implantes de ti cp com osseointegração estabelecida. Estudo radiográfico, biomecânico e histométrico em ratas"* sob a responsabilidade de *Gabriela Giro* e *Silvana Regina Perez Orrico* está de acordo com os Princípios Éticos em Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pelo Comitê de Ética em Experimentação Animal (CEEA) da Faculdade de Odontologia de Araraquara-UNESP em reunião de *28/agosto/2007.*

# CERTIFICATE

We certify that the protocol *15/2004* referring to the research *"Evaluation of the influence of induced osteoporosis and its treatment with estrogen and alendronate on the bone around titanium implants with established osseointegration. Radiological, biomechanical and histometric study in rats"* under responsibility of *Gabriela Giro* and *Silvana Regina Perez Orrico* is in agreement with the Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the Araraquara Dental School-UNESP Ethical Committee for Animal Research (CEEA) in *August 28, 2007.*

Araraquara, 28 de agosto de 2007

*Profa. Dra. Maria Rita Brancini de Oliveira*
Coordenadora do CEEA/FOAr/UNESP

Araraquara, 17 de abril de 2007

Senhores Pesquisadores:

O Comitê de Ética em Experimentação Animal-CEEA desta Faculdade reunido em 16*/04/2007*, após a avaliação do projeto de sua responsabilidade intitulado *"Avaliação dos efeitos da terapia com alendronato de sódio sobre o tecido ósseo ao redor de implantes"* (Proc. CEEA nº 02/2007) **AUTORIZA** a realização da pesquisa, ficando a aprovação vinculada à apresentação do **RELATÓRIO FINAL** em **DEZEMBRO/2009.**

Atenciosamente.

***Profa. Dra. Maria Rita Brancini de Oliveira***
*Coordenadora do CEEA*

Ao
***Profª Drª SILVANA REGINA PEREZ ORRICO***
DD. Pesquisador Responsável
*Prof. Dr. Élcio Marcantonio Junior*
*C.D. Gabriela Giro*
Roberto Sales e Pessoa
Nesta

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# Effect of 17beta-estradiol and alendronate on the removal torque of osseointegrated titanium implants in ovariectomized rats.

Giro G, Sakakura CE, Gonçalves D, Pereira RM, Marcantonio E Jr, Orrico SR.

Department of Oral Diagnosis and Surgery, School of Dentistry of Araraquara, State University of São Paulo, Araraquara, SP, Brazil.

BACKGROUND: This study investigated the influence of estrogen deficiency and its treatment with estrogen and alendronate on the removal torque of osseointegrated titanium implants. METHODS: Fifty-eight female Wistar rats received a titanium implant in the tibia metaphysis. After 60 days, which was needed for implant osseointegration, the animals were randomly divided into five groups: control (CTLE; N = 10), sham surgery (SHAM; N = 12), ovariectomy (OVX; N = 12), ovariectomy followed by hormone replacement (EST; N = 12), and ovariectomy followed by treatment with alendronate (ALE; N = 12). The CTLE group was sacrificed to confirm osseointegration, whereas the remaining groups were submitted to sham surgery or ovariectomy according to their designations. After 90 days, these animals were also sacrificed. Densitometry of femur and lumbar vertebrae was performed by dual-energy x-ray absorptiometry (DXA) to confirm systemic impairment of the animals. All implants were subjected to removal torque. RESULTS: Densitometric analysis of the femur and lumbar vertebrae confirmed a systemic impairment of the animals, disclosing lower values of bone mineral density for OVX. Analysis of the removal torque of the implants showed statistically lower values ($P < 0.05$) for the OVX group in relation to the other groups. However, the group treated with alendronate (ALE group) presented significantly higher torque values compared to the others. CONCLUSION: According to this study, estrogen deficiency was observed to have a negative influence on the removal torque of osseointegrated implants, whereas treatment with alendronate increased the torque needed to remove the implants.

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| **Corresponding Author** | Gabriela Giro (UNESP - Univ Estadual Paulista) |
| **Contributing Authors** | Paulo Coelho , Roberto Pessoa , Rosa Pereira , Elcio Marcatonio Junior , Toshihisa Kawai , Silvana Orrico |
| **Abstract** | Bisphosphonates are widely used in osteoporosis treatment and have been accounted for bone remodeling suppression, potentially affecting the osseointegration of dental implants. This investigation tested the hypothesis that significant differences in biomechanical/histometric parameters would be observed for implants placed before and after the systemic administration of alendronate in a rat model. The following experimental groups were evaluated: animals that received systemic alendronate prior to implant placement, animals that received the treatment after implant osseointegration was established, and a control group (implant placement only). The implants were subjected to biomechanical testing and histologic/metric evaluation. Significant lower biomechanical and histometric measurable parameters were observed for the group that received alendronate prior to implant placement relative to the control group, resulting in partial hypothesis validation. |
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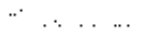

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