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FACULDADE DE ODONTOLOGIA DE ARARAQUARA

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EFEITO DA DEFICIÊNCIA DE MAGNÉSIO SOBRE O METABOLISMO DO TECIDO ÓSSEO

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EFEITO DA DEFICIÊNCIA DE MAGNÉSIO SOBRE O METABOLISMO DO TECIDO ÓSSEO

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RESUMO

Belluci MM. Efeito da deficiência de magnésio sobre o metabolismo do tecido ósseo. [Tese de Doutorado]. Araraquara: Faculdade de Odontologia UNESP; 2012.

O magnésio (Mg^{+2}) é um mineral que possui um importante papel na homeostase mineral, podendo afetar diretamente a função das células ósseas e a formação da hidroxiapatita. Entretanto, até o presente momento existem poucos estudos avaliando os mecanismos relacionados à alteração do metabolismo ósseo frente à deficiência de Mg.

O objetivo desse estudo foi avaliar, in vitro, o efeito da deficiência de magnésio sobre a osteoclastogênese e atividade de osteoclastos. Também foi objetivo avaliar, in vivo, o efeito da deficiência de magnésio na dieta sobre o metabolismo ósseo, doença periodontal e tecido ósseo ao redor de implantes com osseointegração estabelecida.

Para o estudo in vitro, foram avaliadas células indiferenciadas da medula óssea de ossos longos e mandíbula de camundongos. As células foram cultivadas em plástico e sobre tecido ósseo, em meios de cultura com diferentes concentrações de Mg (0.8 Mm que é considerada a quantidade adequada de Mg; e meios com redução do Mg, 0.4 Mm; 0.08 Mm; e 0 Mm). Após 3 dias de cultura, foram avaliadas a viabilidade celular, a taxa de proliferação celular e a expressão gênica, por PCR, de genes relacionadas à osteoclastogênese (c-fms, RANK, DC STAMP, c-fos, TNF-α e IL-1β) e ao metabolismo do Mg (TRPM-7 e MRS2). Após 6 dias de cultura, foram avaliados os parâmetros: número de células osteoclásticas (coloração de TRACP), atividade (liberação de TRACP) e taxa de proliferação celular. Após 8 dias de cultura a atividade dos osteoclastos foi avaliada por análise de pit de reabsorção. Para o estudo in vivo, foram utilizados 30 ratos, divididos em 2 grupos: grupo controle (CTL), que recebeu dieta com conteúdo adequado de magnésio, e grupo Mg, que recebeu dieta com redução de 90% da necessidade diária do mineral. Após um período de adequação ao ambiente do biotério, todos os animais

foram submetidos à cirurgia de instalação de um implante na tíbia direita (dia 0). Decorrido um período de 60 dias, necessário à osseointegração dos implantes, os animais de cada grupo foram aleatoriamente divididos conforme a dieta que receberam (grupo CTL e grupo Mg). Após um período de 60 dias, todos os animais receberam uma ligadura no primeiro molar inferior esquerdo. Os animais foram sacrificados, com aprofundamento da anestesia, após um período de 150 dias do início do experimento. No dia do sacrifício foram coletadas amostras de sangue e urina, para avaliação das concentrações urinária de creatinina e deoxipiridinolina (DPD) e sérica de cálcio (Ca), magnésio (Mg), osteocalcina (OCN) e paratormônio (PTH). Para avaliação sistêmica foram realizadas análises de densitometria óssea (DEXA) do fêmur e vértebras lombares além da análise histológica do fêmur. Para avaliação do tecido mandibular foram realizadas análise da densidade radiográfica, análise histológica, análise macroscópica e PCR (RANKL, OPG e IL-6). E finalmente, para avaliação do tecido ósseo ao redor dos implantes foram realizadas análises de densidade radiográfica, torque de remoção e análise histométrica.

Como resultados foram observadas, no experimento in vitro, um aumento na quantidade e tamanho dos osteoclastos, provenientes de ossos longos e mandíbula, quando cultivados em meio com deficiência de Mg. A maior quantidade de osteoclastos pode ser devido ao aumento na expressão gênica de c-fos, DC-STAMP, TNF-α e IL-1β. Não houve alteração na viabilidade e taxa de proliferação celular na deficiência de Mg. Entretanto houve uma diminuição na atividade osteoclástica na deficiência de Mg. Os estudos in vivo demonstraram a deficiência de Mg resultou em diminuição da concentração sérica de Mg. Foi evidenciado que a deficiência do mineral teve uma influência negativa sobre o tecido ósseo, comprovada pela diminuição da densidade do fêmur e vértebras lombares, aumento na concentração de DPD e de PTH. Em análises específicas da mandíbula foi observada a diminuição na densidade radiográfica e o aumento na

perda óssea alveolar relacionada à indução de doença periodontal. Nas análises específicas dos sítios de instalação dos implantes, apesar de não ter sido observada alteração na quantidade de tecido ósseo ao redor dos implantes, foi constatada a redução na espessura das corticais e diminuição nos valores de torque de remoção.

Assim, dentro das limitações do estudo, pode-se concluir que a deficiência de Mg apresentou uma influência negativa sobre o metabolismo ósseo, agindo diretamente sobre a osteoclastogênese. A restrição de Mg resultou em perda de massa óssea sistêmica, bem como redução da resistência biomecânica dos implantes e perda óssea associada à doença periodontal induzida. Os resultados sugerem que a deficiência de Mg acentua a severidade da doença periodontal e influencia negativamente a manutenção de implantes com osseointegração estabelecida.

Palavras chaves: deficiência de magnésio, doenças periodontais, osteoclastos, osseointegração, implantes dentários, tecido ósseo.

ABSTRACT

Belluci MM. The effect of magnesium deficiency on bone metabolism. [Tese de Doutorado]. Araraquara: Faculdade de Odontologia UNESP; 2012.

Magnesium (Mg) deficiency is very common condition in the world population. This mineral has an important role on bone homeostasis. However, there has been little research regarding the mechanisms in which magnesium deficiency alters bone metabolism. Also, the understanding of the factors associated to bone metabolism is of interesting for the prognosis of oral therapies, such as on periodontal disease and on osseointegrated dental implants.

The aim of this study was to evaluate, in vitro, whether Mg deficiency affects the formation and/or activity of osteoclasts. And also to evaluate, in vivo, the effect of Mg intake deficiency on bone metabolism, periodontal disease and the bone tissue around osseointegrated implants.

For the in vitro study, bone marrow cells from long bone and jaw of mice were seeded on plastic and on bone in medium containing different concentrations of Mg (0.8 mM which is 100% of the normal value, 0.4, 0.08 and 0 mM). The effect of Mg deficiency was evaluated on cell viability after 3 days and proliferation rate after 3 and 6 days, as was mRNA expression of osteoclastogenesis-related genes (M-CSF, RANK, c-fos, DC STAMP, TNF-α and lL-1β) and Mg-related genes (TRPM7 and MRS2). After 6 days of incubation, the number of tartrate resistant acid phosphatase-positive multinucleated cells ($TRACP^{+}$-MNCs) was determined, and the TRACP activity of the medium was measured. Osteoclastic activity was assessed at 8 days by resorption pit analysis. For the in vivo studies, 30 rats received an implant in the right tibial metaphysis. After 60 days for healing of the implants, the animals were divided into groups according to the diet received. Control group (CTL) received a standard diet with adequate Mg content while test group (Mg) received the same diet except for a 90% reduction of magnesium. After 60

days of magnesium deficiency all animals received a ligature in the left lower first molar. The animals were sacrificed after 90 days of the start of the diet. It was evaluated calcium (Ca), magnesium (Mg), osteocalcin (OCN) and parathyroid hormone (PTH) serum levels and the deoxypyridinoline level (DPD) in the urine. The effect of magnesium deficiency on bone mineral density (BMD) was evaluated by densitometry of the lumbar vertebrae and femur and the histological analysis of the femur. The mandible BMD was assessed using digital radiography; alveolar bone loss was evaluated by linear (CEJ-bone crest) and area measurements. We used RT-PCR to assess mRNA expression (RANKL, OPG and IL-6) in the gingival tissues, and histological analysis was carried for the evaluation of inflammation, alveolar bone loss and for the number of osteoclasts. While the effect of bone tissue around titanium implants was evaluated by radiographic measurement of cortical bone thickness and BMD. The effect on biomechanical characteristics was verified by implant removal torque testing and the histometric analysis was carried out to evaluate the bone tissue around the osseointegrated implants.

In vitro results showed that Mg deficiency resulted in increased numbers of osteoclast-like cells; a phenomenon found for both types of marrow. Mg deficiency had no effect on cell viability and proliferation. Increased osteoclastogenesis due to Mg deficiency was reflected in higher expression of osteoclast-related genes. However, resorptions per osteoclast, as well as TRACP activity were lower in the absence of Mg. The in vivo results showed that Mg deficiency was associated with higher concentrations of PTH, DPD and significant decreases on both systemic and mandibular BMD, but not around osseointegrated implants. There was a greater severity of alveolar bone loss, although there were no significant differences on mRNA expression for the target genes. Mg deficiency also resulted in decreased cortical bone thickness and lower values of removal torque of the implants.

In vitro data suggest that altered osteoclast numbers and activity may contribute to the skeletal phenotype on magnesium deficiency. The in vivo study concluded that magnesium deficient diet had a negative influence on bone metabolism as well as on the severity of periodontal disease and on bone tissue around the implants.

Keywords: magnesium deficiency, periodontal diseases, osteoclasts, osseointegration, dental implants, bone tissue.

INTRODUÇÃO

A importância da dieta na prevenção ou manutenção de patologias que interferem no metabolismo ósseo é um assunto cada vez mais estudado[10, 75, 107].

A nutrição é um dos fatores que podem ser modificados durante o desenvolvimento e manutenção da massa óssea objetivando a prevenção e tratamento de doenças que atingem o metabolismo ósseo[45]. Nutrientes presentes na dieta apresentam uma influência direta sobre o metabolismo ósseo e, como exemplo, pode ser citada a ação de minerais sobre hormônios que controlam o metabolismo ósseo [107].

Dentre os principais determinantes nutricionais para manutenção da saúde óssea estão o cálcio (Ca), fósforo (P) e o magnésio (Mg) [45].

O magnésio é o quarto mineral mais abundante no organismo, sendo o mais abundante cátion divalente encontrado nas células em vertebrados [59, 88].

Essencial ao organismo, o Mg tem como principais funções a manutenção de músculos e nervos, regulação do ritmo cardíaco, síntese de nucleotídeos, manutenção do metabolismo energético (geração e atividade de ATP) e manutenção do tecido ósseo [10].

Cerca de 50 a 65% da quantidade total de Mg presente no organismo encontra-se no tecido ósseo, o qual serve como reservatório para manutenção da homeostase mineral no organismo [59, 80].

A ingestão diária de Mg recomendada (Recommended Daily Allowance – RDA) é de 420mg/dia para homens adultos e 320mg/dia para mulheres adultas[80-84]. Entretanto, apesar de ser um mineral facilmente encontrado em alimentos, a ingestão diária de Mg é abaixo do recomendado, principalmente nos Estados Unidos e países Europeus [32, 80, 81, 93, 101].

A deficiência de Mg ocorre tanto por hábitos alimentares inadequados quanto por alterações nas condições sistêmicas, que podem levar à má absorção ou à excessiva excreção do mineral. Estudos demonstram que alimentos orgânicos apresentam 29% mais Mg quando comparados a não-orgânicos [18, 112]. Além disso, a ingestão excessiva de certas substâncias, como álcool, chá, sal, acido fosfórico e açúcar, pode provocar a eliminação excessiva do mineral pelos rins [49].

Como alterações sistêmicas, pode ser citado o uso de medicamentos (ciclosporinas, antibióticos e diuréticos), que irão promover a excreção excessiva de Mg, e doenças, como o Diabetes mellitus e o alcoolismo, que resultarão em má absorção do mineral [10, 49].

Influência do magnésio sobre o metabolismo ósseo

O Mg é um mineral presente em grande quantidade no tecido ósseo, atrás apenas do Ca e fósforo [45]. Assim, apresenta um papel

importante sobre esse tecido e a homeostase mineral, podendo afetar diretamente a função das células ósseas e a formação da hidroxiapatita.

Estudos epidemiológicos demonstram uma correlação positiva entre pessoas com dieta deficiente de magnésio e aumento na perda de massa óssea e/ou diminuição da densidade óssea, o que sugere que a deficiência de magnésio pode ser um fator de risco para a osteoporose [44, 71, 89, 102].

Já estudos em animais utilizando dietas com diferentes concentrações de Mg (10% do NR - Nutrition Requirement, 25% do NR e 50% do NR), por diferentes períodos, demonstraram que aos 6 meses, mesmo com menor restrição do nutriente, houve perda de massa óssea caracterizada por diminuição do volume ósseo trabecular e fragilidade óssea. Essa perda de massa óssea foi seguida por aumento na liberação de substância P, fator de necrose tumoral (TNF-α) e interleucina 1β (IL1β), substâncias essas responsáveis pelo aumento da osteoclastogênese e reabsorção óssea[82-84]. Além disso, foi observada uma alteração na secreção e ação do paratormônio (PTH), contribuindo para a diminuição na formação óssea[82-84].

A dieta deficiente de Mg por um período prolongado (um ano) foi capaz de induzir a perda de massa óssea de vértebras lombares e fêmures, diagnosticada por densitometria óssea, além de promover alterações biomecânicas e histomorfométricas no tecido ósseo [97].

Estudos demonstram ainda, que a deficiência de Mg pode alterar a mineralização do tecido ósseo, pela ação direta sobre formação dos cristais de hidroxiapatita. O Mg não é parte integral da estrutura dos cristais de hidroxiapatita, mas é absorvido à superfície do cristal durante sua formação. Segundo estudos, durante a deficiência desse mineral, pode haver um retardamento na formação dos cristais de hidroxiapatita o que resulta em cristais maiores em dimensão, porém mais friáveis [11, 81]. Além disso, a deficiência de Mg retarda a mineralização da cartilagem e do tecido ósseo recém formados[81].

Influência do magnésio sobre osteoclastos

O Mg é o cátion divalente mais abundante nas células, sendo que da quantidade total de Mg presente no meio intracelular, a maior concentração é encontrada nas mitocôndrias e retículo endoplasmático [79].

Este mineral apresenta funções versáteis sobre as células, participando tanto de atividades estruturais como dinâmicas. Dentre as funções estruturais estão incluídas a manutenção da estabilidade das camadas lipídicas da membrana extracelular, manutenção das estruturas protéicas e manutenção da dupla hélice da molécula de DNA [79, 109, 111].

Como função dinâmica é relatada sua participação em mais de 300 reações enzimáticas celulares. Como exemplo temos que todas as enzimas que utilizam ATP necessitam de Mg para a formação do

substrato (Mg/ATP), o que torna esse mineral essencial também no metabolismo energético e de proliferação celular [79, 111].

Apesar do Mg ser de grande importância para o metabolismo celular, há poucos estudos na literatura demonstrando seu efeito sobre as células ósseas.

O metabolismo ósseo normal é o resultado de um processo de remodelação óssea ou *turnover* ósseo [103], o qual envolve células especializadas como osteoblastos (formação óssea), osteoclastos (reabsorção óssea) e osteócitos (manutenção do tecido ósseo), podendo ser influenciado por fatores locais e sistêmicos [41].

Tanto a formação como a reabsorção óssea dependem da adequada proliferação, migração, diferenciação e função de osteoclastos e osteoblastos, os quais possuem uma relação interdependente [100]. A alteração da função, diferenciação e sobrevida de qualquer uma dessas células pode levar a estados patológicos, como osteoporose e osteopetrose [103].

Essas células são reguladas por uma complexa rede sinalizadora, mediada por receptores das membranas celulares e várias citocinas[41].

Estudos sugerem ainda que há diferenças entre as subpopulações de osteoclastos, tanto em relação à formação como à atividade de reabsorção. Uma possível explicação para essas diferenças pode estar relacionada ao tipo de célula progenitora ou ao micro-ambiente

local, como o tipo de tecido ósseo, de origem endocondral (ossos longos) ou intramembranosa (ossos curtos) [26, 27, 73, 104]. Segundo Everts et al.[27] (1999) a função de reabsorção por osteoclastos derivados da calvária, portanto de origem intramembranosa, depende da atividade de catepsinas e metaloproteinases, enquanto que para os osteoclastos derivados de ossos longos, portanto de origem endocondral, a função de reabsorção depende somente de catepsinas. No estudo de Faloni et al.[20] (2011) foi demonstrado ainda que osteoclastos derivados da mandíbula e ossos longos apresentam diferentes precursores, o que altera o potencial de formação dessas células.

Dentre os fatores sistêmicos que podem interferir de forma direta ou indiretamente no metabolismo ósseo, os nutrientes presentes na dieta são considerados de grande importância. Nutrientes como Ca e P são imprescindíveis para o metabolismo ósseo, por representarem de 80 a 90% do conteúdo mineral desse tecido. Já as proteínas são incorporadas à matriz orgânica, fazendo parte das estruturas colágenas do tecido ósseo, que posteriormente será mineralizado. Outros minerais e vitaminas são considerados essenciais para reações metabólicas desse tecido [45].

Em estudo realizado em mulheres na pós-menopausa foi observada associação significativa e consistente entre nutrientes, como ferro e magnésio, e a manutenção da densidade mineral óssea. Além disso, foi demonstrada associação positiva entre minerais, como cálcio,

zinco, fósforo e potássio, e a manutenção da densidade mineral óssea, independente da dieta ou métodos avaliados[28].

Quanto ao efeito da deficiência de magnésio sobre as células ósseas, foi constatado, em animais, que esta pode alterar a proporção de osteoblastos e osteoclastos no tecido ósseo, resultando em maior quantidade osteoclastos e sem alteração na quantidade de osteoblastos, além de um possível aumento na atividade dos osteoclastos [81, 84].

As alterações sobre as células ósseas foram confirmadas em estudos *in vitro,* onde foi demonstrado que a deficiência extracelular de Mg apresenta uma influência negativa sobre essas células. Nesses estudos, osteoblastos cultivados em meio deficiente de Mg apresentaram redução na taxa de proliferação e diferenciação [2, 4]. Entretanto, não há estudos *in vitro* na literatura que demonstrem a influência do Mg sobre osteoclastos.

Portanto, apesar de constatada que a deficiência de magnésio resulta em perda de massa óssea, deve-se ressaltar que os mecanismos relacionados ainda são desconhecidos. Além disso, pouco é conhecido sobre o efeito da deficiência sobre os osteoclastos e se a origem dessas células pode influenciar na resposta à deficiência do mineral.

Influência da deficiência de magnésio sobre a doença periodontal

A doença periodontal é uma doença inflamatória multifatorial induzida por microrganismos, que afeta as estruturas de suporte do dente. Por apresentar essa característica, fatores etiológicos, comportamentais e genéticos, como resposta do hospedeiro, podem modificar a severidade, suscetibilidade e progressão da doença [53, 65].

O processo imunoinflamatório da doença periodontal é regulado por quimiocinas, citocinas e prostaglandinas [46]. Durante o processo de doença periodontal, células de defesa como, macrófagos e neutrófilos estão ativados e presentes em grande quantidade no tecido gengival inflamado. Essas células secretam mediadores inflamatórios como, interleucinas 1β, 2, 4, 6 e 10 (IL1β, IL2, IL4, IL6, IL10), fator de necrose tumoral alfa (TNFα) e prostaglandina E_2 (PGE_2), entre outros. A liberação desses mediadores induz uma resposta inflamatória exacerbada, a qual leva à destruição dos tecidos periodontais [46, 53].

Muitos desses mediadores estão aumentados na deficiência de Mg. Estudos demonstraram que a deficiência experimental de magnésio apresenta um efeito sobre o processo inflamatório e a resposta imunológica, tendo sido verificado um aumento dos níveis séricos de citocinas pró-inflamatórias como TNF-α, IL1β, PGE_2 e IL-6 [60, 82-84].

Além disso, as principais células de defesa, encontradas no tecido periodontal, estão alteradas em animais com algum grau de

deficiência do mineral. Dentre as principais alterações causadas pela deficiência de Mg, estão o aumento no número de neutrófilos e macrófagos, seguida de maior ativação dessas células, havendo portanto, um aumento na atividade fagocítica e maior produção de espécies reativas de oxigênio, responsáveis pela maior destruição tecidual [13, 60, 64].

Um estudo realizado em humanos evidenciou que a deficiência de Mg está relacionada à maior severidade da doença periodontal, demonstrado pela correlação significativa entre níveis séricos de Mg e parâmetros periodontais. Nesse estudo foi demonstrada a correlação entre a redução dos níveis séricos de Mg com maior profundidade de sondagem e nível de inserção clínica. Alem disso, por uma análise de regressão múltipla, foi observado que o Mg apresentou resultados semelhantes a fatores de risco já conhecidos, como idade, educação e fumo, enquanto que a suplementação nutricional desse mineral foi relacionada à saúde periodontal [65].

Assim, alterações no metabolismo ósseo e no processo imunoinflamatório, causadas por doenças sistêmicas ou deficiências nutricionais, dentre elas a deficiência de magnésio, podem ser consideradas como um fator modificador da progressão da doença periodontal [34, 92].

Influência da deficiência de magnésio sobre a osseointegração

O entendimento de fatores associados à modificação do metabolismo ósseo é de extremo interesse para o tratamento com implantes osseointegrados, visto que muitas situações como osteoporose, doenças infecciosas e/ou inflamatórias, que afetam o tecido ósseo e prejudicam seu metabolismo, podem influenciar no processo de osseointegração[70].

Em implantes intraorais, o sucesso do tratamento, ou seja, a obtenção e a manutenção da osseointegração depende de diversos fatores. Dentre eles podem ser citados, o trauma cirúrgico e as condições anatômicas, fatores responsáveis pela perda primária de implantes; e a qualidade óssea, o volume ósseo e a sobrecarga dos implantes, fatores determinantes na perda tardia [66].

A falha de um implante anteriormente estável e ancorado pode estar relacionada ainda a problemas na mineralização do tecido ósseo em contato direto com a superfície do implante, já que mecanicamente a interface implante/osso depende de uma estrutura mineralizada e intacta[50].

Assim, todas as alterações ósseas, relatadas anteriormente, provocadas pela deficiência de Mg podem ser consideradas um fator de risco para o tratamento com implantes.

Poucos estudos, entretanto, têm demonstrado o efeito das doenças que causam perda de massa óssea sobre implantes com osseointegração já estabelecida. Em estudos realizados em ratas ovariectomizadas foi observada a redução da massa óssea ao redor de implantes osseointegrados instalados na tíbia, assim como a diminuição da força de torque de remoção desses implantes, mostrando que, mesmo em implantes com osseointegração já estabelecida, as alterações sistêmicas podem exercer um efeito negativo sobre sua manutenção[35, 36, 90].

Apesar da deficiência de Mg atingir uma parcela considerável da população mundial e de terem sido constatadas alterações sistêmicas e ósseas causadas por essa deficiência, estudos avaliando a deficiência de Mg como um fator de interferência sobre o metabolismo de células ósseas, a doença periodontal e a osseointegração de implantes ainda são escassos.

PROPOSIÇÃO

A hipótese deste estudo é que a deficiência de Mg pode ter um efeito importante sobre os osteoclastos e, conseqüentemente, influenciar a severidade da doença periodontal induzida e a manutenção de implantes osseointegrados. Para avaliar esta hipótese, os objetivos específicos deste estudo foram:

Objetivo 1: Avaliar in vitro, o efeito da restrição de magnésio sobre a formação e atividade de osteoclastos.

Objetivo 2: Avaliar in vivo, o efeito da deficiência do magnésio na dieta sobre a perda óssea sistêmica e relacionada à doença periodontal induzida em ratos.

Objetivo 3: Avaliar in vivo, o efeito da deficiência de Mg sobre a perda óssea sistêmica e o tecido ósseo ao redor de implantes com osseointegração estabelecida.

CAPÍTULO 1

Magnesium deficiency results in an increased formation of osteoclasts

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Magnesium deficiency results in an increased formation of osteoclasts

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Abstract

Magnesium (Mg^{+2}) deficiency is a frequently occurring disorder that leads to loss of bone mass, abnormal bone growth and skeletal weakness. Until now, it is not clear whether Mg^{2+} deficiency affects the formation and/or activity of osteoclasts. We evaluated the effect of Mg^{2+} restriction on these parameters. Bone marrow cells from long bone and jaw of mice were seeded on plastic and on bone in medium containing different concentrations of Mg^{2+} (0.8 mM which is 100% of the normal value, 0.4, 0.08 and 0 mM). The effect of Mg^{2+} deficiency was evaluated on cell viability after 3 days and proliferation rate after 3 and 6 days, as was mRNA expression of osteoclastogenesis-related genes and Mg^{2+}-related genes. After 6 days of incubation, the number of tartrate resistant acid phosphatase-positive multinucleated cells ($TRACP^{+}$-MNCs) was determined, and the TRACP activity of the medium was measured. Osteoclastic activity was assessed at 8 days by resorption pit analysis. Mg^{2+} deficiency resulted in increased numbers of osteoclast-like cells; a phenomenon found for both types of marrow. Mg^{2+} deficiency had no effect on cell viability and proliferation. Increased osteoclastogenesis due to Mg^{2+} deficiency was reflected in higher expression of osteoclast-related genes. However, resorption per osteoclast as well as TRACP activity were lower in the absence of Mg^{2+}. In conclusion, Mg^{2+} deficiency augmented osteoclastogenesis but appeared to decrease the activity of these cells.

Together, our in vitro data suggest that altered osteoclast numbers and activity may contribute to the skeletal phenotype as seen in Mg^{+2} deficient patients.

Key words: Magnesium deficiency, osteoclast, osteoclastogenesis, osteoclast activity, bone metabolism, bone marrow.

1. Introduction

The importance of intake of minerals in the maintenance of bone homeostasis has been demonstrated in various studies [1-5]. Of these minerals magnesium (Mg^{2+}) has been shown to play a crucial role in hormone action (e.g. parathyroid hormone), expression of some neurotransmitters and, more in general, in regulating cellular activity [6, 7].

Mg^{2+} is the most abundant divalent cation in cells and serves as an essential structural element in the maintenance of phospholipid bilayers, DNA double helices and protein structure. One of its most important functions is related to the enzymatic activity of cells since all enzymes utilizing ATP require Mg^{2+}. It also acts as an allosteric activator of enzyme action including adenylate cyclase and phospholipase C. This could consequentially facilitate the activation/deactivation of signal transduction pathways [8, 9].

It is estimated that 2.5% to 15% of the world population suffers from some level of hypomagnesia [10]. According to [2], the mean value of daily Mg^{2+} intake in the North American human population is below recommendation. A similar low intake is present in some European countries [10-13].

The normal adult body Mg^{2+} content is about 25g. Bone can be regarded as the major storage compartment of the body, containing 60-65% of the total Mg^{2+} content [14]. Part of this magnesium resides on bone surfaces, and it is hypothesized that its main function is to maintain the extracellular Mg^{2+} concentration [14, 15].

A study conducted in rats with severe restriction of magnesium intake (0.04% of the Nutrient Requirement – NR) resulted in increased skeletal fragility, increased bone resorption, decreased bone formation, increased expression of the neuropeptide substance P (SP), tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β). This finally resulted in an osteoporotic phenotype [16]. Other studies were conducted with a less severe mineral restriction (10%, 25% and 50% of NR), and showed that even under these conditions bone loss occurred [1, 2, 17]. In addition, the Mg^{2+} deficient diet had a negative influence on the maintenance of osseointegrated implants [18, 19].

Mg^{2+} deficiency also potentiates the inflammatory response by increasing the release of cytokines and growth factors that mediate the

formation and activity of the body's bone degradation specialist cell, the multinucleated osteoclast [20, 21]. Osteoclasts are multinucleated cells from the macrophage lineage, specialized in degrading mineralized matrices, such as bone. The formation and activity of these cells is mediated by cytokines like macrophage colony stimulating factor (M-CSF) and the osteoclast differentiation factor RANKL and their receptors, c-fms and RANK, respectively [22]. Deregulation of osteoclast differentiation, function and survival may lead to pathological conditions, such as osteoporosis [23] or osteopetrosis [24, 25].

Although Mg^{2+} deficiency leads to loss of bone mass it is not known whether Mg^{2+} influences osteoclast formation and activity. Therefore, the aim of this study is to evaluate the effect of magnesium restriction on osteoclast formation and activity. Precursors were obtained from marrow of long bone and jaw and cultured in the presence of M-CSF and RANKL. Precursor cells from two different sites were analyzed because several studies have suggested phenotypic differences among osteoclast subpopulations, such as, functional differences in formation and resorption activity [26-29].

2. Materials and Methods

2.1 Mice

Prior to their sacrifice, six weeks old male C57BL/6 mice (Harlan, Horst, The Netherlands) were maintained for one week at the animal facility of the VU University. Permission for the experiments was obtained from the animal ethical committee (DEC) of the VU University, Amsterdam.

2.2 Bone marrow isolation

Bone marrow cells from long bones (femur and tibia) and jaws were isolated as described before [30]. The cells were resuspended through a 21-gauge needle and filtered over a 100 µm pore size cell strainer filter (Falcon/ Becton Dickinson, Franklin Lakes, NJ, USA) and kept on ice in culture medium without magnesium (MEM alpha without nucleotides, with L-glutamine; Promocell, Heidelberg, Germany) supplemented with 5% FCI (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (antibiotic antimyotic solution; Sigma-Aldrich, St. Louis, MO, USA) until further use.

2.3 Osteoclastogenesis

Bone marrow cells were plated in 96-well flat-bottom tissue culture-treated plates (Costar, Cambridge, MA, USA) at a density of 1.0×10^5 cells/well in

150 µl of culture medium containing 30 ng/ml recombinant murine M-CSF (R&D Systems, Minneapolis, MN, USA) and 20 ng/ml recombinant murine RANKL (RANKL-TEC, R&D Systems, Minneapolis, MN, USA). Cells were cultured in 4 different concentrations of magnesium: 100% Mg^{2+} or 0.8mM Mg^{2+}, as supplied with standard alpha-MEM. The other concentrations, 50% (0.4 mM) and 10% (0.08 mM) were made by mixing Mg^{2+}-free alpha-MEM (0%) with 100% Mg^{2+} containing alpha-MEM. Cells were cultured on plastic or on 650-µm-thick bovine cortical bone slices. Culture media were replaced every 3 days. After either 3 or 6 days, cells were washed with PBS and harvested for PCR and DNA analysis. Supernatants were collected for viability assay. On day 6, wells were washed with PBS and fixed in 4% PBS-buffered formaldehyde, stored at 4°C and stained for tartrate-resistant acid phosphatase (TRACP). On day 8, water was added to osteoclast cultures on bone slices for bone resorption analysis.

2.4 TRACP staining

Cells from femur/tibia and jaw were cultured for 6 days on plastic or on bone and stained for TRACP activity using the leukocyte acid phosphatase kit (Sigma-Aldrich, Saint Louis, MO, USA). Nuclei were stained with diamidino-2-phenylindole dihydrochloride (DAPI). To evaluate osteoclast formation, the number of TRACP-positive multinucleated cells

(TRACP$^+$-MNC) was counted. These multinucleated cells were categorized in cells containing 3–5, 6–10, or more than 10 nuclei.

2.5 Magnesium and calcium measurements

To determine the concentration of magnesium and calcium in the culture medium, the supernatants of the 4 different magnesium containing media after cell culture were collected after 6 days of culturing on plastic and on bone (n=6). The media were also incubated at the same conditions but without cells (n=3). Magnesium and calcium concentration were analyzed by atomic absorbance spectrometry (Analyst100; Perkin-Elmer Corporation, Norwalk, CT, USA).

2.6 DNA quantification

Cell proliferation was measured using DNA quantification. The bone marrow cells of 6 mice were collected on day 0 at a density of 1.0 x 10^5 cells and after 3 and 6 days of incubation on plastic in the 4 different magnesium concentration media with M-CSF and RANKL. The amount of DNA was determined using the CyQuant cell proliferation assay kit (Invitrogen Corporation, Carlsbad, CA, USA) and measured in the Synergy HT plate reader (BioTek Instruments; Winooski, VT; USA).

2.7 Citotoxicity

To determine the cell viability, the supernatant of cells cultured on plastic were collected after 3 days. The cytotoxicity assay was performed according to the manufacturer's instructions (ToxiLight BioAssay kit, Lonza Rockland Inc., Rockland, ME, USA). This assay quantitatively measures the leakage of adenylate kinase from damaged cells.

2.8 Real-time quantitative PCR

For real-time qPCR analysis, bone marrow samples of 6 mice were collected after 3 days culture with M-CSF and RANKL. RNA isolation and real-time quantitative PCR was performed as described [31]. Real-time PCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) (Table 1). To avoid amplification of genomic DNA, each amplicon spanned at least 1 intron. To test for PCR efficiencies, one of the osteoclast samples was used to generate a standard curve for all of the genes studied. The PCR reactions of the different amplicons had equal efficiencies. Expression of porphobilinogen deaminase (PBGD) was not affected by the different Mg^{2+} concentrations and was used as housekeeping gene. Samples were tested for TRPM-7 (Transient Receptor Potential-Melastin-like 7), MRS2, c-fos, RANK, c-fms, DC STAMP (dendritic cell-specific transmembrane protein), m-calpain, μ-calpain, IL1-β and TNF-α. Subsequently, expression was normalized for

the expression of PBGD by calculating the ΔCt ($Ct_{gene\ of\ interest}$ - Ct_{PBGD}), and expression of the different genes is expressed as $2^{-(\Delta Ct)}$.

2.9 TRACP activity assay

The supernatant of cells obtained from 6 mice seeded on plastic cultured in 4 different magnesium concentrations in the presence of M-CSF and RANKL was collected after 6 days of incubation. TRACP activity was assayed in 96-well plates with *p*NPP as substrate in 5µl of supernatant with final concentrations of: 10 mM *p*NPP, 1 mM ascorbic acid, 0.1 mM $Fe(NH_4)_2(SO_4)_2$, 0.1 M NaAc, 0.15 M KCl, 10 mM disodium tartrate, 0.1% (v/v) Triton X-100. The *p*-nitrophenol liberated after 30min of incubation at 37 °C was converted to *p*-nitrophenolate by addition of 100µl of 0.3 M NaOH. The absorbance was measured at 405 nm in a Synergy HT plate reader (BioTek Instruments; Winooski, VT; USA). 1 unit of TRACP activity corresponds to 1 µmol of *p*-nitrophenol formed per min.

2.10 Bone resorption

Bone marrow cells from long bone of 6 mice were cultured on bone slices for 8 days in 4 different magnesium concentration media with M-CSF and RANKL. After this period, the cells present on the bovine cortical bone

slices were removed with 0.25 M NH_4OH. The slices were washed in distilled water, incubated in a saturated alum ($KAl(SO_4)_2.12(H_2O)$ solution, washed in distilled water, and stained with coomassie brilliant blue. The area of resorption pits were measured using Image Pro-Express 6.3 software (Olympus, Tokyo, Japan). The bone slices were analyzed in triplicate for each animal. Five fields per bone slice were examined. Bone resorption was assessed by calculating the percentage of surface area of resorbed bone.

2.11 Statistical analysis

Unless otherwise stated, most experiments were performed with bone marrow from 6 mice. Statistical analyses were performed using GraphPad Prism (GraphPad Prism 5.0, San Diego, CA, USA). The parametric test ANOVA followed by Tukey multicomparison test was conducted for comparisons between groups for all analyzed parameters. Effects were considered statistically significant when $p<0.05$.

3. Results

3.1 Increased osteoclast formation due to magnesium deficiency.

Magnesium deficiency had no effect on the morphological aspects of $TRACP^+$-MNCs derived from long bone marrow after 6 days of culturing on plastic (Fig. 1).

Low magnesium concentrations resulted in increased numbers of $TRACP^+$-MNCs after 6 days of culturing on plastic (Fig. 1). Compared to the control (0.8 mM) concentration, in Mg^{2+}-free culture medium (the 5% FCS contributing trace amounts of Mg^{2+}) the highest number of $TRACP^+$-MNCs were generated (Fig. 1).

When cells were cultured on bone, again there was no effect on the morphological aspect of $TRACP^+$-MNCs among the Mg^{2+} concentrations (Fig. 2). However, a comparison of $TRACP^+$-MNCs cultured on plastic by those cultured on bone revealed differences in their shape. $TRACP^+$-MNCs cultured on plastic proved to be more elongated whereas those cultured on bone were round (compare Figs 1 and 2).

With respect to the number of cells, an effect was observed comparable to the one found on plastic. On bone, however, the highest number was found with a concentration of 0.08 mM Mg^{2+} concentration (Fig. 2).

Since marrow cells from different skeletal locations such as long bone and jaw, respond differently to osteoclastogenesis inducing cytokines M-CSF and RANKL, [29, 32], it is possible that these marrow cells also respond differently to magnesium deprived conditions. As the number of cells with more than 6 nuclei is higher in jaw cultures [29], only cells with 6-10 and >10 nuclei were counted. Also with marrow cells obtained from the jaw a response was seen similar to the one found with marrow cells from long bones. When the cells were seeded on plastic or on bone we found a statistically significant increased number of $TRACP^{+}$ MNCs cultured in the presence of lower Mg^{2+} concentration (Figs 3A, B, C, D).

Together these data show that Mg^{2+} deficiency leads to an increase in osteoclast formation, regardless the bone site used to retrieve precursors from and regardless of the substratum. Given the fact that long bone and jaw marrow reacted similarly, and since long bone marrow contains approximately 20-fold higher cell numbers, the remaining assays were conducted with long bone marrow cell cultures only.

3.2 Mg^{2+} did not alter the medium concentration of Ca^{2+}.

As Mg^{2+} and Ca^{2+} are antagonists, we evaluated the concentration of both ions to assess whether the presence of cells and/or bone would influence their concentration.

The analysis revealed similar concentrations of Mg^{2+} in the medium of each group, regardless of the presence of bone and/or cells. Only when the bone was added to the culture, the 0.8 mM Mg^{2+} concentration showed a significant decrease of Mg^{2+}; a phenomenon found only in the absence cells (Fig. 4A). The Ca^{2+} measurement showed a similar distribution for all conditions studied (Fig.4B), indicating that none of the Mg^{2+} culture conditions resulted in alterations of Ca^{2+} in the medium.

3.3 Mg^{2+} deficiency does not influence osteoclast precursor viability or proliferation

We attempted to explain the increased osteoclast formation by analyzing cell proliferation. Mg^{2+} deficiency could result in an increased proliferation of osteoclast precursors, hence leading to more osteoclasts. The DNA measurement showed an increase in the level of DNA from day 0 to day 3 of precursor cells in all Mg^{2+} concentrations, but there were no differences among the Mg^{2+} concentrations. From day 3 to day 6 there were no differences in the DNA amount (Fig. 5A).

Next, an increase in osteoclast formation under Mg^{2+} restrictive concentrations could be explained by increased cell survival. The cell cytotoxicity assay revealed, however, no effect of the different Mg^{2+}-concentration on cell viability (Fig. 5B).

3.4 Mg^{2+} altered the expression patterns of osteoclastogenesis-related genes

In order to evaluate the influence of different Mg^{2+} concentrations on osteoclast differentiation, the expression of osteoclastogenesis-related genes was analyzed.

After 3 days of culture on plastic, the gene expression of DC-STAMP, c-fos, TNF-α and IL-1β was significantly increased in the cultures of 0.08 mM Mg^{2+} (Figs 6C, D, E, F).

In contrast to cultures on plastic, gene expression of cultures on bone was hardly affected by the different Mg^{2+} regimes (Fig. 7). Here, only RANK expression was significantly increased in 0.08 mM Mg^{2+} cultures (Fig. 7B).

3.5 Mg^{2+} altered the expression patterns of magnesium-related genes

Since it was previously shown for osteoblast-like MG-63 cells that Mg^{2+} deprivation caused an increased expression of receptors for Mg^{2+} [33], we analyzed the expression of Mg^{2+}-related genes. Those were TRPM-7 (transient receptor potential calcium channel, subfamily M), which is the receptor responsible for the maintenance of intracellular Mg^{2+} concentration and MRS2 which is a Mg^{2+} transporter in the mitochondria that is important for the viability of the cell.

When the cells were cultured on plastic, the expression of TRPM-7 as well as MRS2 was increased in 0.08 mM Mg^{2+} cultures (Figs 8A, B). The expression of these receptors followed the same pattern as found for the gene expression of osteoclastogenesis markers.

When the cells were cultured on bone, there was an increase in the expression of MRS2 in the presence of 0.4 mM Mg^{2+} (Fig. 8D).

3.6 Low Mg^{2+} causes a decreased osteoclast activity.

Two parameters of osteoclastic activity were assessed: TRACP activity in the conditioned medium and resorption of bone. These values were correlated to the number of osteoclasts. When analyzing bone resorption, there was no difference in number of resorption pits among the various Mg^{2+} concentrations (Fig. 9A). However, when expressing resorption per $TRACP^{+}$ MNC, there is a tendency to a decreased resorptive activity per $TRACP^{+}$ MNC in Mg^{2+}-poor media (Fig. 9B).

Mg^{2+}-poor cultures secreted significantly less TRACP (Fig. 9C). When expressing TRACP secretion per $TRACP^{+}$ MNC, this result is even more obvious (Fig. 9D).

4. Discussion

The results of the present study show that deficiency of Mg^{2+} is inversely proportional to the number of osteoclasts generated from bone marrow precursors: the lower the extracellular Mg^{2+} concentration, the higher the number of osteoclasts. It is remarkable to note that the higher number of osteoclasts does not lead to a higher resorptive activity; in fact per osteoclast less activity was apparent. Thus our data indicate that a lower level of Mg^{2+} stimulates the formation of osteoclasts but appears to decrease their activity. The mechanisms underlying this effect of Mg^{2+} on osteoclasts are not known. We assume that the deficiency of this mineral activates some transcription factors and proteins such as c-fos and DC-STAMP. Both genes are key regulators of osteoclastogenesis [34, 35]; DC-STAMP being required for fusion of osteoclast precursors. The present data indicate that the higher number of cells with higher number of nuclei per cell in Mg^{2+} deficient medium correlates to some extent with the expression of osteoclastogenesis-related genes. Moreover, an increased expression of cytokines that stimulate osteoclastogenesis, e.g. TNF-α and IL-1β, likely results in an enhanced formation of osteoclasts [36, 37]. In general, it appeared that the higher expression was only seen in 0.08 mM of Mg^{2+}, suggesting this being an optimum concentration for the activation of osteoclastogenesis gene expression.

An intriguing finding of the present study was the lack of an effect on mRNA expression when cells were seeded on bone. Except for RANK, all other genes analyzed were less or not affected by the different Mg^{2+} regimes. This indicates that cells on bone respond differently to Mg^{2+} regiments than cells attached to plastic. It is not unlikely to assume that the low amount of Mg^{2+} released from the bone is sufficient to partially overcome the deficiency. Yet, higher numbers of osteoclasts were still formed; a situation comparable to the cells seeded on plastic. The lower levels of Mg^{2+} did result in increased numbers of osteoclasts, but resorption per osteoclast was decreased.

Not only osteoclastogenesis-related genes were affected by the lower levels of Mg, also the expression of genes related to the cellular exchange of Mg proved to be sensitive to this metal. The expression of Mg^{2+} related genes (TRPM7 and MRS2) was higher in 0.08 mM of Mg^{2+}. This may suggest that the cells attempt to maintain the intracellular concentration of Mg^{2+} by upregulating the expression of TRPM7 and MRS2.

As Ca^{2+} is another divalent cation that plays an important role in cellular metabolism and it is suggested that Mg modulates the influx of Ca^{2+} in the cells [38], the level of Mg^{2+} and Ca^{2+} in the medium was analyzed. Our findings showed that the medium concentration of Mg^{2+} is proportional to the amount of Mg^{2+} added to the medium and that the concentration of Ca^{2+} did not change under Mg^{2+} deficient conditions.

These findings strongly suggest that the effects seen were not due to changes in the level of Ca, but probably directly related to Mg^{2+} levels. This is in agreement with [39], who showed that the osteoclast activity in Mg^{2+} deficient animals is not responsible for the increased amount of Ca^{2+} in the serum.

Mg^{2+} also participates as a key factor in the proliferation process, as it is essential to sustain protein synthesis prior to cell division. It is also involved in DNA duplication, cytoskeleton re-arrangement and the upregulation of energy metabolism prior to cell division [9]. Cell proliferation is differently affected by Mg^{2+} deprivation in different cell types. Endothelial cells are highly sensitive, HC11 mammary epithelial cells are less sensitive and HL-60 leukemic cells are resistant to Mg availability [9]. Interestingly, the latter cell line was used as osteoclast precursor in various studies [40-43]. In the present study, it was shown that Mg^{2+} deficiency did not alter the rate of proliferation of bone marrow cells after 3 and 6 days of culture, which suggests that the proliferative effect of M-CSF and RANKL treated bone marrow is less sensitive to alterations in Mg^{2+} concentrations. The lack of an increased cell proliferation between day 3 and 6 may be related to the differentiation of the cells, which is in agreement with a previous study that showed similar findings with cultures of bone marrow as osteoclast progenitors treated with RANKL next to M-CSF [31].

This decreased resorption might be caused by the hampered activity of these cells due to the lack of a proper amount of Mg^{2+}. Resorption of bone requires high energy levels, which is provided by the high numbers of mitochondria. The majority of enzymes involved in energy metabolism are Mg^{2+}-dependent, especially by the high affinity of Mg^{2+} to ATP (Mg/ATP, which is the only active form of ATP) [9]. The restriction of Mg impairs the optimal cellular Mg/ATP levels that mainly reverberate on the proper function of cells, because of the importance of cellular Mg/ATP level on protein synthesis [38], so Mg^{2+} deficiency could depress cell metabolism [9].

The higher number of osteoclasts may be due to an attempt of the cells to compensate for their impaired ability to resorb. This compares with some genetic models for osteopetrosis (e.g. *oc/oc* mice) in which more but less functional osteoclasts are found [25, 44]. The higher number of osteoclasts seen in our in vitro study coincides with in vivo findings. [2] showed that in rats Mg^{2+} deficiency leads to higher number of osteoclasts. Although in vivo such an increase in higher number can be due to numerous other indirect factors [20], our in vitro findings suggest a direct effect on formation of osteoclasts.

Several studies have suggested functional and phenotypic differences among osteoclast subpopulations [26, 27, 29, 32]. Yet, deficiency of Mg^{2+} had a similar effect on precursors obtained from long

bone or from the jaw. This suggests that Mg^{2+} deficiency has a similar effect on bone marrows from different skeletal locations.

5. Conclusions

In conclusion, we have shown, that Mg^{2+} deficiency promotes osteoclastogenesis thus resulting in an increased number of osteoclasts. These osteoclasts were, however, less active than the control cells. We propose that the increased number of osteoclasts in medium depleted of Mg results from an enhanced fusion of precursors. Our findings may help to better understand the mechanisms by which Mg^{2+} affects osteoclast differentiation, maturation and activity and thus provide important information on the mechanisms by which this mineral influences bone metabolism.

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Figures

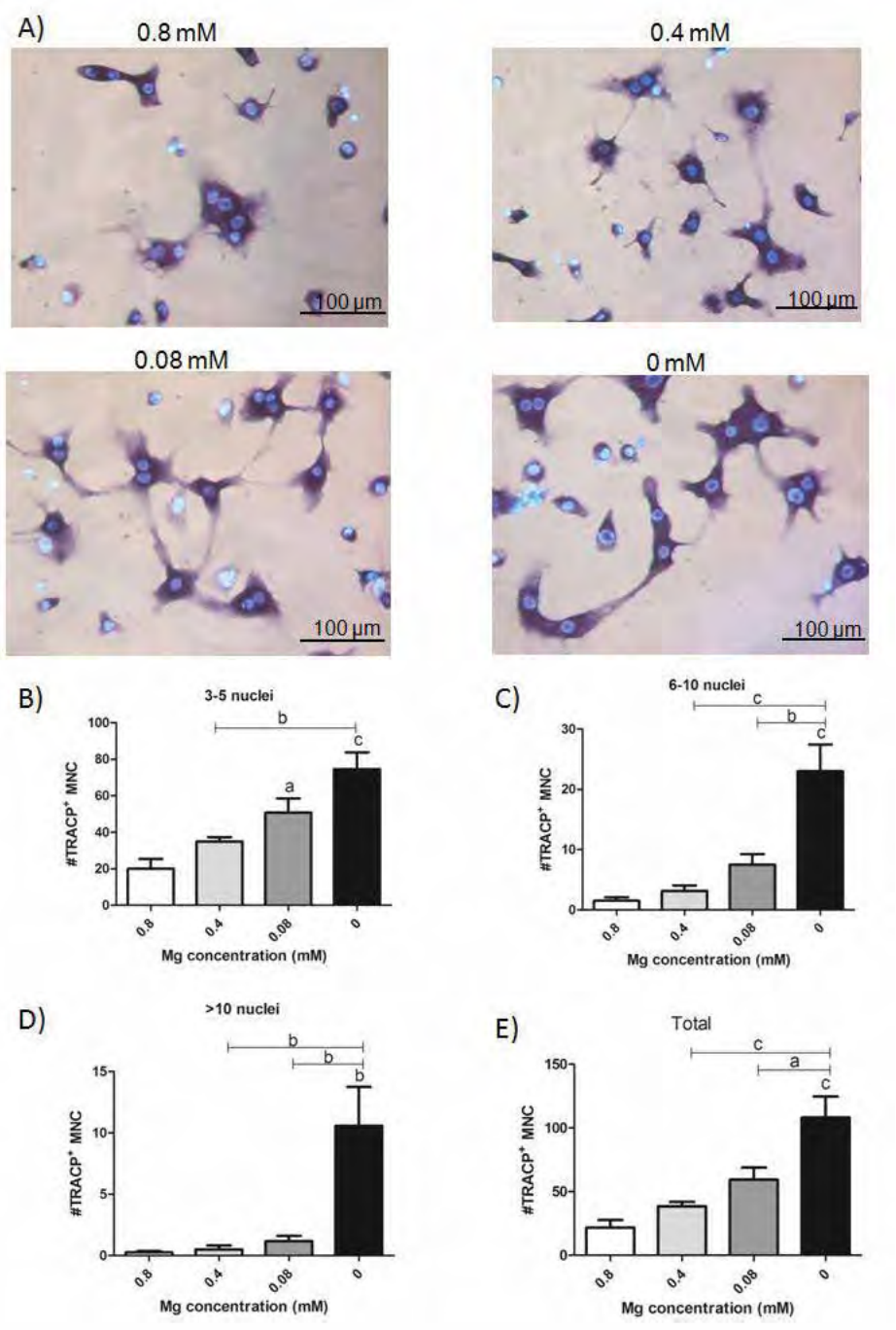

Figure 1 (A) Morphological aspects of TRACP+ multinucleated cells formed from precursors obtained from long bone marrow after 6 days of culture on plastic in four different Mg^{2+} concentrations in the

presence of M-CSF and RANKL (n=6). The cells show an elongated shape in all Mg^{2+} concentrations. Higher number of nuclei per cell were observed under Mg^{2+} deprivation. (B-D) Number of $TRACP^{+}$ multinucleated cells ($TRACP^{+}$-MNC) with 3-5 nuclei (B), 6-10 nuclei (C), > 10 nuclei (D) and total number of $TRACP^{+}$ MNC (E) formed under the four Mg^{2+} culture regimes (C) Data are expressed as mean ± SD. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.0001$.

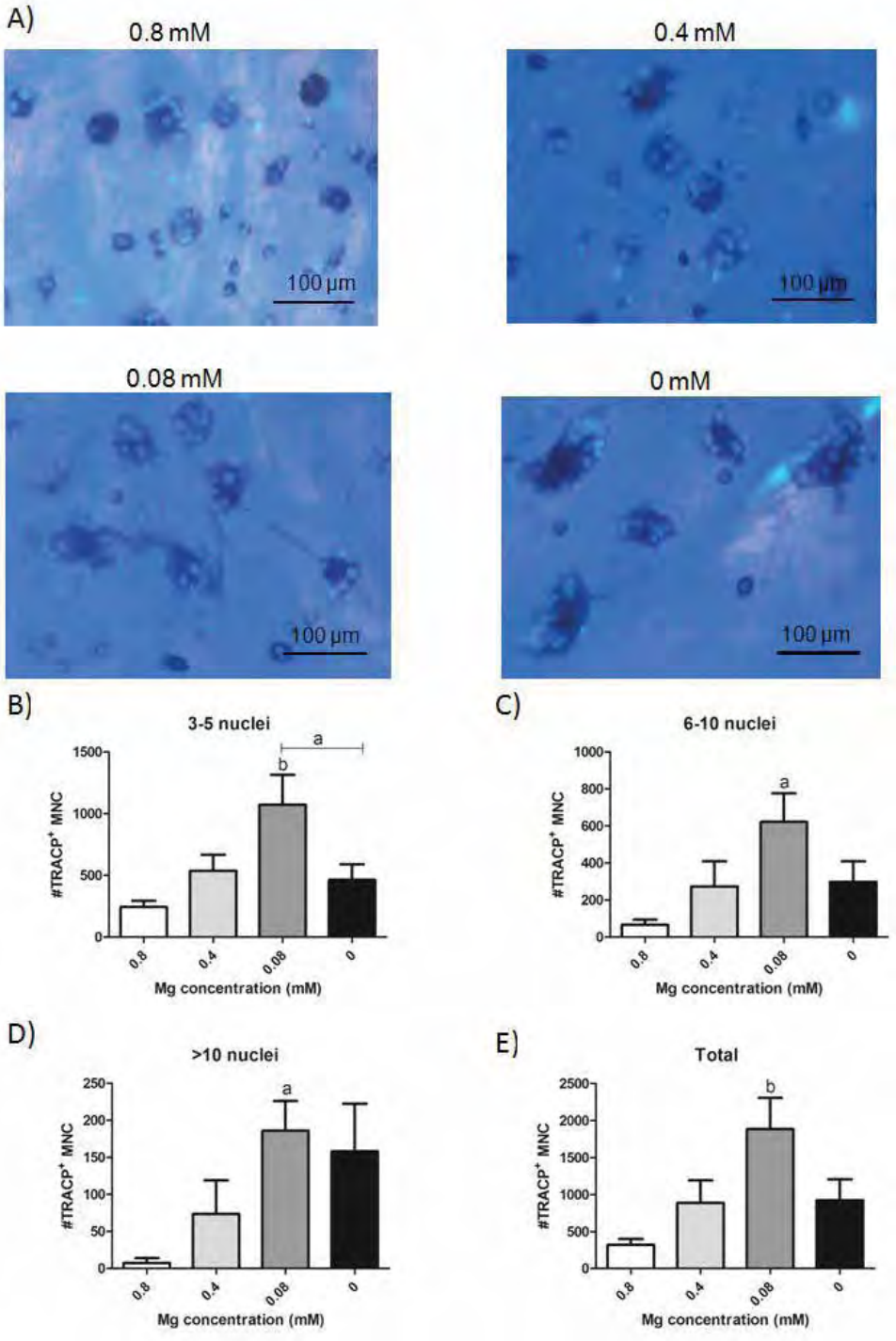

Figure 2 (A) Morphological aspects of $TRACP^+$ multinucleated cells formed by long bone marrow cells after 6 days of culture on bone in four different Mg^{2+} concentrations in the presence of M-CSF and RANKL (n=6). $TRACP^+$ MNC on bone had a round shape in all

Mg^{2+} concentrations. Higher number of nuclei per cell were observed under Mg^{2+} deprivation (B-D). Number of $TRACP^{+}$ multinucleated cells with 3-5 nuclei (B), 6-10 nuclei (C), > 10 nuclei (D) and total number of $TRACP^{+}$ MNC (E) formed under the four Mg^{2+} culture regimes (C). Data are expressed as mean ± SD. $^{a}p<0.05$, $^{b}p<0.01$.

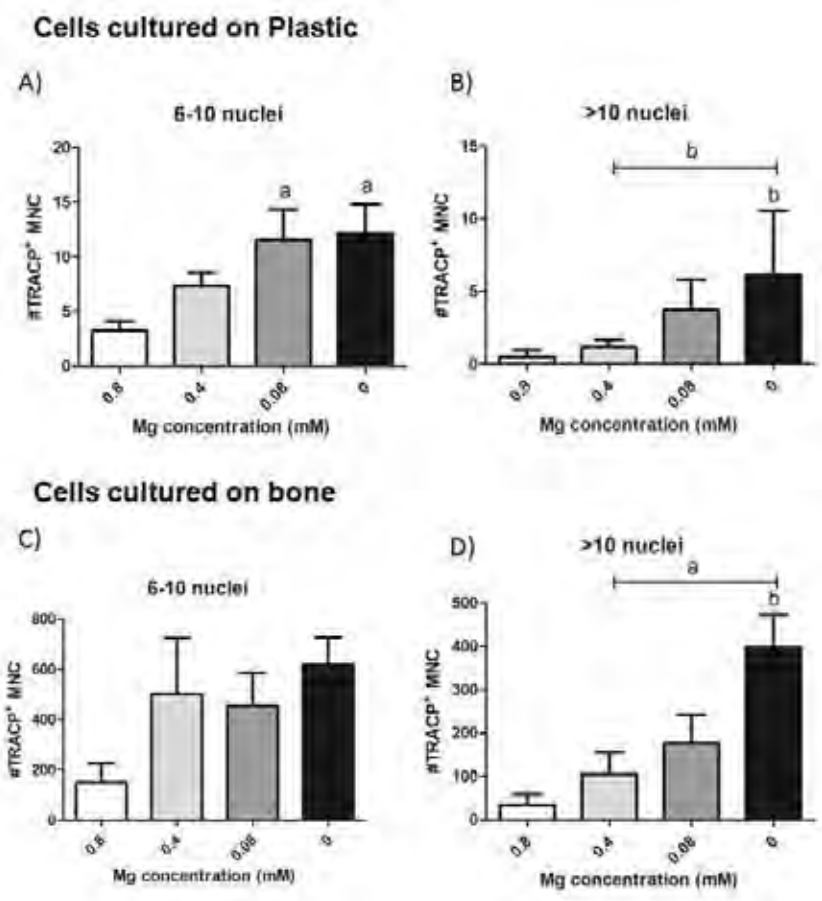

Figure 3 Number of $TRACP^{+}$ multinucleated cells formed by jaw marrow cells after 6 days of culture on plastic (A-B) and on bone (C-D) in four different Mg^{2+} concentrations in the presence of M-CSF and RANKL (n=6). Number of $TRACP^{+}$ multinucleated cells with 6-10 nuclei (A), > 10 nuclei (B) cultured on plastic. Number of $TRAP^{+}$

multinucleated cells with 6-10 nuclei (C), > 10 nuclei (D) cultured on bone. Data are expressed as mean ± SD. [a]p<0.05, [b]p<0.01.

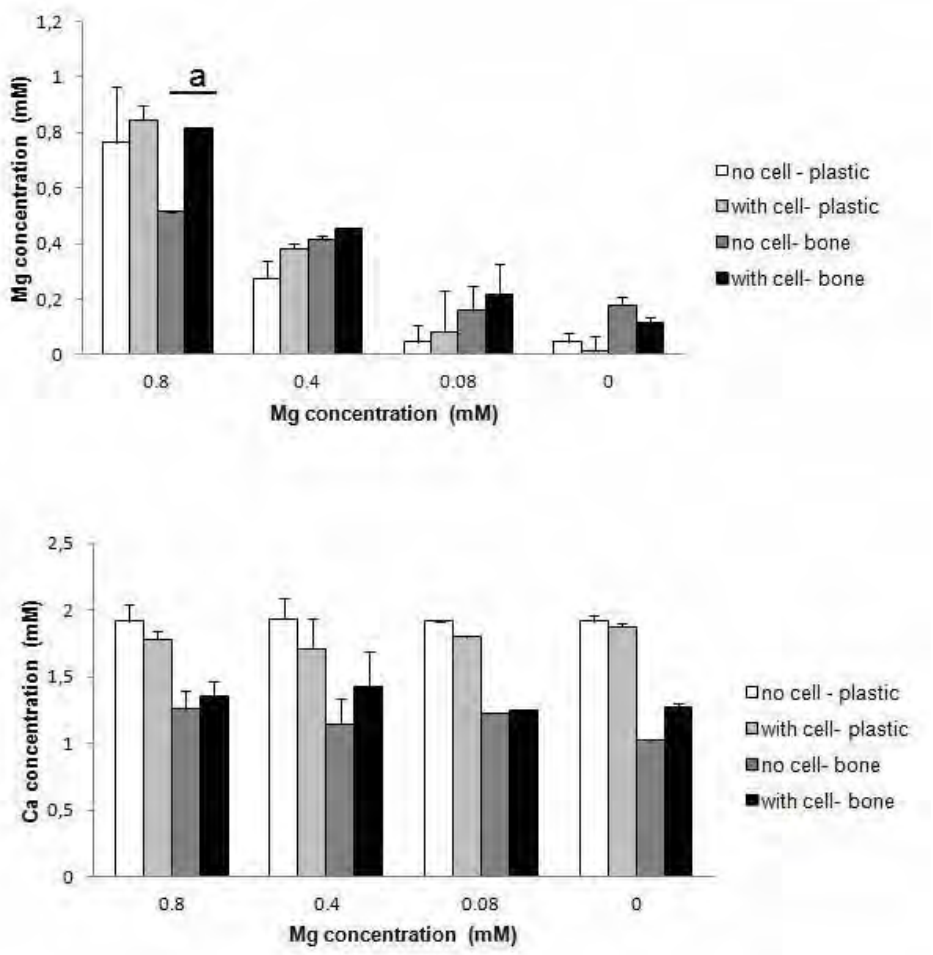

Figure 4 (A) Mg^{2+} concentration in the medium of cultures with and without cells (n=6). Cells were cultured on plastic or on bone for 6 days. (B) The Ca^{2+} concentration in the medium with and without cells cultured on plastic or on bone for 6 days (n=6). Data are expressed as mean ± SD. [a]p<0.05.

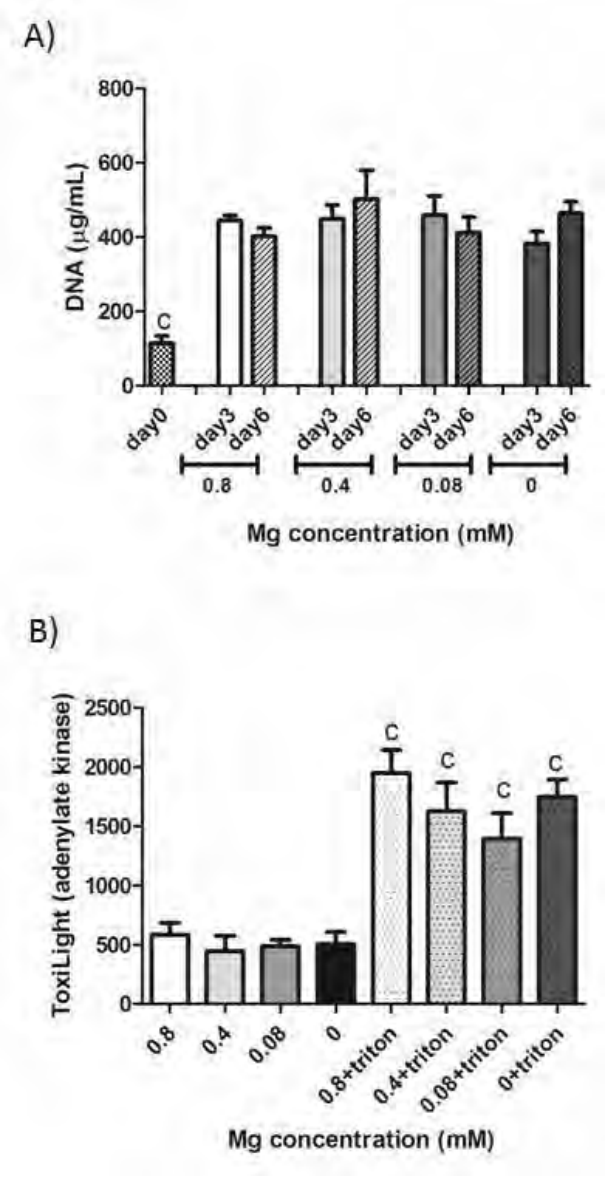

Figure 5 (A) The amount of DNA of cells from long bone marrow on day 0, 3 and 6 (n=6). $^{c}p<0.0001$. (B) The released amount of adenylate kinase was not altered under the different Mg concentrations. Triton X100 was added to the wells as a positive control. Data are expressed as mean ± SD. $^{c}p<0.0001$.

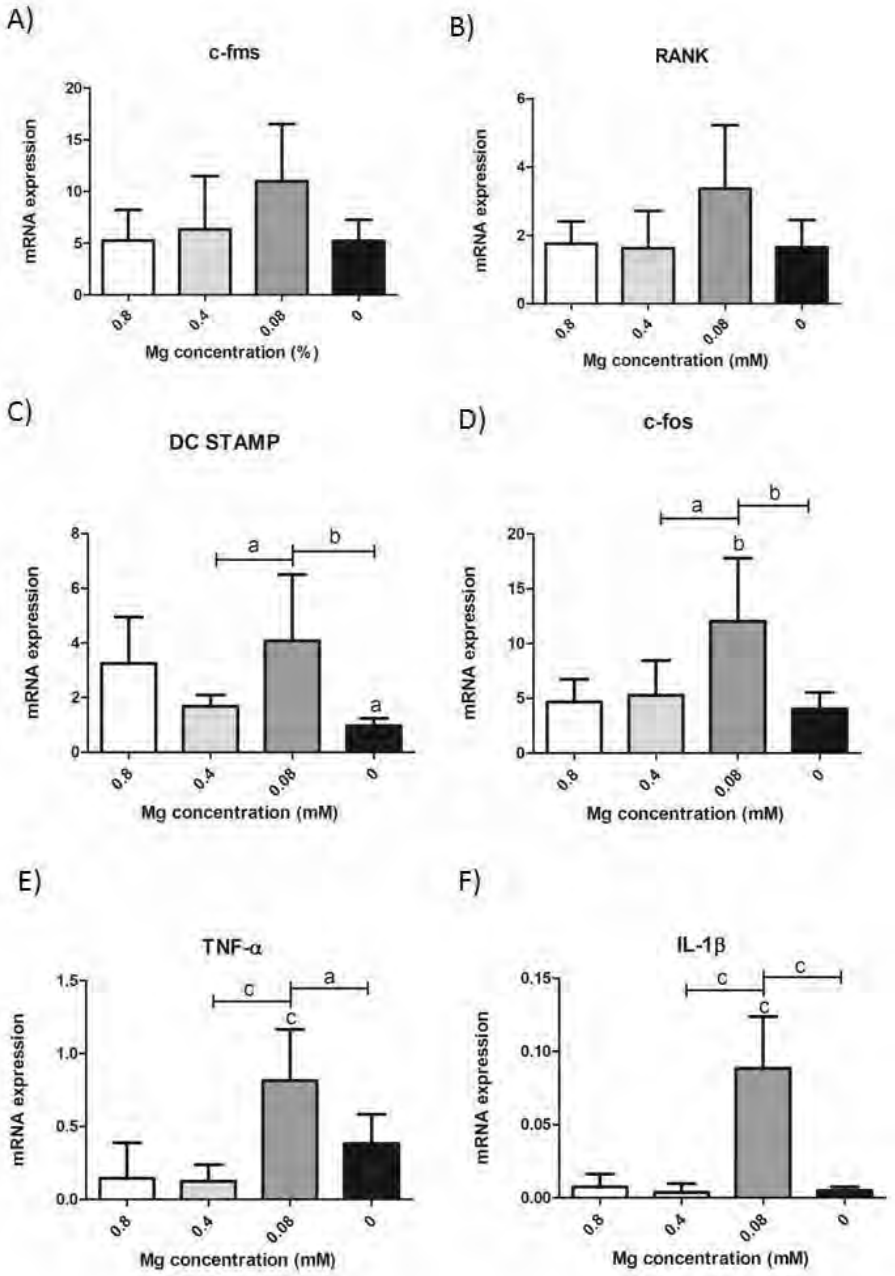

Figure 6 Gene expression of c-fms (A), RANK (B), DC-STAMP (C), c-fos (D), TNF-α (E) and IL1-β (F) by precursor cells from long bone marrow after 3 days of culture on plastic (n=6). The gene expression was normalized for the housekeeping gene PBGD. Data are expressed as mean ± SD. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.0001$.

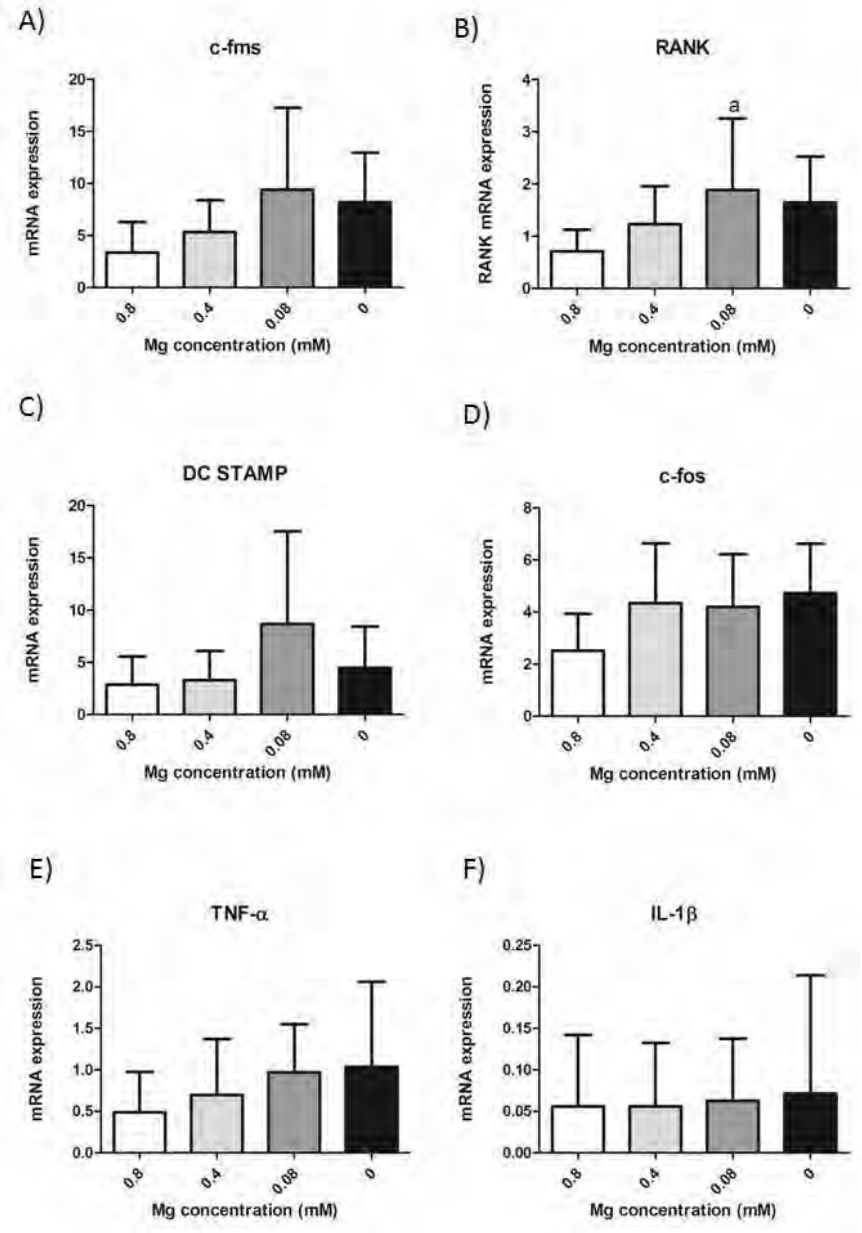

Figure 7 Gene expression of c-fms (A), RANK (B), DC-STAMP (C), c-fos (D), TNF-α (E) and IL1-β (F) by precursor cells from long bone marrow after 3 days of culture on bone (n=6). The gene expression was normalized for the housekeeping gene PBGD. Data are expressed as mean ± SD. $^{a}p<0.05$.

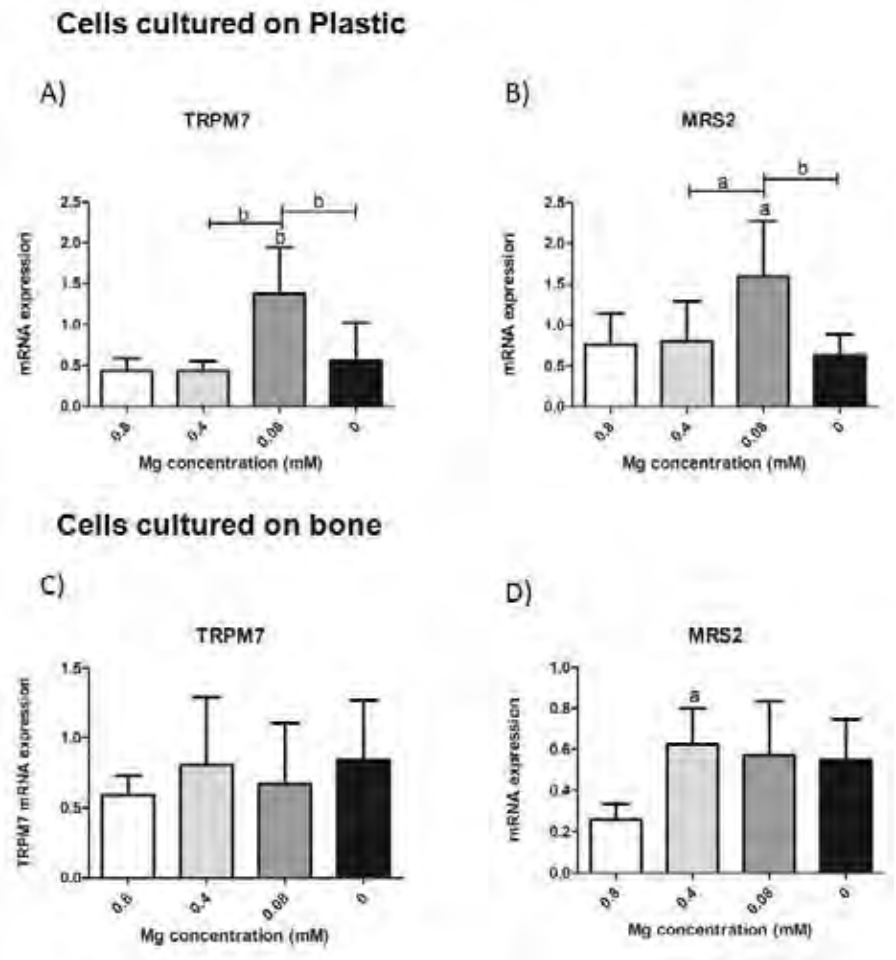

Figure 8 Gene expression of TRPM7 (A, C) and MRS2 (B, D) by precursor cells from long bone after 3 days of culture. Gene expression was assessed from cells cultured on plastic (A-B) and on bone (C-D), respectively (n=6). The gene expression was normalized for the housekeeping gene PBGD. Data are expressed as mean ± SD. $^{a}p<0.05$, $^{b}p<0.01$.

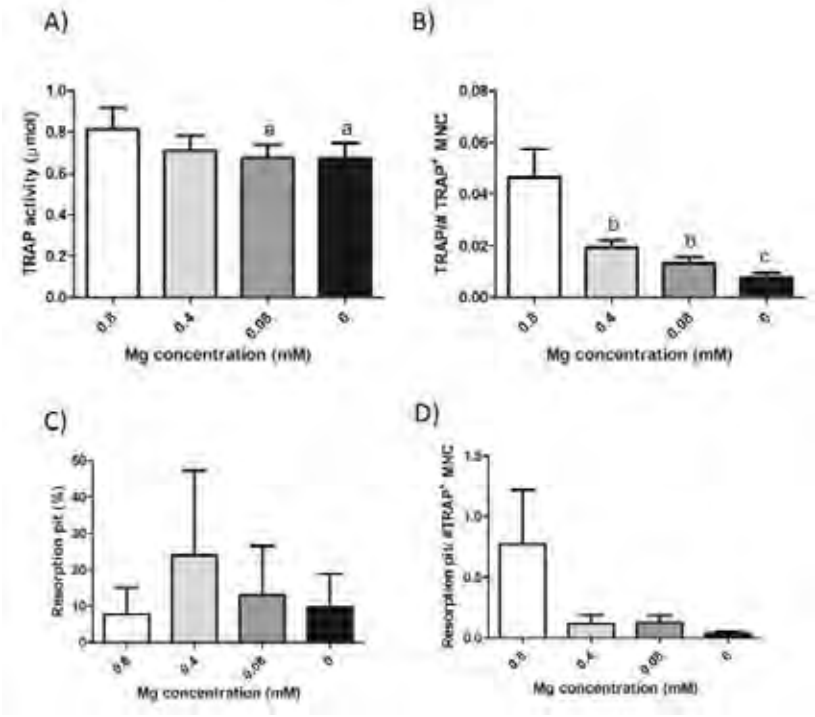

Figure 9 (A) Bone resorption after 8 days of culture. (B) Bone resorption corrected for the number of osteoclasts. (C) Levels of TRACP enzyme secreted in the medium. (D) Levels of TRACP corrected for the number of osteoclasts. Data are expressed as mean ± SD. [a]p<0.05, [b]p<0.01.

Table

Table 1 – Primer sequences used in the study.

Primer	Sequence	Amplicon length (pb)	Accession #
c-fms	GAAACGCGACCTTCAAAAACA GGCCGGATCTTTGACATACAA	100	ENSMUSG00000024621
c-fos	TCACCCTGCCCCTTCTCA CTGATGCTCTTGACTGGC TCC	64	ENSMUSG00000021250
DC STAMP	TGTATCGGCTCATCTCCTCCAT GACTCCTTGGGTTCCTTGCTT	100	ENSMUSG00000022303
IL-1β	GGACCCATATGAGCTGAAAGCT TGTCGTTGCTTGGTTCTCCTT	100	ENSMUSG00000027398
MRS2	GGGTCCTGATTGACGATTCG TCACATTGCGATGGCTGTCT	62	ENSMUSG00000021339
TNF-α	GCCACCACGCTCTTCTGTCT GTCTGGGCCATAGAACTGATGAG	100	ENSMUSG00000024401
TRPM-7	CAACCGGAGCTTGGATTTTAAC TGAGGGCATCACCAACATGT	68	ENSMUSG00000027365
RANK	TGGGCTTCTTCTCAGATGTCTTT TGCAGTTGGTCCAAGGTTTG	59	ENSMUSG00000026321
PBGD	AGTGATGAAAGATGGGCAACT TCTGGACCATCTTCTTGCTGA	122	ENSMUSG00000032126

CAPÍTULO 2

Magnesium intake deficiency and its effects on systemic and periodontal bone loss.

Marina M. Belluci, Gabriela Giro, Elaine M. S. Massucato, Rosa M. R. Pereira, Paulo S. Cerri, Elcio Marcantonio Junior, Carlos Rossa Junior, Silvana R. P. Orrico

Magnesium intake deficiency and its effects on systemic and periodontal bone loss.

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Running title: Mg deficiency and periodontal bone loss

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Abstract

Background and Objective: Magnesium (Mg) has an important role on bone homeostasis and its deficiency may result in bone mass loss. There has been little research regarding the effect of this deficiency on periodontal disease. The aim of this study was to evaluate the effect of Mg intake deficiency on alveolar bone resorption associated with periodontal disease.

Material and Methods: Thirty rats were randomly divided into two groups: control - animals fed a standard diet; Mg- animals fed a diet with 90% Mg deficiency. After 60 days on the diets, all animals received ligature on the lower left first molars to induce periodontal disease, and were euthanized after 30 days. Blood and urine were collected for determination of serum concentrations of Mg, calcium (Ca), osteocalcin (OCN), interleukin-6 (IL-6) and parathyroid hormone (PTH), as well as the urinary concentration of deoxypyridinoline (DPD). We determined the effect of Mg deficiency on systemic bone mineral density (BMD) evaluating dual energy x-ray absorptiometry (DXA) in femurs and vertebrae. Mandible BMD was assessed using digital radiography. Alveolar bone loss was evaluated by linear (CEJ-bone crest) and area measurements. We used RT-PCR to assess mRNA expression of RANKL, OPG and IL-6 in the gingival tissues.

Results: Mg deficiency was associated with higher concentrations of PTH, DPD and significant decreases on both systemic and mandibular BMD, as

well as greater severity of alveolar bone loss. There were no significant differences on mRNA expression for the target genes.

Conclusion: Severe deficiency of Mg reduces BMD and aggravates alveolar bone loss associated with ligature-induced periodontal disease.

Key words: periodontitis; osteoporosis; periodontal-systemic disease interactions; magnesium deficiency.

Introduction

Magnesium (Mg) is one of the main minerals present in the organism, playing a key role in several metabolic, regulatory and structural functions (1).

Epidemiological studies have demonstrated a positive correlation between individuals with Mg-deficient diet and increased bone mass loss and/or decrease of bone mineral density (BMD), which suggests that Mg deficiency can be a risk factor for osteoporosis (2-5).

Animal studies have shown that Mg plays an important role on bone tissue and that its deficiency may cause alterations such as greater bone fragility, reduction in the number and volume of bone trabeculae, uncoupling, deposition of disorganized bone matrix, and decrease of mineral content (6-9). Other studies using animal models with different levels of Mg deficiency showed that bone loss was characterized by a decreased volume of trabecular bone followed by increased release of substance P

and TNFα. These factors are believed to mediate the increased osteoclastic activity and bone resorption associated with Mg deficiency. In addition, decreased secretion of PTH has been observed in animals with concomitant calcium (Ca) deficiency, which may contribute to the decrease of bone formation (7, 10, 11). There also seems to exist an interference in the RANK-RANKL-OPG (Receptor activator of nuclear factor kβ- Receptor activator of nuclear factor kβ Ligant-Osteoprotegerin) axis, with predominance of RANKL over OPG under conditions of severe Mg deficiency, which would favor bone resorption (12). On the other hand, supplementation of Mg and Ca increased serum OPG levels, with consequent inhibition of bone resorption in ovariectomized rats (13).

Deficiency of Mg also affects the inflammatory process and immunological response, with increased production of inflammatory mediators, such as IL-1β and TNF-α. Even short-term (4 days) deficiency of Mg increased activation of macrophages and also serum levels of IL-6, indicating a role of Mg in the innate immune response (14).

Periodontitis is a multifactorial inflammatory disease initiated and maintained by microorganisms of the dental biofilm, which affects the supporting structures of the teeth. In spite of its infectious nature, behavioral, genetic and host-related factors ultimately determine the severity, susceptibility and progression of the disease (15, 16). The immunoinflammatory process of periodontal disease is regulated by chemokines, cytokines and prostaglandins (17). Activated lymphocytes,

macrophages and neutrophils present in the inflamed gingival tissue, secrete inflammatory mediators such as IL1β, TNFα, PGE_2, IL2, IL4, IL6, IL10 (16, 17). Animals fed a Mg-deficient diet had increased plasma levels of several of these mediators, namely IL1β, TNFα, PGE_2 and IL6 (5, 7, 12, 18). In addition, the nature of the immune cells present in the gingival tissues of animals on a Mg-deficient diet were increased in number and activity (18-21), suggesting that it might alter the progression of periodontal disease.

The relevant role played by Mg in the inflammatory process and bone metabolism raised the hypothesis that deficiency of Mg could also influence the inflammation-associated alveolar bone loss that is the hallmark of destructive periodontitis. In this study we determine the effect of severe Mg deficiency on both systemic bone homeostasis and alveolar bone loss associated with ligature-induced periodontitis.

Materials and methods

Animals

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

This study included 30 rats (*Rattus Norvegicus, albinus, Holtzman*), 60 days old, weighing approximately 180g that were randomly divided into two groups (n=15) according to their diet. The control group (CTL) was fed regular rat chow with normal concentration of magnesium (507 mg/kg) (22); and the magnesium deficient group (Mg) was fed rat chow with 90% reduction in the normal Mg content (50.7 mg/kg). The animals were kept in individual stainless steel cages under controlled environmental conditions. Animals received a daily ration of 25g of standard or Mg deficient rat chow (23) and distilled water ad libitum.

The rat chow used in this study was prepared according to AIN-93 (American Institute of Nutrition) for maintenance of rodents (22, 24). It was formulated by a specialized company (Pragsoluções Biociência, Jaú, SP, Brazil) and was chemically analyzed for determination of the centesimal composition to ensure the Mg content.

Experimental protocol

The animals received the standard (CTL) or the Mg deficient rat chow for 60 days previously to the beginning of the experimental period in order to induce systemic magnesium deficiency in the test group (Mg) on Day 0 (start of the experimental period) (11).

The experimental period (day 0, after 60 days on the assigned rat chow) the animals were anaesthetized with a combination of

ketamine chloridrate (0.08 mL/100g body weight) (Ketamina Agener, Agener União Ltda, São Paulo, SP, Brazil) and xylazine chloridrate 2% (0.04 mL/100g) (Rompum, Bayer S.A., São Paulo, SP, Brazil) and the lower left first molars received a cotton thread ligature tied around the cervical portion of the teeth. The first molars on the right side were kept as healthy controls. Thirty days after ligature placement (90 days of the beginning of the study), all animals were euthanized by an overdose of general anesthetics.

Biochemical evaluations

Blood samples were collected from caudal artery early in the morning on the day of euthanasia. Serum levels of Magnesium (Magnésio, Labtest Diagnostica SA, Lagoa Santa, MG, Brazil) and Calcium (Cálcio Arsenazo Liquiform, Labtest Diagnostics SA, Lagoa Santa, MG, Brazil) were determined with a colorimetric method. Enzyme-linked immunosorbent assays (ELISA) determined the serum concentrations of osteocalcin (OCN) (Rat Osteocalcin EIA Kit, Biomedical Technologies Inc., Stoughton, MA, USA) and parathyroid hormone (PTH) (Rat Total Intact PTH ELISA Kit, Scantibodies Laboratory Inc., Santee, CA, USA). Deoxypyridinoline (DPD) levels in the urine were measured by ELISA (DPD Metra®, Quidel Corporation, San Diego, CA, USA) and corrected for urinary concentration of creatinine (Creatinina ref. 35-100, Labtest, Lagoa Santa, MG, Brazil) (DPyr/creatinine; nM/mM) that was determined with a colorimetric method.

Bone densitometry

Bone mineral density (BMD) of the femur and lumbar vertebrae were measured by Dual Energy X-Ray Absorptiometry (DXA) using a densitometer (Discovery QDR, Hologic, Bedford, MA, USA) in the high-resolution mode and the software “Small Animal” supplied by the densitometer’s manufacturer. Data area was expressed in g/cm^2 (Figures 2A, B and C).

Alveolar bone density

The right (no ligature control) hemimandibles from 10 rats of each group were radiographed with a direct digital imaging system – CDR (Shick Technologies Inc., Long Island, NY, USA). Exposure of the digital sensor was set to 70 KVp and 10 mA/15 pulses per second. Specimens were positioned perpendicular to the central beam and parallel to the sensor at a standardized focus to sensor distance of 40 cm. An aluminum step-wedge was positioned on the sensor, next to the specimens as a reference for relative densitometry. TIFF (Tagged Image File Format) image files without compression were used with the image analyzer software (Pragsoluções Biociência, Jaú, SP, Brazil).

A single trained and calibrated examiner blind to the experimental group coding of the images evaluated all the digital radiographies. The pixel

intensity on the image of each step of the aluminum reference was used to determine the relative bone density (expressed in aluminum equivalents, Al.Eq.) in each of the three regions of interest (ROIs) presented at Figure 2D and E.

Measurement of periodontal bone loss

Gingival soft tissues of the left hemimandibles (ligature side) were removed, stored in trizol in -80ºC and used for subsequent RT-PCR analysis. The specimens were soaked in 3% hydrogen peroxide for 24 h in order to remove remaining soft tissues and subsequently stored in 70% ethanol. Linear and area measurements of periodontal bone loss were performed on the buccal side of the molars on the defleshed left hemimandibles of 10 rats of each group. The distance from cemento-enamel junction to the alveolar crest (CEJ to AC) was measured on the long axis of each root of the first molar (Figure 3A) and the alveolar bone loss area was assessed including first and second molars (Figure 3B), under magnification of x25. Measurements were made by using image analyzer software (ImageJ – 1.40g/Java 1.6.0, National Institute of Health, USA – http://rsb.info.nih.gov/ij/).

Histological analysis

The left mandibles of 5 animals per group were fixed in 4% neutral buffered formalin for 48 hours and decalcified for 28 days in 10% ethylenediamine tetra-acetic acid (EDTA, pH 6.9), changing the solution every 24 hours. After decalcification, the samples were dehydrated in graded concentrations of ethanol and embedded in paraffin. Serial sections (5 µm thick) were obtained on the sagittal plane. After staining with hematoxylin and eosin (HE), digital images were obtained with a Olympus BX-51 light microscope and analyzed. A single trained examiner who was blind to the experimental groups analyzed these images (Image Pro-Express 6.0; Olympus, Tokyo, Japan) measuring the linear distance between the cementum-enamel junction (CEJ) and the junctional epithelium (JE)

RNA isolation and reverse transcription– polymerase chain reaction (RT-PCR)

Gingival tissues surrounding both lower first molars of 10 animals were collected separately, and homogenized with a dounce homogenizer in Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA).

The mRNA expression of receptor-activor of nuclear factor-kB ligand (RANKL), osteoprotegerin (OPG) and interleukin 6 (IL-6) was assessed by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from tissue homogenates in Trizol according to the manufacturer's instructions (Promega Corporation, Sunnyvale, CA, USA). The quantity

and purity of total RNA were determined on a Biomate 3 (Thermo Fisher Scientific Inc, Waltham, MA, USA) spectrophotometer. Isolated RNA (700 ng/µL) was reverse transcribed using the Improm II system (Promega Corporation, Sunnyvale, CA, USA) in the presence of random primers (Invitrogen Corporation, Carlsbad, CA, USA). The subsequent complementary DNA was amplified by PCR reaction, in a MyCycler thermocycler (Bio-Rad Laboratories Inc., Hercules, CA, USA), with Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA, USA) using previously optimized conditions. The primer sequences used for DNA amplification and PCR conditions are shown in Table 1. Amplification of the β-actin gene (Invitrogen Corporation, Carlsbad, CA, USA) was used as an internal control. The PCR products were resolved by electrophoresis on 1.5% (w/v) agarose gels containing ethidium bromide (0.5 mg/mL) to visualize the PCR products. The amplified DNA bands were analyzed densitometrically after digital imaging capture (Image Quant 100 – GE Healthcare Fairfield, CT, USA), using Image J 1.32j software (National Institute of Health, USA – http://rsb.info.nih.gov/ij/). The density of the bands corresponding to RANKL, OPG and IL6 mRNA in each sample was normalized to that of the band for the housekeeping gene (β-actin).

Data analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Prism 5.0, San Diego, CA, USA). Mann Whitney's test was conducted for

comparisons between groups for all analyzed parameters. Significance level was set at 5%.

Results

Bone remodeling biomarkers in serum and urine

The effectiveness of the experimental model is confirmed by statistically significant decrease of the serum concentration of Magnesium (Figure 1A) on the experimental (Mg) group when compared to control (CTL) ($p<0.0001$). There was no difference between groups on serum levels of Calcium (Figure 1B).

Magnesium deficiency was associated with significant increases on serum levels of PTH ($p=0.037$) and urinary DPD ($p=0.030$) (Figure 1C and 1D, respectively). Although serum concentration of OCN was reduced in the Mg group (Figure 1E), the difference was not statistically significant ($p=0.0906$).

Bone densitometry

Bone mineral density in the Mg group was significantly reduced for both femur ($p=0.0079$) and lumbar vertebrae ($p=0.0010$) (Figure 2C). The relative radiographic density of the alveolar bone on right side (no ligature control) of the mandible was also significantly lower values in the Mg group on ROI 1 ($p=0.0076$). There were no statistically significant differences between groups on ROI 2 ($p=0.3207$) and ROI 3 ($p=0.2238$) (Figure 2E).

Inflammation-associated alveolar bone resorption

Both linear (Figure 3C; p=0.007) and area (Figure 3D; p=0.0379) measurements indicate a significantly greater resorption of alveolar bone in the left (ligature side) hemimandibles on the Mg deficient group. Figures 3E and 3F are representative of the macroscopic bone loss pattern for CTL and Mg groups, respectively.

Morphological Findings

In HE-stained sections, the healthy periodontium (right side, no ligatures) exhibited a typical interdental papilla between the molars for both groups. Usually, the apical portion of the junctional epithelium was situated at the cementum-enamel junction; bundles of collagen fibers were subjacent to the junctional epithelium. In the narrow periodontal space several collagen bundles and fibroblasts were observed. On the other hand, evident destruction and inflammatory infiltrate were observed in the periodontium with ligature-induced periodontal disease (left side) on both CTL (Figure 4A-4C) and Mg groups (Figures 4D-4E). The interdental papilla was severely inflamed with extensive degradation and disorganization of collagen and loss of epithelium; the junctional epithelium was juxtaposed to the cementum surface, apically to the cementum-enamel junction (Figures 4A, 4B and 4D). Moreover, the alveolar bone crest was partially resorbed (Figures 4A and 4D); several osteoclasts were observed in

eroded bone sufaces of the alveolar process in the rats from the Mg group (Fig. 4F). In fact, resorption of the alveolar bone crest was significantly ($p=0.0286$) greater on the Mg group, as indicated by linear measurements between the CEJ and the bone crest.

An inflammatory infiltrate was found in the gingival mucosa (Figures 4D and 4E) and on the periodontal space on the furcation region in both CTL and Mg groups; but the severity of the inflammation was markedly greater on the Mg group (Figures 4A, 4C and 4D).

Regulation of genes related with bone tissue turnover

Although the mRNA expression levels of RANKL, OPG and IL6 were not significantly different between Mg and CTL groups in both healthy (right side) and diseased (left side) gingival tissues, Figure 5 indicates a discrete increase in RANKL and OPG mRNAs and a decrease of IL6 mRNA expression on the healthy gingival tissues (right side) from Mg group. Similarly, a distinct (albeit not statistically significant) decrease on OPG and increase on IL-6 mRNA is observed on periodontal disease gingival tissues (left side).

The regulation of the expression of these candidate genes was also evaluated compared between healthy (right side) and diseased (left side) gingival tissues within the same experimental group (Mg and CTL). There were no differences between healthy and diseased tissues on the mRNA

expression of these genes in CTL group (RANKL p=0.2197; OPG p=0.591; IL6 p=0.9733). However, mRNA expression of OPG (p=0.0061) was significantly reduced and the mRNA expression of IL6 (p=0.0089) was significantly increased in the diseased tissues of the Mg group, whereas RANKL mRNA expression was not significantly regulated (p=0.0978, Figure 5C).

Discussion

Magnesium deficiency is more prevalent in industrialized countries, where diet habits negatively affects the intake amount and absorption of this mineral (21, 25, 26) . Under physiological conditions, Magnesium plays a critical role in regulating bone and mineral metabolism (5-7, 10-12), which suggests that its deficiency may have deleterious effects on the homeostasis of mineralized tissues. In this study, we show that magnesium deficiency has a direct effect on bone metabolism, as supported by a significant decrease of bone mass in the femur and lumbar vertebrae of experimental animals. Furthermore, we also present data indicating that Mg deficiency augments the severity of alveolar bone loss induced by experimental periodontal disease, demonstrating that Mg plays a role not only in bone homeostasis but also in the modulation of bone turnover associated with an immunoinflammatory process. It is well known that macro and micronutrients modulate the inflammatory and immune response (21, 27, 28). Several studies reported that experimental

magnesium deficiency in rats induced clinical inflammatory syndrome after a few days of deficiency, mainly characterized by the increased number and activation of leukocytes and neutrophils (18, 19, 21, 29). One of the mechanisms related to the increased bone loss associated with Mg deficiency is its indirect modulation of PTH secretion and action. Depletion of Mg was shown to disturb Calcium homeostasis, which is followed by an increase of PTH secretion and subsequent mobilization of Calcium from the bones (7, 30, 31). In the present study, serum level of magnesium was significantly reduced in the Mg group, demonstrating the effectiveness of our experimental protocol for a diet-induced deficieny of Mg; however Calcium serum levels were not altered. We did observe a significant increase on serum levels of PTH in the Mg group. PTH is considered a bone-resorbing factor when present in sustained elevated systemic levels (32), and this role may be related to the maintenance of the serum Calcium levels in the Mg group, due to increased mobilization of Ca from the bones. The differences between our findings and previous reports indicating a change in Ca levels with Magnesium deficiency may be attributed to the severity of the deficiency and other experimental model characteristics. The role of Magnesium deficiency on the decrease of bone mass is further supported by the changes on bone turnover markers: a significant increase of urinary DPD and a clear, albeit not statistically significant, decrease for serum OCN levels on Mg group when compared to the CTL group. The net result of these findings suggests uncoupling of

bone turnover, with increased bone resorption and impaired bone formation in the Mg group.

Several studies have shown that magnesium deficiency is associated with reduced bone mass (2-4, 7, 11, 12, 33, 34), and it is suggested that Mg deficiency may lead to osteoporosis (5-8). Our findings support this hypothesis, as we verified a significant loss of bone mass, demonstrated by decreased BMD values in femur and lumbar vertebrae of the experimental animals 90 days after the onset of magnesium deficiency.

Alveolar bone may be less sensitive to Magnesium deficiency effects, since we observed a significant decrease on relative bone density on only one region of interest, out of three different areas assessed on the mandible. The fact that BMD was significantly affected by Mg deficiency on femur and lumbar vertebrae, but not on the mandible suggests a site-specific effect that may be related with anatomical features of different bones or load-bearing characteristics of these bones. Even though we have to consider the different experimental approaches used to evaluate BMD on the femur, vertebrae (DXA) and alveolar bone (digital radiography), it is tempting to speculate that the irregular shape, occlusal forces generated by gnawing behavior and the type of mechanical stress transmitted to the mandible of the rodents plays a role bettering the improved maintenance of the BMD in the mandible (35).

Alveolar bone loss is the hallmark event in periodontitis. Our model induced alveolar bone loss, which was of significantly greater severity in the Mg group, represented by greater distance between the cementum-enamel junction and the bone crest (vertical bone loss) and by a greater area of exposed root surface (area of bone loss). This greater bone loss appears to be at least in part caused by increased resorptive activity, since the histomorphological findings show an increased prevalence of osteoclasts adjacent to resorption surfaces of the alveolar process in the Mg group in comparison with the CTL group. The morphological findings indicate a marked increase of the linear distance between the cementum-emanel junction and the apical limit of the junctional epithelium in the Mg group, supporting the findings of increased bone loss in the macroscopic analysis.

Since alveolar bone loss was affected by Mg deficiency, we evaluated the regulation of bone-related cytokines IL-6, RANKL and OPG. There was no difference on the expression of these genes when comparing healthy and diseased sites between the experimental groups (CTL and Mg). However, there was a significant increase on IL-6 and decrease on OPG mRNA expression in the periodontal disease sites of the Mg- group. This suggests that Mg deficiency may aggravate the inflammatory reaction associated with periodontal disease. This increased severity of inflammation could be associated with the greater loss of alveolar bone observed in the Mg group. Indeed, the histomorphological analysis

indicated a greater severity of the inflammatory infiltrate, in support to this hypothesis. It is important to bear in mind the limitations associated with the evaluation of gene expression only at the mRNA level (e.g., limited half-life of the mRNA, lack of correspondence between mRNA and protein levels) and also to consider that the RNA used for this analysis was obtained from the connective tissues overlaying the alveolar bone. These limitations may account for the lack of regulation on RANKL mRNA. With these methodological limitations in mind, the increase on IL-6 mRNA in the periodontal disease sites of the Mg group may be associated with enhanced resorptive activity and indirectly also with the impaired expression of OPG. Liu et al. (36) reported a cross-talk between IL-6 and PGE2 on osteoclast activation and suppression of OPG production by osteoblasts. This indirect effect of IL-6 on bone homeostasis may be one of the mechanisms that could explain the increased bone resorption presented in Mg group.

In summary, our findings indicate that magnesium deficiency is associated with systemic loss of bone mass and increased resorption of alveolar bone in the presence of periodontal disease-associated inflammation. These results suggest that Mg deficiency may influence on severity of periodontal disease. Further studies are necessary to obtain insight into the biological mechanisms involved in the modulation of bone turnover both in the presence and in the absence of inflammation. This knowledge may

disclose a relevant and therapeutically accessible factor involved in the control of bone metabolism.

Acknowledgments

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Figures

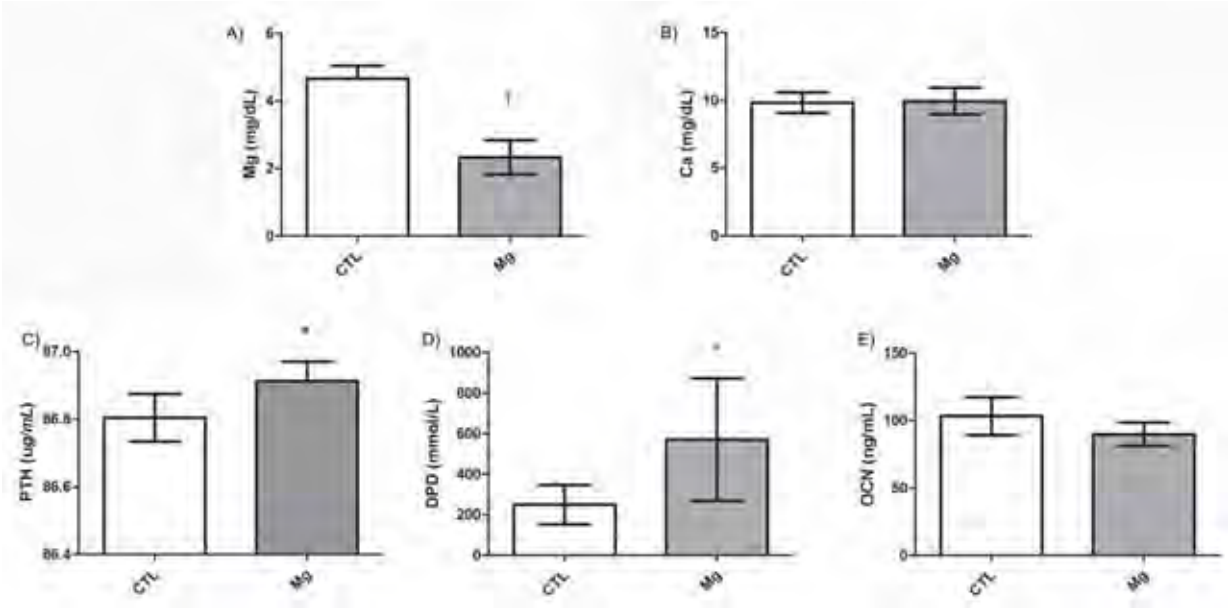


Figure 1. Biochemical evaluation of serum (Mg, Ca, PTH and OCN) and urine (DPD) concentrations for the different groups of the study.

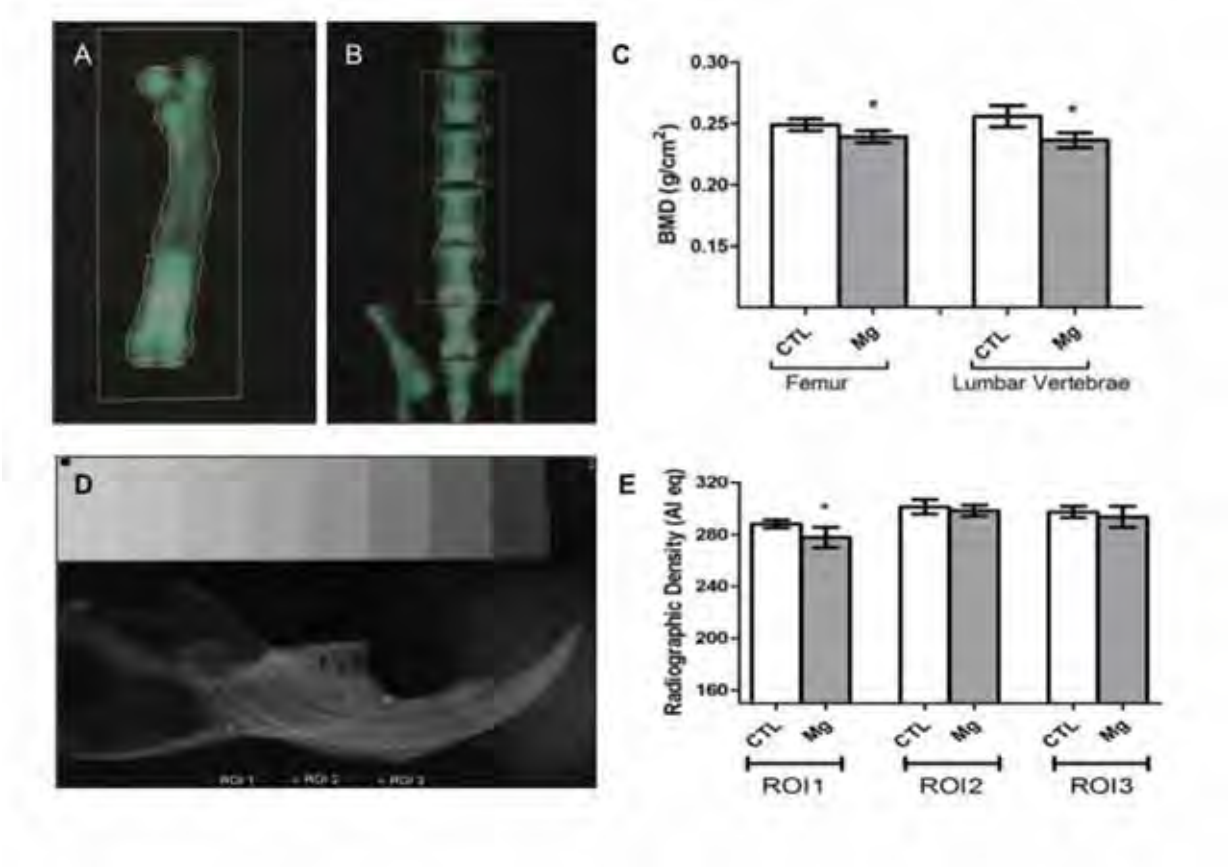


Figure 2. Bone densitometry of femur (A) and lumbar vertebrae (B). (C) graphical representation of mean ± 95% confidence interval of BMD values. In this figure is also presented a representative radiograph showing the regions of interest (D) in which was measured the mandible radiographic bone density (E). * $p<0.05$ in relation to the CTL group.

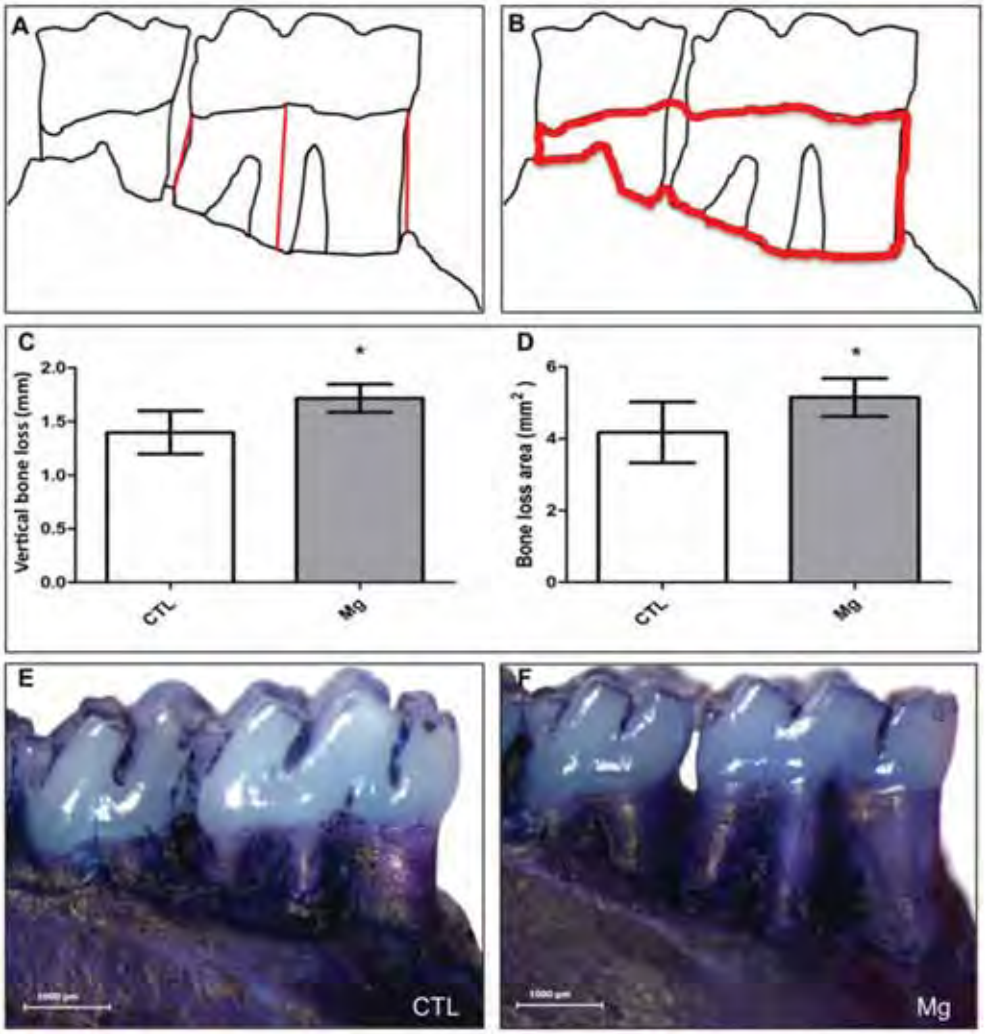
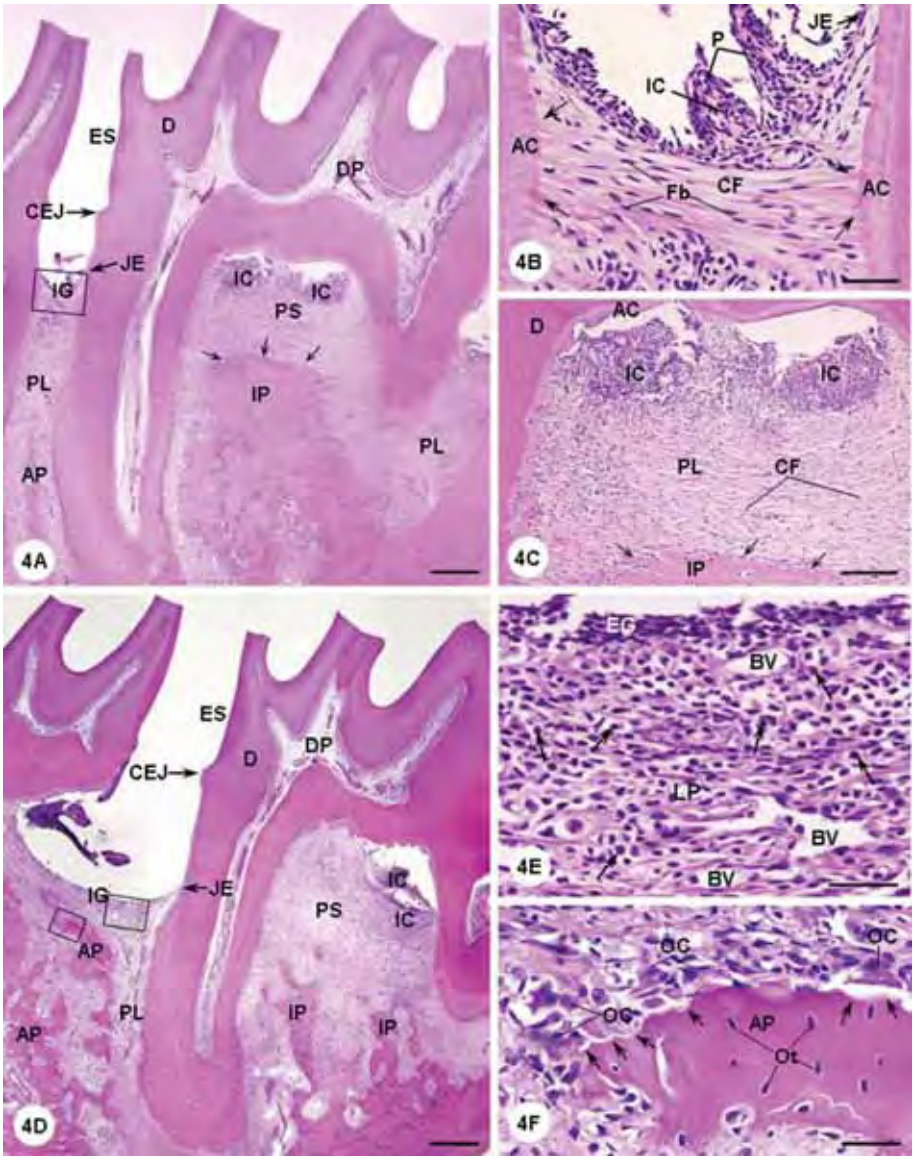


Figure 3. Representative diagram of vertical bone loss (A) and bone loss area (B) measured under x25 magnification. (C) and (D) represent mean ± 95% confidence interval of both measurements, while (E) and (D) represent periodontal bone loss for CTL and Mg groups respectively.



Figures 4A-4C – Light micrograhs of a sagittal section of the periodontal disease site of the CTL group. In **4A**, the interdental papilla located between the first and second molars is destroyed. The junctional epithelium (JE) is juxtaposed to the acellular cementum surface, apical to the cemento-enamel junction (CEJ). The alveolar interdental process (AP) is formed by a thin bone septa. At furcation region, numerous inflammatory cells (IC) are present in the wide periodontal space (PS) next

to root surface. The regular surface of alveolar interradicular process (IP) partially reabsorbed (arrows) is covered by osteoblasts. IG, interdental gengiva; PL, periodontal ligament; ES, enamel space; DP, dental pulp; D, dentine. Bar, 350µm. In **4B**, outlined area of 4A, the gingival mucosa exhibits some protrusions (P) towards to the interdental space; inflammatory cells (IC) are observed in the lamina of the gingival mucosa. Note the presence of bundles of collagen fibers (CF) subjacent to the gingival mucosa; some of these fibers bundles (arrows) penetrate into the acellular cementum (AC) surface. Bar, 50µm. The **figure 4C**, furcation region of the figure 4A, shows numerous inflammatory cells densely aggregated (IC) located next to the acellular cementum (AC) surface. Some collagen fibers (CF) are observed in the periodontal ligament (PL) subjacent to the inflammatory process (IC). Osteoblasts (arrows) cover the bone surface of the interradicular process (IP). D, dentine. Bar, 150µm.

Figures 4D-4F – Light micrograhs of a sagittal section of lower molars with induced periodontal disease in Mg group. In **4D**, likewise in the CTL group, the interdental papilla located between the first and second molars is destroyed; interdental gingiva (IG) exhibits a thin layer of epithelial cells covering the lamina propria. The apical end of the junctional epithelium (JE) is away from the cemento-enamel junction (CEJ). The alveolar interdental process (AP), partially reabsorved, shows irregular bone trabeculae. The periodontal space (PS), at furcation region, shows evident enlargement; inflammatory cells (IC) are present adjacent to the root

surface. The alveolar interradicular process is restricted to some thin trabeculae (IP). PL, periodontal ligament; ES, enamel space; D, dentine; DP, dental pulp. Bar, 350µm. In **4E**, outlined area of 4D, shows a portion of gingival mucosa. The flattened epithelial cells (EG) cover the lamina propria (LP) which contains numerous inflammatory cells (arrows) and blood vessels (BV). The **figure 4F**, area outlined of the alveolar interdental process (AP) in 4D, shows several osteoclasts (OC) adjacent to resorption sufaces of the alveolar process (arrows). Ot, osteocytes. Bar, 50µm.

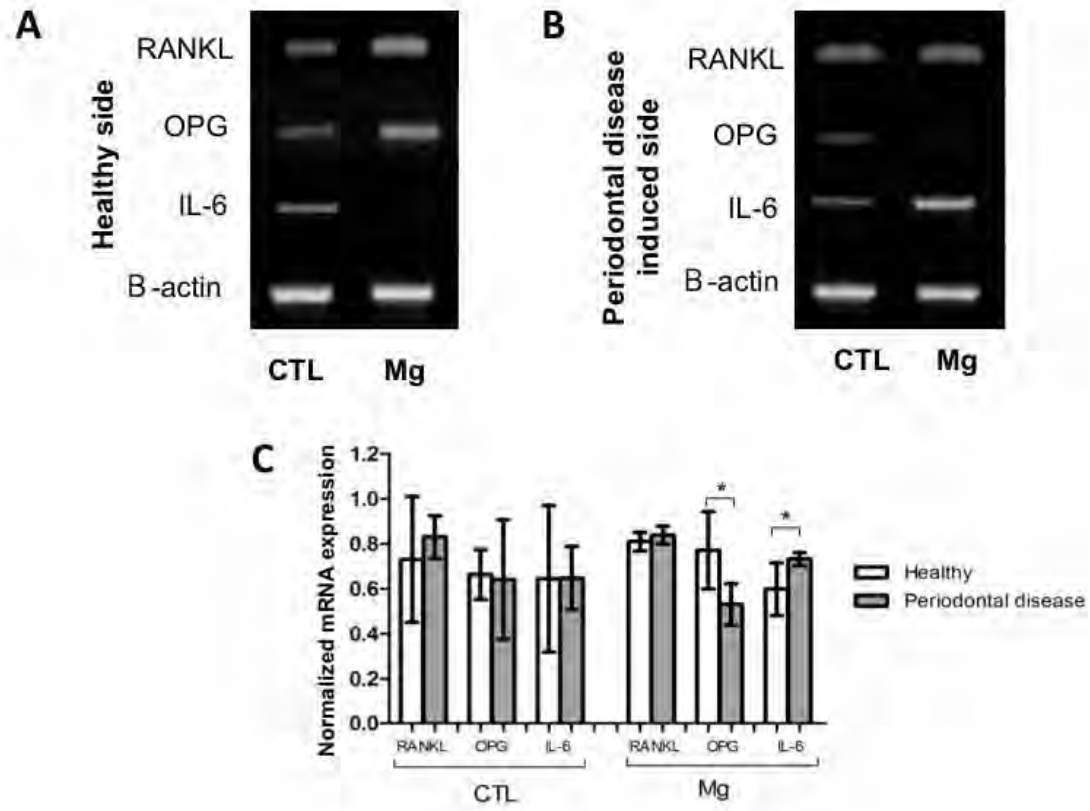


Figure 5. Normalized mRNA expression for RANKL, OPG and IL6 among the different groups of the study on the healthy (A) and the periodontal disease side (B) and the comparison of the expression of the normalized mRNA among the healthy and periodontal disease side on the same group (C).

TABLE

Table 1 – Primer sequences and PCR conditions used in the study.

Gene	Primers (5' – 3') Sense (S) and antisense (AS)	Acession#	Ta (^{0}C)	Amplicon	Cicles
β-actin	TCTCAGCTGTGGTGGTGAAG– S TGTCACCAACTGGGACGATA– AS	NM031144	57	437 bp	30
RANKL	ACGCAGATTTGCAGGACTCGAC – S TTCGTGCTCCCTCCTTTCATC – AS	F019048	60	493 bp	36
OPG	TCCTGGCACCTACCTAAAACAGCA – S CTACACTCTCGGCATTCACTTTGG - AS	U94330	57	578 bp	36
IL-6	CCGGAGAGGAGACTTCACAG – S GAGCATTGGAAGTTGGGGTA - AS	NM012589	56	428 bp	30

CAPÍTULO 3

Effects of magnesium intake deficiency on bone metabolism and bone tissue around osseointegrated implants.

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Effects of magnesium intake deficiency on bone metabolism and bone tissue around osseointegrated implants.

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Running title: Effects of magnesium deficiency intake on bone tissue

Key words: magnesium deficiency, dental implants, bone markers, rat.

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Abstract

Objectives: This study evaluated the effect of magnesium dietary deficiency on bone metabolism and bone tissue around implants with established osseointegration.

Materials and Methods: For this, 30 rats received an implant in the right tibial metaphysis. After 60 days for healing of the implants, the animals were divided into groups according to the diet received. Control group (CTL) received a standard diet with adequate magnesium content while test group (Mg) received the same diet except for a 90% reduction of magnesium. The animals were sacrificed after 90 days for evaluation of calcium, magnesium, osteocalcin and parathyroid hormone serum levels and the deoxypyridinoline level in the urine. The effect of magnesium deficiency on skeletal bone tissue was evaluated by densitometry of the lumbar vertebrae while the effect of bone tissue around titanium implants was evaluated by radiographic measurement of cortical bone thickness and bone density. The effect on biomechanical characteristics was verified by implant removal torque testing.

Results: Magnesium dietary deficiency resulted in a decrease of the magnesium serum level and an increase of parathyroid hormone and deoxypyridinoline levels ($p \leq 0.05$). The Mg group also presented loss of

systemic bone mass, decreased cortical bone thickness and lower values of removal torque of the implants ($p \leq 0.01$).

Conclusions: The present study concluded that magnesium deficient diet had a negative influence on bone metabolism as well as on the bone tissue around the implants.

Introduction

Magnesium is one of the most abundant minerals in the body and is essential for several enzymes and cell functions, acting as an important modifier of the inflammatory and immune response (Maguire & Cowan, 2002). It also plays a relevant role on bone tissue and mineral homeostasis and may directly affect the function of bone cells and the hydroxyapatite crystal growth (Creedon et al. 1999).

It is estimated that from 2.5% to 15% of the world population suffers from some form of hypomagnesemia (Sabbagh et al. 2008). Magnesium dietary deficiency may be common in industrialized countries, as reported in the United States. (Ford & Mokdad 2003, Marx & Neutra 1997) and European countries (Schimatschek et al. 2001; Touvier et al. 2006). Although it is a mineral found in many foods, its absorption requires ideal conditions and may be easily inhibited by several factors. Even after being absorbed in the body, several substances contribute to increased kidney excretion of

magnesium such as excessive alcohol intake, diuretics, coffee, tea, salt, phosphoric acid and sugar, all common in diets nowadays (Johnson 2001). Some manifestations may be related to a deficiency such as hypertension, vascular function alteration, insulin resistance and/or altered insulin secretion (Evangelopoulos et al. 2008).

Some epidemiological studies show a positive correlation between a magnesium deficient diet and increase in loss of bone mass and/or decrease in bone density, which suggests that this mineral deficiency may be a risk factor for osteoporosis (New et al. 1997; Tucker et al. 1999; Wang et al. 1999). Animal studies with different levels of deficiency showed bone loss, characterized by decrease of trabecular bone volume, followed by increase in the release of pro-inflammatory cytokines and alteration in secretion and action of the parathyroid hormone (PTH), contributing to the decrease in bone formation (Rude et al. 2004; Rude et al. 2005; Rude et al. 2006). For a prolonged period (12 months), a magnesium deficient diet induced the bone mass loss of lumbar vertebrae and femurs in addition to biomechanical and histomorphometric changes of bone tissue in rats, similar to those in human osteoporosis (Stendig - Lindberg et al. 2004). For this reason it was hypothesized that bone changes caused by magnesium deficiency could be a risk factor for maintenance of the implants to the extent that they affect bone remodeling.

The purpose of this study was to evaluate the effect of magnesium deficiency on systemic bone metabolism and bone tissue around implants with established osseointegration.

Materials and Methods

Animals

This study was approved by the Animal Experimentation Ethics Committee of the Araraquara Dental School - UNESP (Protocol# 20/2006).

Thirty 60 days-old rats (*Rattus Norvegicus, albinus, Holtzman*) were used in this study. The animals were kept in individual stainless steel cages in the animal facility with controlled temperature, humidity and light exposure.

Animals were divided into two groups (n = 15) according to their diet. The control group (CTL) was fed on a diet with standard daily magnesium content (507 mg/kg of Mg) according to AIN-93 (American Institute of Nutrition) for maintenance of rodents (Reeves et al. 1993; Reeves 1997). The test group (Mg) was fed on a diet with 90% reduction in the magnesium content (50.7 mg/kg of Mg). Body weight was monitored weekly to assess the animal's growth.

Study Design

On day 0, all animals underwent surgery for implants placement in the left tibia. The animals received the standard diet during the 60 days required to the healing of the implants (Clokie & Warshawasky 1995). For the next 90 days the control group continued to receive the standard diet while the Mg group was given the standard diet with magnesium reduction. At 150 days, all animals were sacrificed by deep anesthesia (Figure 1).

Surgical procedure

The animals were anaesthetized with a combination of ketamine chloridrate (Ketamina Agener, Agener União Ltda, São Paulo, SP, Brazil) at a concentration of 0.08 ml/100g body weight and 2% xylazine chloridrate (Rompum, Bayer S.A., São Paulo, SP, Brazil) at a concentration of 0.04 ml/100g. Next, the animals were submitted to preoperative trichotomy in the inner region of the leg and asepsis with povidone iodine solution.

An incision was made in layers on the tibial metaphysis. Underlying bone was subjected to osteotomy under abundant irrigation, carried out with a start drill of 1.8 mm for accommodation of the titanium implant (aluminum sand-blasting and acid etched surface), 4.0 mm long and 2.2 mm in thick

(Conexão Sistemas de Proteses Ltda., Aruja, SP, Brazil). The tissue was sutured with silk thread 4-0 (Ethicon * - Division of Johnson & Johnson Medical Limited, Sao Jose dos Campos, SP, Brazil).

The animals received a intramuscular dose of penicillin associated with streptomycin (Pentabiotic Pequeno Porte, Fort Dodge®, Campinas, SP, Brazil), 0.1 ml / kg of bodyweight and 5 mg / kg of dexamethasone intramuscular (Dexter - Agener ®, Agener União Ltda., Sao Paulo, SP, Brazil) immediately after surgery.

Biochemical evaluations

The animals were kept in metabolic cages for urine collection during 24 hours before sacrifice, for later dosage of deoxypyridinoline (DPD) (DPD Metra ® - Quidel Corporation, San Diego, CA, USA) level by the ELISA method.

At sacrifice, a blood sample was taken by caudal artery puncture for determination of magnesium (Magnésio - Labtest Diagnostica SA, Lagoa Santa, MG, Brazil) and calcium (Calcio Arsenazo Liquiform - Labtest Diagnostics SA , Lagoa Santa, MG, Brazil) serum levels by colorimetric method. Osteocalcin (OCN) (Rat Osteocalcin EIA Kit - Biomedical Technologies Inc., Stoughton, MA, USA) and parathyroid hormone (PTH)

(Rat Total Intact PTH ELISA Kit - Scantibodies Laboratory Inc., Santee, CA, USA) serum levels were evaluated by ELISA method.

Bone densitometry

Bone densitometry was obtained by Dual-energy X-ray Absorptiometry (DXA) using a densitometer (Discovery-A SN: 80999 Hologic, Bedford, MA, USA) in "High Resolution" mode with analysis by “Small Animal" software, supplied by the equipment manufacturer. For this overall BMD measurements were taken as well as those of the lumbar vertebrae 2 (L2), 3 (L3) and 4 (L4).

DXA accuracy for determination of BMD was evaluated by the coefficient of variation, expressed as a percentage of the average (Grier et al. 1996). For this, five consecutive measurements of each anatomical region of the same sample were made. The coefficient of variation obtained was 1.9%.

Image acquisition

Radiographic images of the implants were obtained by a direct digital imaging system - CDR (Computed Dental Radiography for Microsoft

Windows - Shick ® Technologies Inc., Long Island, NY, USA). In order to standardize images, tibia with implants and sensor were placed into a rigid positioning device where the long axis of the implant were perpendicular to central X-ray beam and parallel to sensor at a focus distance of 40 cm. The sensor was exposed to X-rays of 70 KVp and 10 mA for a 15 pulses per second exposure period. Images were stored in TIFF (Tagged Image File Format) without image compression and analyzed by image analysis software (Adobe® Photoshop® CS2 9.0, Adobe System Incorporated, San Jose, CA, USA).

Radiographic bone density

Tibia radiographies were evaluated by a single blinded and calibrated examiner. Radiographic bone density was obtained by measuring the gray level (histogram) in an area of 5x5 pixels at six different points: cortical (upper and lower) and medullar region, on both sides of the implant (Figure 2).

Calculation of bone density was performed by first obtaining the average of gray level values in each region of interest and then the values of gray level of the implant. The value of the regions of interest was divided by the

relative value of the implant to compensate small differences among radiographs.

Cortical bone thickness

Thickness of the tibial cortical (upper and lower) was assessed by radiographic images by a blinded calibrated examiner. The measured region was standardized at 1 mm from the implant. An image analyzer software (Image Tool) was used for linear measurement in millimeters of each cortical on both sides of the implant. Mean values were obtained for each cortical bone.

Removal torque test

Analysis of the removal torque of implants was immediately performed after the euthanization of the rats. The tibial implant was exposed, and the bone block attached to a vise for stabilization. A 0.88 mm wrench was adapted to the internal connection of the implant and torque was measured with a torque gauge (ATG24CN-S, Tohnichi MFG Co. LTD. Tokyo, Japan) on a scale of 0.5 N.cm with force ranging from 3 to 24 N.cm. A reverse force was applied until complete rupture of the bone-

implant interface, and the force needed to cause displacement of the implant in the bone tissue was recorded.

Data analysis

Body weight, biochemical evaluation, radiographic bone density, bone densitometry, cortical bone thickness and removal torque data were submitted to Mann Whitney (Graphpad Prism 5, San Diego, USA). Significance level was 5%.

Results

Body weight

Analysis of the animal weight shows that there was a measurable gain for both groups during the experimental period. At study onset a significant difference between groups was shown ($p=0.0048$). However, at baseline and the end of the experiment, no significant difference was found between groups (Table 1).

Biochemical evaluations

Results showed a statistically significant decrease in magnesium serum levels for the Mg group as compared to control ($p<0.0001$). However, for calcium serum levels no difference was found (Figure 3).

Serum levels of OCN showed no significant difference between groups; however there was a tendency ($p=0.0906$) for reduction in marker concentration when comparing groups. Regarding PTH ($p=0.0377$) and DPD ($p=0.0303$) levels, there were statistically significant differences between groups disclosing higher values for the Mg group (Figure 3).

Bone densitometry

Bone densitometry showed significantly lower BMD values for lumbar vertebrae in the Mg group as compared to control group for all regions studied, L2 ($p=0.0040$), L3 ($p=0.0028$), L4 ($p=0.0009$) and global ($p=0.0010$) (Figure 4).

Analysis of radiographic bone density and cortical bone thickness

There was no difference between groups in radiographic bone density around implants (Table 2). On the other hand, upper ($p<0.0001$) and lower

(p=0.0436) cortical thickness was shown to be significantly reduced for the Mg group compared to the control group (Figure 5).

Removal torque

Removal torque for the Mg group was significantly lower than control group (p=0.0105) (Figure 6).

Discussion

Systemic conditions, particularly those resulting in bone tissue changes are viewed as important factor for implant treatment predictability (Marco et al. 2005). Within this respect, magnesium deficiency could be considered as a risk factor for osseointegrated implant, as it is recognized to affect bone metabolism (Creedom et al. 1999; Rude et al. 2004; Rude & Gruber 2004; Stendig-Lindberg et al. 2004).

In the present study magnesium deficiency was induced for three months of dietary reduction of mineral content, demonstrated by its decreased serum levels. Calcium is a mineral antagonist to magnesium and in animals there may be a tendency to increase serum level in proportion to

magnesium deficiency (Meisel et al. 2005; Bussière et al. 2002), which agrees to this study (Figure 3).

Bone turnover is a physiological complex process that involves not only interaction among cells and bone matrix, but also a variety of systemic and local regulation factors that coordinate cell proliferation and activity (Marco et al. 2005; Takayanagi 2005). Many studies report that magnesium deficiency has a particular influence on bone mass loss (Creedon et al. 1999; Rude et al. 2003; Rude et al. 2004; Rude et al. 2005; Rude et al. 2006; Rude & Gruber 2004). This effect was observed in diets with different magnesium contents ranging from lesser (50% of NR) to the most severe restrictions (0.04% of NR), showing higher bone mass loss when the deficiency is increased (Rude et al. 2003; Rude et al. 2004; Rude et al. 2005; Rude et al. 2006; Rude & Gruber 2004; Del Barrio et al.). In the present study, bone densitometric analysis of lumbar vertebrae disclosed bone mass loss statistically significant for the Mg group as compared to control (Figure 4), agreeing with Rude et al. (2004).

Successful treatment with osseointegrated implants depends, among other factors, on formation of a rigid anchorage to the bone, which provides biomechanical stability (Shibata et al. 2008). Different geometries and properties of cortical and cancellous bone may affect this stability by deformation of the bone crest around endosteal implants (Petrie & Williams, 2007).

The effect of magnesium deficiency on bone tissue around implants with established osseointegration was evaluated in this study by radiographic bone density, measurement of cortical bone thickness and removal torque. Results showed that magnesium deficiency had a negative effect on the peri-implant cortical bone significantly reducing tibial cortical thickness (Table 2 and Figure 5).

Changes in cortical thickness greatly influences deformation of the peri-implant bone crest when submitted to occlusal load. By finite element analysis, it was found that low density cancellous bone and cortical bone thinning is more likely to undergo resorption when compared to bone tissue with thicker cortical and high density cancellous bone, which could interfere in the treatment predictability (Petrie & Williams 2007).

However, assessment of radiographic bone density around implants showed no significant difference between groups (Table 2). Although this methodology has been previously used to evaluate peri-implant bone tissue (Sakakura et al. 2006; Giro et al. 2008; Sakakura et al. 2008), in this study the absence of difference between groups might be due to technique limitations when compared with DXA or the relatively short period of deficiency, resulting in changes not detected by this analysis.

Although no difference was found between groups for radiographic bone density, changes in bone morphology and size of hydroxyapatite crystals, affecting bone architecture may have taken place around implants in Mg

group (Boskey et al. 1992; Creedon et al. 1999; Rude et al. 2005; Rude et al. 2006). This uneven configuration of bone structure may cause structural weakness, creating microfractures of the trabeculae and changing biomechanical behavior. Such a structural change in hydroxyapatite crystals could not be detected by the related method, since the crystals affected geometry may be masked under a bone structure with a radiographic density similar to that of the control group. However, such morphological changes together with decreased cortical thickness may explain the difference in biomechanical behavior in view of the reduced implant removal torque for the Mg group (Figure 6).

Several mechanisms may induce bone mass decrease related to this deficiency, such as hormone regulation (PTH), stimulation of pro-inflammatory cytokines (IL-1β and TNFα) (Rude et al. 2003; Rude et al. 2004; Rude et al. 2005; Rude et al. 2006; Rude & Gruber, 2004) and change in hydroxyapatite crystal formation (Boskey et al. 1992; Creedon et al. 1999; Rude et al. 2005; Rude et al. 2006). Increased release of PTH is related to an increase in calcium resorption by the kidneys, increase in paracellular calcium and magnesium resorption through stimulation of calcium channels. Thus, bone resorption as a result of stimulation of new osteoclasts and increased activity of mature osteoclasts, leading to imbalance of bone turnover (Goltzman 2008; Holtrop et al. 1979; Mosekilde 2008). A statistically significant difference between groups was found in this study by assessment of this pathway, with higher values of

serum PTH concentration for animals with deficiency (Figure 3). Increased release of PTH was observed previously in mice with this deficiency, in short periods of reduced mineral intake (Rude et al. 2004; Rude et al. 2005; Rude et al. 2006). Significantly higher values of urine deoxypyridinoline, an important bone resorption marker, were also verified in the group with magnesium deficiency. This data confirms the increased activity of bone resorption in this group (Figure 3). Osteocalcin expression, an important bone formation marker, is regulated by several calciotropic hormones and 1.25 dihydroxyvitamin D3, PTH, glucocorticoids and also by growth factors such as bone morphogenetic proteins (BMPs), fibroblast 2 growth factor (FGF-2) and TNF-α (Jiang et al. 2004). The decreased serum concentration showed in the Mg group, although not statistically significant ($p=0.0906$) may be related to a decrease of its synthesis in magnesium deficiency (Carpenter et al. 1992) (Figure 3). These findings express an imbalance on bone turnover in animals with magnesium deficiency, showing higher resorption activity without a concomitant increase in bone formation activity.

Within the limitations of this study it was concluded that magnesium deficiency may lead to alterations on systemic bone metabolism, reduction of cortical bone thickness and lower removal torque of implants with established osseointegration. However, the related mechanisms to bone loss require further clarification.

Acknowledgments

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Table 1 – Mean and standard deviation (SD) for body weight of animals during experiments periods ($^{‡}p \leq 0.01$).

	Initial	**Baseline**	**Final**
CTL	181,1 ± 6,0	382,8 ± 34,5	472,8 ± 71,5
Mg	172,7 ± 5,5‡	397,1 ± 28,6	468,2 ± 47,4

Table 2 - Values (mean ± SD) of radiographic bone density around implants in the upper cortical, cancellous and lower cortical regions for groups CTL and Mg.

	Superior cortical	**Cancellous**	**Inferior cortical**
CTL	0,70 ± 0,04	0,65 ± 0,03	0,66 ± 0,04
Mg	0,72 ± 0,06	0,66 ± 0,06	0,65 ± 0,05

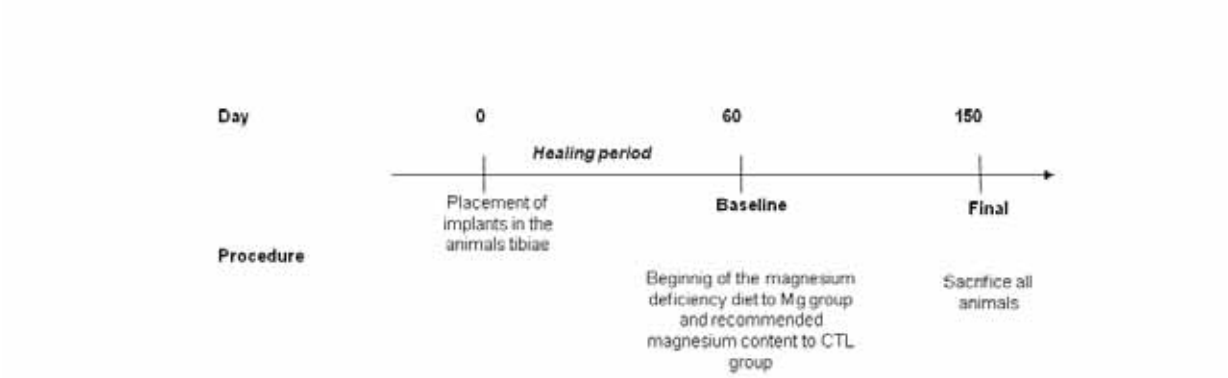

FIGURE 1 – Experimental design

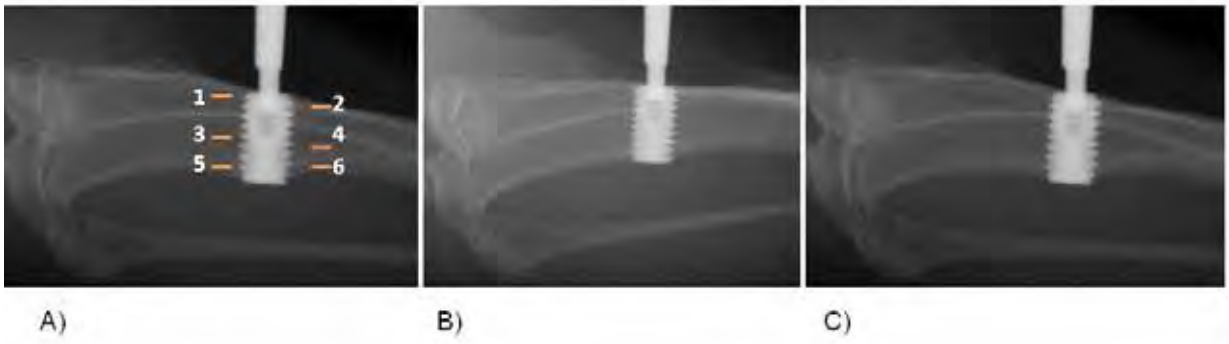

FIGURE 2 - Regions of interest for analysis of radiographic bone density (1 and 2 – upper cortical; 3 and 4 – cancellous; 5 and 6 – lower cortical).

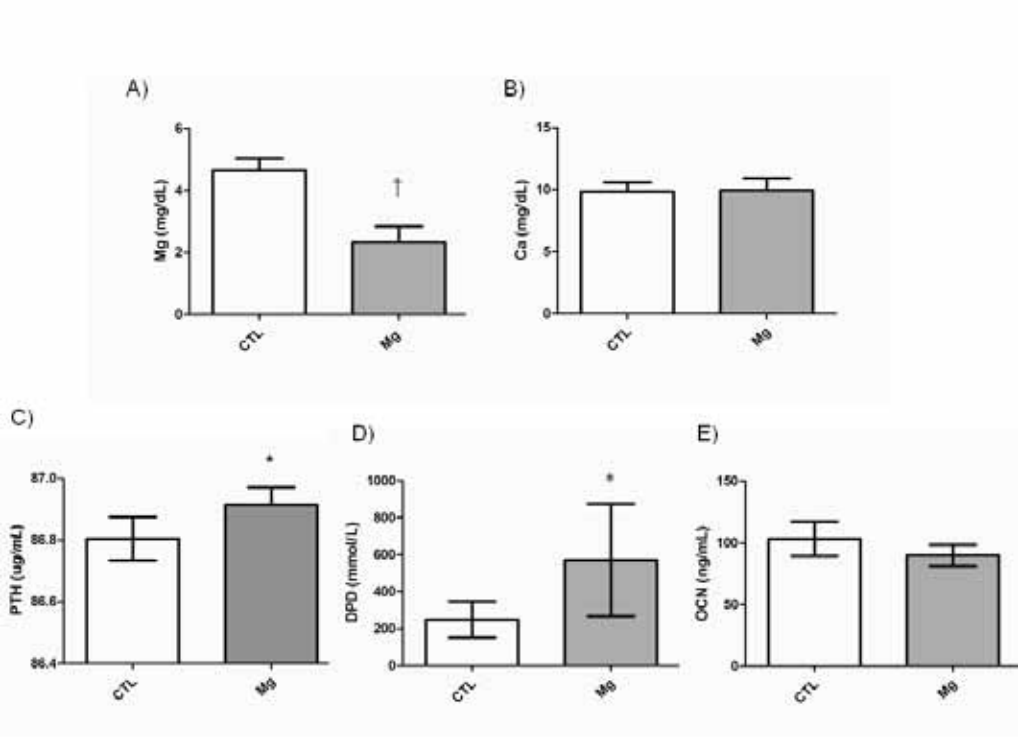

FIGURE 3 - (A) Serum levels of Mg (†p≤0.0001), (B) Ca, (C) PTH (*p≤0.05) and (D) OCN and urine concentration of (E) DPD (*p≤0.05) for groups CTL and Mg.

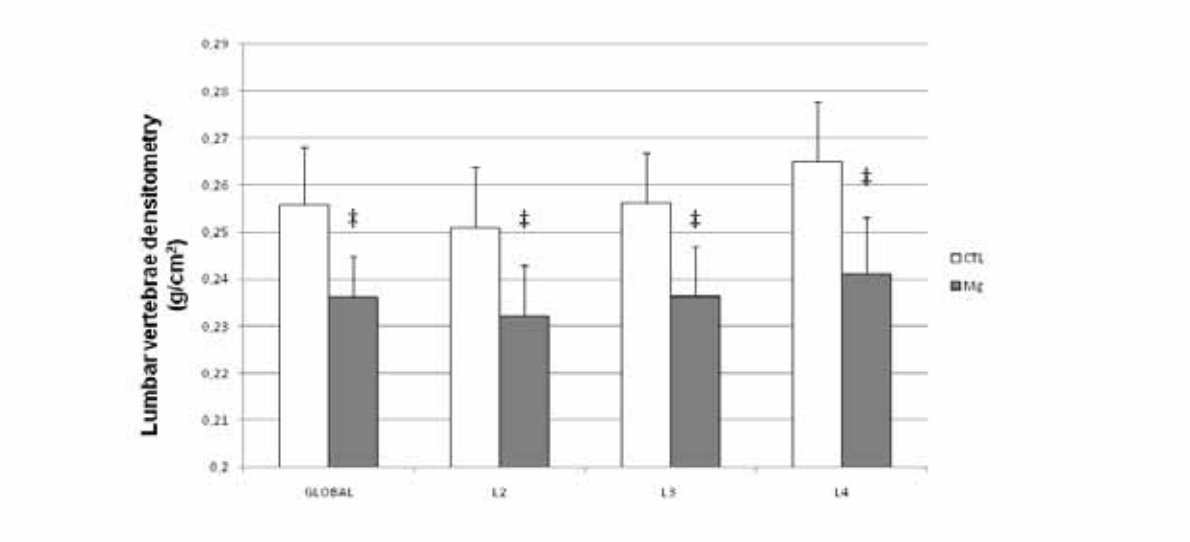

FIGURE 4 - Bone densitometry of the lumbar vertebrae in L2, L3, L4 and global, for groups CTL and Mg (‡p≤0.01).

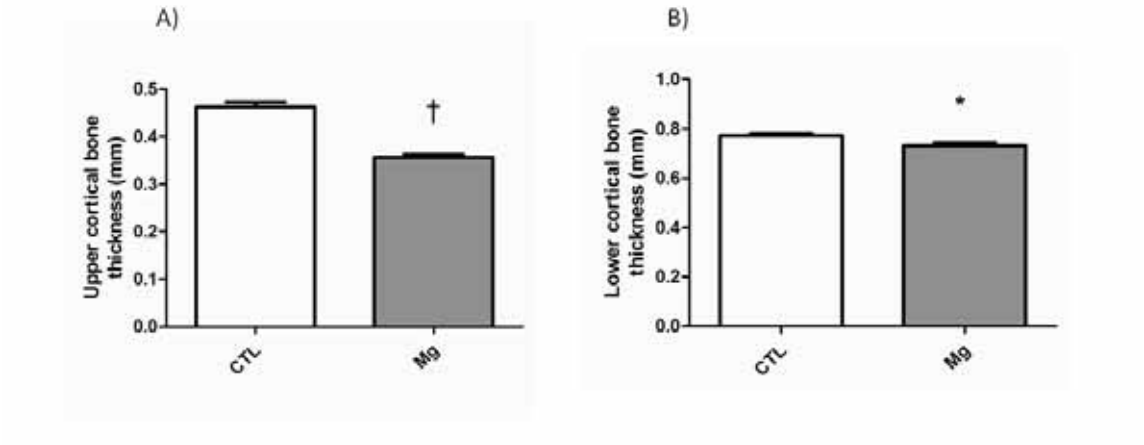

FIGURE 5 –Thickness of (A) upper ($^{\dagger}p \leq 0.0001$) and (B) lower ($^{*}p \leq 0.05$) cortical bone for groups CTL and Mg.

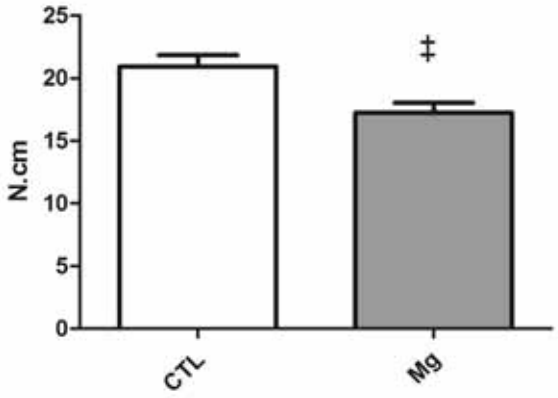

FIGURE 6 - Removal torque of the implants for groups CTL and Mg ($^{‡}p \leq 0.01$).

DISCUSSÃO

Os resultados do presente estudo demonstraram que a deficiência de Mg, devido a uma ação direta sobre células ósseas, apresenta um efeito negativo sobre o metabolismo ósseo levando à perda de massa óssea sistêmica. Além disso, a deficiência desse mineral demonstrou influência sobre a severidade da doença periodontal induzida e a manutenção de implantes osseointegrados.

Nesse estudo, a deficiência de magnésio (hipomagnesemia) foi comprovada após um período de três meses de restrição mineral na dieta, resultando em diminuição da concentração sérica do mesmo, o que confirma a instalação da deficiência. Essa deficiência, em animais, resultou na perda de massa óssea, demonstrada pela diminuição significativa da densidade mineral óssea dos fêmures e vértebras lombares, como demonstrado nos capítulos 2 e 3.

Os mecanismos relacionados à perda de massa óssea pela deficiência de Mg ainda é um assunto muito discutido na literatura. A redução da massa óssea pode ocorrer por vários mecanismos, como regulação hormonal (PTH), estímulo de citocinas pro-inflamatórias (IL-1β e TNFα)[81-84, 86], alteração na formação dos cristais de hidroxiapatita durante a mineralização [11, 82, 83] e/ou mesmo pela ação direta do Mg sobre as células ósseas[1-4, 81].

O aumento da liberação de PTH está relacionado ao aumento na reabsorção de cálcio pelos rins, aumento da reabsorção paracelular de cálcio e magnésio devido à estimulação dos canais de

cálcio e reabsorção óssea, como resultado da estimulação de osteoclastos novos e aumento da atividade de osteoclastos maduros, o que leva a um desequilíbrio no *turnover* ósseo[42, 67]. No presente estudo, a avaliação desta via demonstrou que os animais com deficiência apresentaram concentração sérica de PTH significativamente maior em relação ao controle (Capítulos 2 e 3). Esse aumento da liberação de PTH foi verificado anteriormente em ratos com essa deficiência, em períodos curtos de redução do conteúdo do mineral[82-84]. Foram verificados ainda, valores significativamente maiores de concentração urinária de deoxipiridinolina, um importante marcador de reabsorção óssea, para o grupo com deficiência de magnésio, o que confirma a maior atividade de reabsorção óssea para esse grupo (Capítulos 2 e 3).

A expressão de osteocalcina, um importante marcador de formação óssea, é regulada por vários hormônios calciotrópicos, como 1,25 dihidroxivitamina D_3, glucocorticóides e PTH, e também por fatores de crescimento, como as proteínas ósseas morfogenéticas (BMPs), fator de crescimento de fibroblasto 2 (FGF-2) e TNF-α [47]. No presente estudo foi constatada uma diminuição da concentração sérica de osteocalcina para o grupo teste, embora não significante estatisticamente, que pode estar relacionada à diminuição de sua síntese na deficiência de magnésio[15] (Capítulos 2 e 3).

Estes achados demonstram um desequilíbrio do *turnover* ósseo em animais com deficiência de Mg, com maior atividade de

reabsorção sem o concomitante aumento na atividade de neoformação óssea.

O *turnover* ósseo é um processo complexo, que envolve não somente as interações entre células e matriz óssea, mas também uma variedade de fatores reguladores sistêmicos e locais que coordenam a proliferação e atividade celular[61, 98].

No estudo *in vitro* (Capítulo 1), foi demonstrado, pela primeira vez, que a deficiência de Mg tem ação direta sobre os osteoclastos, tendo sido constatado que a deficiência é inversamente proporcional ao número de células, especialmente as de maior tamanho (maior número de núcleos por osteoclasto). Como há estudos na literatura demonstrando que as células respondem, de acordo com a origem do osso, de maneira diversa ao estímulo indutor de osteoclastogênese pelas citocinas M-CSF e RANKL[7, 20], o presente estudo foi realizado com células provenientes de ossos longos e da mandíbula. Entretanto como não houve diferença de resposta entre os precursores frente à deficiência de magnésio e devido à maior quantidade de células presentes na medula de ossos longos, os resultados aqui apresentados são representativos destas células.

Foi possível demonstrar neste estudo que há uma ativação da expressão gênica de fatores de transcrição e proteínas presentes na transmembrana das células, como c-fos e DC-STAMP, genes esses essenciais para a formação dos osteoclastos[12, 63]. Além disso, houve um

aumento na expressão gênica de citocinas como, IL1β e TNF-α, considerados importantes estimuladores de formação dessas células [55, 99].

Apesar de ter sido observado um aumento do número de osteoclastos em meio com deficiência de Mg, uma redução na atividade de reabsorção também foi verificada. Uma possível explicação para esse achado pode estar relacionada à função do Mg no metabolismo energético, visto que a atividade de reabsorção requer um alto consumo de energia pela célula promovida pela mitocôndria. É na mitocôndria que se encontra a maior quantidade de Mg, sendo este mineral essencial para a adequada função e transporte de energia dentro das células[111]. Além disso, grande parte das enzimas envolvidas no metabolismo energético são Mg dependentes[109-111]. Sendo assim, apesar da maior quantidade de osteoclastos, a baixa concentração de Mg dentro das células pode influenciar na atividade das mesmas.

Por outro lado, o alto número de osteoclastos presente na deficiência de Mg pode estar relacionado à tentativa de compensar a baixa atividade de reabsorção. Esse fenômeno foi demonstrado em alterações genéticas, como por exemplo a osteopetrose[25, 29].

Apesar de todas as alterações sistêmicas provocadas pela deficiência de Mg, como fragilidade esquelética, perda de massa óssea, exacerbação da resposta inflamatória e imunológica[60, 64, 81, 84], ainda há poucos estudos na literatura relacionando a deficiência do mineral a

patologias de alta prevalência na população mundial, como a doença periodontal.

A doença periodontal é umas das desordens mais prevalentes em humanos, causada pela presença de bactérias periodontopatogênicas e cuja resposta do hospedeiro a esses agentes resulta em uma doença inflamatória infecciosa crônica, caracterizada pela perda das estruturas dentárias de suporte[5, 33].

Estudos demonstram que para a instalação da doença periodontal é necessária não somente a presença de periodontopatógenos, mas a persistência da resposta imunoinflamatória contra esses patógenos, responsável pela destruição dos tecidos periodontais de suporte[33, 39, 58].

É estabelecido na literatura que macro e micronutrientes podem modificar a resposta inflamatória e imunológica, agindo como moléculas essenciais na modulação da expressão gênica e protéica[17, 19, 64]. Dentre os macronutrientes, estudos demonstram que a deficiência de Mg em ratos foi capaz de induzir uma síndrome inflamatória caracterizada pelo aumento no número e atividade de neutrófilos e eosinófilos e pela ativação de macrófagos em apenas poucos dias de deficiência, além de induzir uma maior liberação de citocinas inflamatórias como TNF-α, IL-1β e IL6 [13, 14, 60, 64].

No presente estudo, a análise macroscópica demonstrou que nos animais com deficiência de Mg houve maior perda óssea linear e

de área de tecido ósseo alveolar entre as raízes do primeiro molar inferior com indução de doença periodontal. A análise histológica da mesma região confirmou a presença de área de reabsorção e evidenciou a ocorrência de maior inflamação para os animais com deficiência de Mg (Capítulo 2). Pela coloração de TRACP, foi observado ainda uma tendência ao maior número de osteoclastos no grupo Mg (Resultados adicionais). Todos esses resultados demonstram maior severidade da doença periodontal induzida em animais com deficiência de Mg.

A análise da densidade óssea alveolar demonstrou que houve uma diminuição na densidade radiográfica somente na área de interesse localizada abaixo da raiz distal do primeiro molar inferor. A forma irregular da mandíbula, a força de oclusão durante a mastigação, os hábitos dos roedores e o tipo de estresse mecânico sofrido pela mandíbula durante a mastigação podem ser responsáveis pela manutenção da densidade do tecido ósseo mandibular[24].

Apesar da carência de estudos na literatura a respeito da influência da deficiência de minerais sobre a doença periodontal, estes resultados estão de acordo com estudo em humanos, onde foi demonstrado que a alteração da razão entre Ca e Mg, com diminuição nos níveis séricos de Mg, está relacionada ao aumento da severidade da doença periodontal[65]. Em estudo longitudinal avaliando a influência do conteúdo de Ca e Mg de japoneses idosos, fumantes e não fumantes, sobre a progressão da doença periodontal, foi observado um aumento

significativo do risco para pacientes fumantes com redução dos níveis de Mg [114]. Segundo Schifferle (2009)[92], a influência de uma dieta saudável sobre a doença periodontal está na adequada resposta do hospedeiro, bem como, na manutenção da integridade dos tecidos periodontais.

Apesar de ter sido constatado que a deficiência de Mg apresenta uma influência negativa sobre o metabolismo ósseo, até o presente momento não existem na literatura estudos sobre a influência da deficiência de Mg no tratamento com implantes osseointegrados.

O sucesso do tratamento com implantes osseointegrados depende, entre outros fatores, da formação de uma ancoragem rígida com o tecido ósseo, o que proporciona a estabilidade biomecânica. Diferentes geometrias e propriedades do tecido ósseo cortical e trabecular podem ter influência na estabilidade de implantes. Em relação ao tecido ósseo trabecular, quando este apresenta baixa densidade há maior sobrecarga de força sobre a crista óssea (tecido ósseo cortical), podendo causar maior deformação dessa crista. Nesse caso há a necessidade de maior espessura da cortical para melhor manutenção do implante. Já no tecido trabecular mais denso, a dissipação de força é mais equilibrada com a cortical óssea, o que proporciona melhor manutenção do implante, sem a necessidade de uma cortical espessa[74]. Assim, as alterações sistêmicas, particularmente as que resultam em modificações no tecido ósseo, são consideradas um importante fator de influência para o sucesso do tratamento com implantes osseointegrados[61].

No presente estudo (Capitulo 3), o efeito da deficiência de magnésio sobre o tecido ósseo ao redor de implantes com osseointegração estabelecida foi avaliado. Os resultados relativos à mensuração da espessura das corticais demonstraram que a deficiência do mineral teve efeito negativo sobre o osso cortical periimplantar, levando a uma redução significativa da espessura das corticais tibiais para o grupo Mg.

Segundo Petrie e Williams (2007)[74], a variação da espessura da cortical tem grande influência sobre a deformação da crista óssea periimplantar quando este é submetido à carga oclusal. Por meio da análise de elementos finitos os autores observaram que o tecido ósseo de baixa densidade em conjunto com espessura fina de cortical tem maior probabilidade de sofrer reabsorção quando comparado a um tecido ósseo com maior espessura de cortical e tecido medular de alta densidade, interferindo, portanto, na longevidade do tratamento.

Entretanto, a avaliação da densidade óssea radiográfica ao redor dos implantes não demonstrou diferença significante entre grupos controle e Mg (Capítulo 3). Embora esta metodologia tenha sido anteriormente utilizada na avaliação do tecido ósseo periimplantar[35, 90], no presente estudo a ausência de diferença entre os grupos pode ser devido à limitação da técnica quando comparada a outras consideradas padrão-ouro, como DXA, ou ao tempo relativamente curto de indução da

deficiência, resultando em alterações, mas com menor detecção por esse tipo de análise.

A análise histomorfométrica do tecido ósseo ao redor dos implantes (resultados adicionais), demonstrou a ausência de diferença significativa na quantidade de tecido ósseo em contato com o implante. Pode ser visto, entretanto, que há uma tendência de menor área de tecido ósseo e maior superfície de reabsorção ao redor dos implantes no grupo de animais com deficiência de Mg.

Apesar de não ter sido demonstrada diferença entre os grupos experimentais com as análises acima citadas, o tecido ósseo ao redor dos implantes no grupo Mg pode ter sofrido alterações na morfologia e dimensão dos cristais de hidroxiapatita, afetando a arquitetura óssea[11, 82, 83]. Esta variabilidade na conformação da estrutura óssea pode resultar em fragilidade estrutural, criando microfraturas das trabéculas e alteração de seu comportamento biomecânico. A avaliação da densidade óssea radiográfica não permite diferenciar tal modificação estrutural nos cristais de hidroxiapatita, uma vez que a geometria comprometida dos cristais pode estar mascarada sob uma estrutura óssea com densidade radiográfica semelhante ao grupo controle. Entretanto, tais alterações morfológicas juntamente com a redução da espessura das corticais podem explicar a diferença no comportamento biomecânico. No presente estudo, a avaliação do comportamento biomecânico foi realizada por meio do torque de remoção, pelo qual é

possível avaliar o grau de osseointegração dos implantes[31, 36, 38, 48, 54, 62, 69]. Os resultados demonstraram que, apesar de não haver diferença entre os grupos quanto à densidade, foi constatada uma diminuição significativa do torque de remoção para o grupo com deficiência de Mg, o que pode representar um risco para a manutenção de implantes a longo prazo (Capítulo 3).

CONCLUSÃO

Dentro das limitações do estudo foi possível concluir que:

- Foi demonstrado pela primeira vez, *in vitro*, que a deficiência de Mg estimula a osteoclastogênese, vista pelo aumento no número de osteoclastos. Apesar desse aumento há diminuição na atividade de reabsorção da célula.

- A deficiência de magnésio resultou em perda de massa óssea, local e sistêmica, em um curto período de tempo. Entretanto os mecanismos relacionados precisam ser melhor elucidados.

- A deficiência de Mg induz uma maior perda óssea alveolar relacionada à doença periodontal induzida, podendo ser um fator modificador da severidade da doença.

- A deficiência de Mg resulta em redução da espessura das corticais e do torque de remoção de implantes osseointegrados, podendo ser um fator de risco para sua manutenção da osseointegração.

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APÊNDICE 1

Resultados adicionais

ANÁLISE HISTOLÓGICA DO FÊMUR

A análise do disco epifisário demonstrou que não houve diferença estatisticamente significante entre os grupos quanto aos parâmetros avaliados, nas diferentes áreas de interesse (Figura 1).

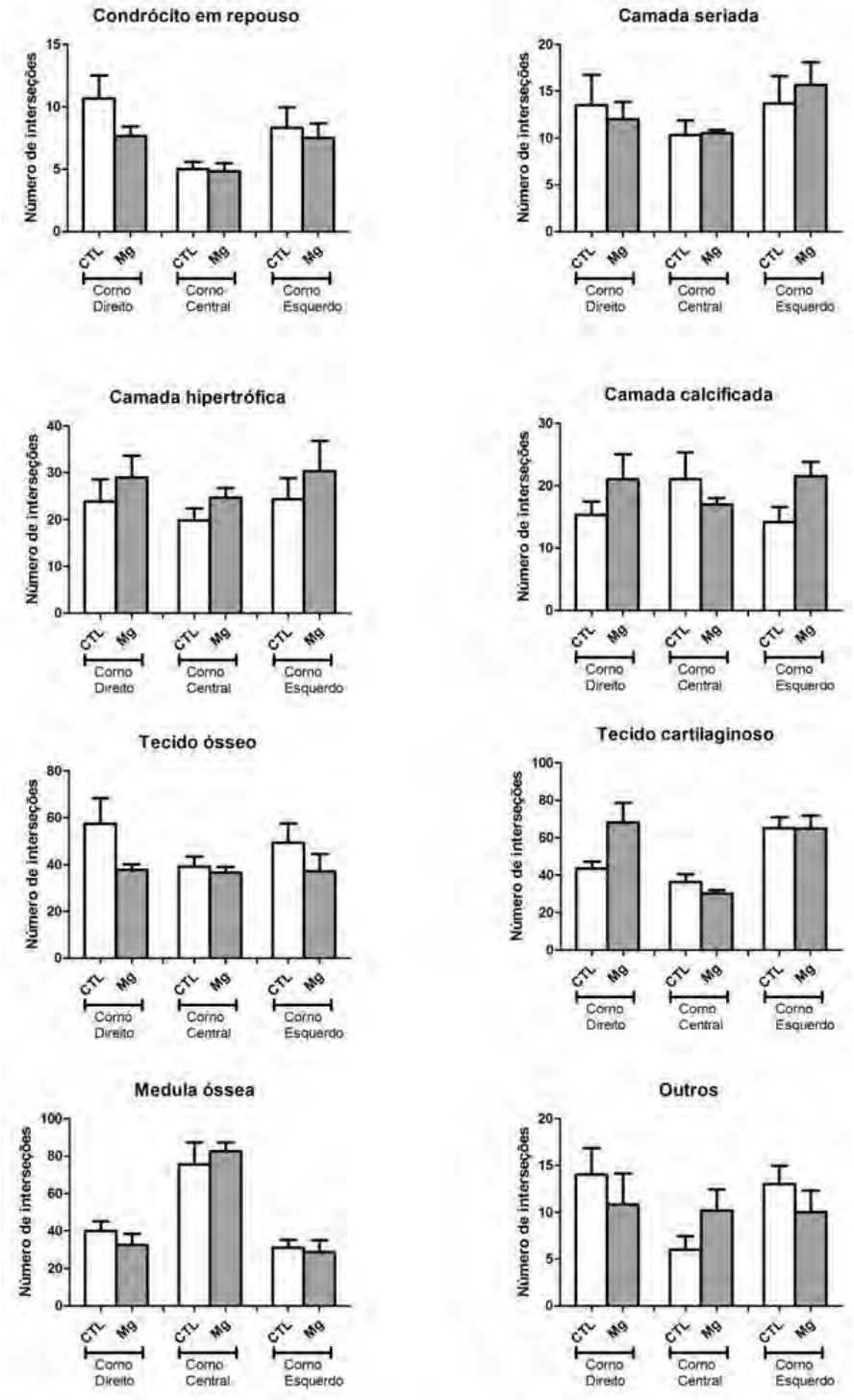

FIGURA 1 – Representação gráfica dos parâmetros avaliados em 3 regiões da epífise distal do fêmur. Nivel de significância estabelecido p menor que 5%.

ANÁLISE HISTOMORFOMÉTRICA DA TÍBIA

A análise dos dados demonstrou uma tendência de diminuição da quantidade de tecido ósseo em contato direto com a superfície do implante, tanto na análise de extensão linear de tecido ósseo em contato direto com a superfície do implante como de área entre as roscas ocupada por tecido ósseo (Figuras 2C e 2D). As análises de superfície de reabsorção e área total de tecido ósseo não demonstraram diferença estatisticamente significante entre os grupos (Figuras 2E e 2F).

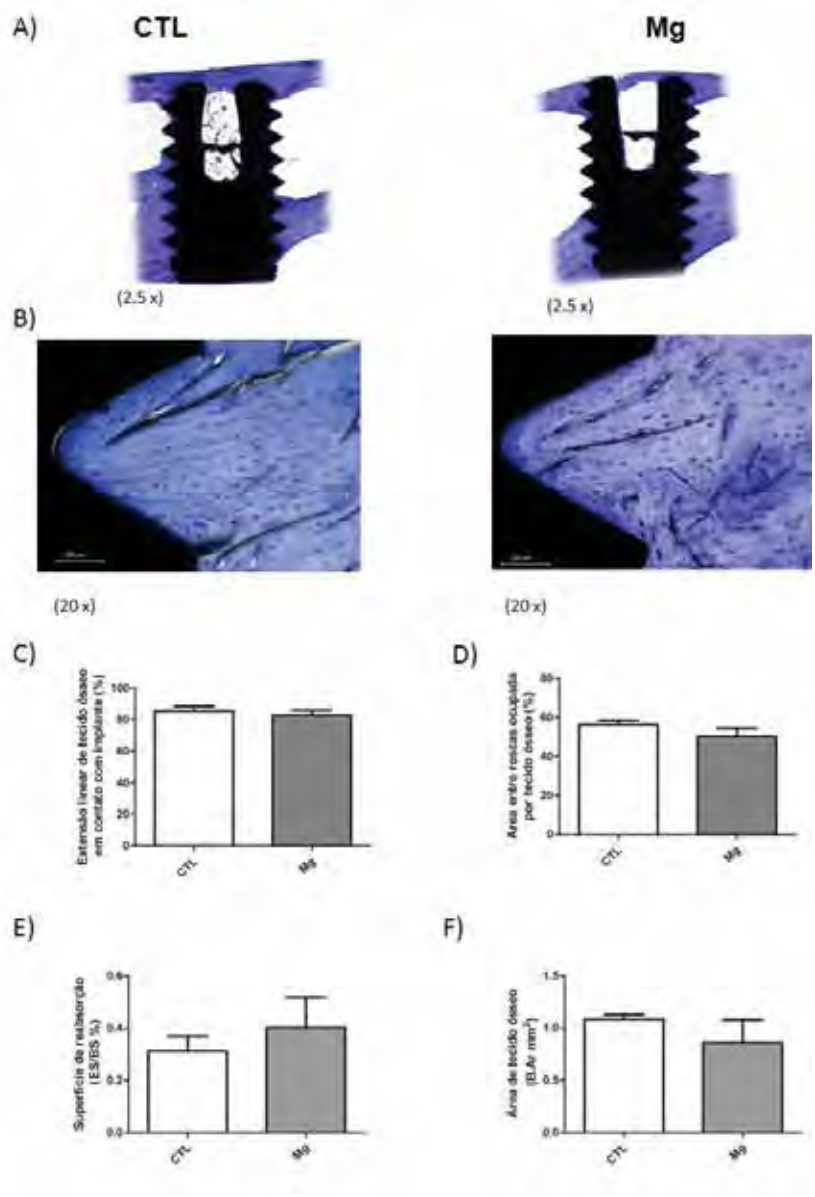

FIGURA 2 – (A) Figura representativa dos implantes instalados em ambos os grupos (2.5X). (B) Figura representativa do tecido ósseo entre a segunda e terceira rosca da cortical inferior do lado direito (20x). Representação gráfica da extensão linear de tecido ósseo em contato direto com a superfície do implante (C), área entre roscas ocupada por tecido ósseo (D), superfície de reabsorção (E) e área de tecido ósseo (F).

AVALIAÇÃO DA QUANTIDADE DE OSTECLASTO NA MANDÍBULA

A avaliação da quantidade de osteoclastos presentes na região de furca dos molares inferiores demonstrou que não houve diferença estatisticamente significante entre os grupos, tanto para o primeiro, como para o segundo molar (Figura 3, 4 e 5).

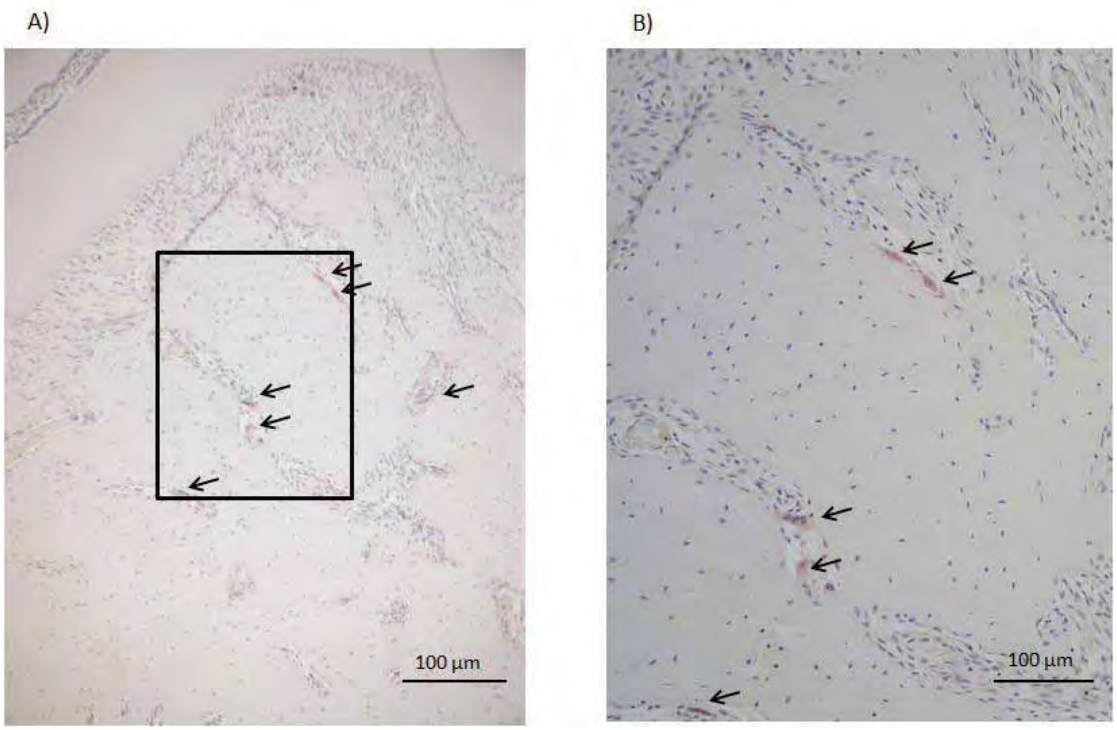

FIGURA 3 – A) Figura ilustrativa da região de furca do 1º molar. Setas indicam presença de osteoclastos corados por TRACP (10x). B) Figura ilustrativa em maior aumento (20x). Setas indicam em maior detalhe a presença de osteoclastos.

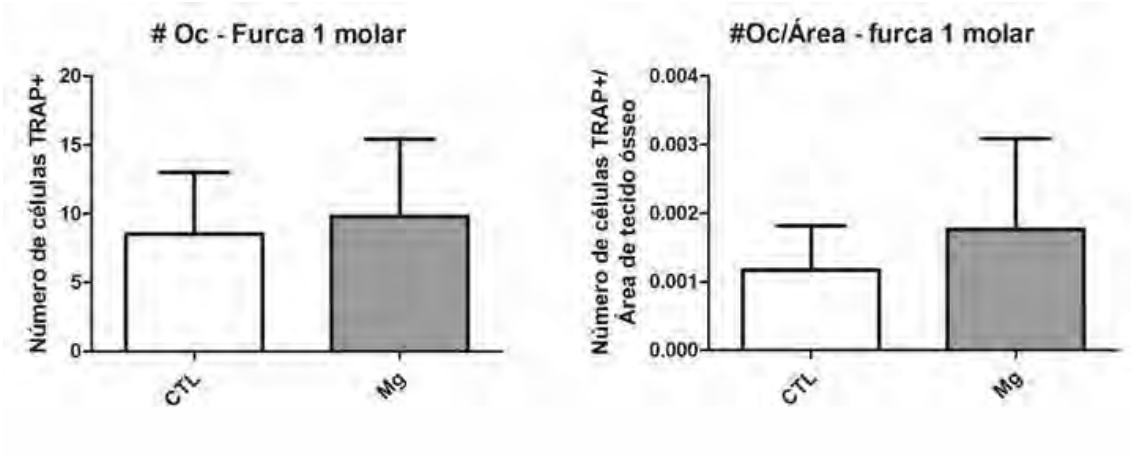

FIGURA 4 – Representação gráfica da contagem de osteoclastos e número de osteoclastos por área de tecido ósseo do primeiro molar inferior.

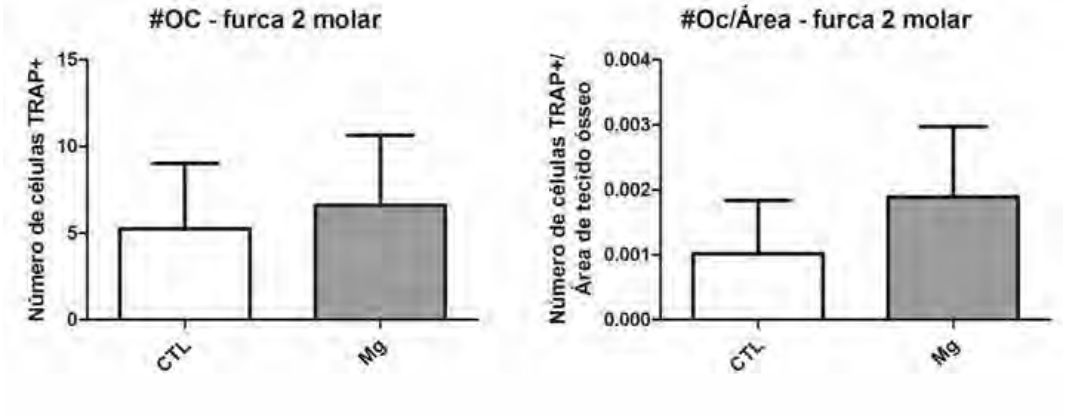

FIGURA 5 – Representação gráfica da contagem de osteoclastos e número de osteoclastos por área de tecido ósseo do segundo molar inferior.

APÊNDICE 2

Materiais e métodos

ANIMAIS - GRUPOS

O presente estudo foi aprovado pelo Comitê de Ética no Uso de Animais (CEUA), processo nº 20/2006.

Neste estudo foram utilizados 30 ratos (*Rattus Norvegicus, albinus, Holtzman*) machos, com aproximadamente 150 dias de idade (peso médio=380g), provenientes do Biotério do Campus de Araraquara – UNESP. Os animais foram mantidos em gaiolas individuais, confeccionadas em aço-inox, em ambiente com temperatura, umidade e luz controlados.

Os animais foram alocados em 2 grupos, conforme a dieta que receberam :

- Controle (CTL): animais com acesso à ração com quantidade diária ideal de Mg[76, 77], por um período de 90 dias. (n=15)
- Mg : animais com acesso à ração com quantidade reduzida de Mg em 90% [84], por um período de 90 dias. (n=15)

Para hidratação, os animais receberam água destilada *ad libitum*.

DIETA

A confecção da dieta foi baseada no AIN-93 (American Institute of Nutrition)[76, 77] para manutenção de roedores, como mostrado nas tabelas abaixo:

Tabela 1: Formulação da dieta para manutenção de roedores (AIN – 93M) sendo a caseína usada como fonte de proteína.

Ingredientes	**AIN – 93M (g/kg dieta)**
Amido de milho	465.69
Caseína (> 85% proteína)	140.00
Malto dextrina	155.00
Sacarose	100.00
Óleo de soja	40.00
Fibra	50.00
Mistura Mineral	35.00
Mistura Vitamínica	10.00
L-Cistina	1.80
Bitartarato de colina (41.1% colina)	2.50

Tabela 2: Mistura mineral com as concentrações minerais recomendadas para a manutenção de roedores (grupo controle)[76, 77] e animais sob dieta com redução de magnésio (grupo Mg). (AIN – 93M – MX)

Ingredientes	**Controle** (g ou Mg/kg)	**Mg** (g ou Mg/kg)
Carbonato de cálcio anidro (40.04% Ca)	375.00	375.00
Fosfato de potássio monobásico (22.76% P, 28.73% K)	250.00	250.00
Citrato de Potássio, tripotássico monohidratado (36.16% K)	28.00	28.00
Cloreto de sódio (39.34% Na, 60.66% Cl)	74.00	74.00
Sulfato de potássio (44.78% K, 18.39% S)	46.60	46.60
Oxido de magnésio (60.32% Mg)	24.00	2.4
Citrato férrico (16.5% Fe)	6.06	6.06
Carbonato de zinco (52.14% Zn)	1.65	1.65
Metassilicato de sódio $9H_2O$ (9.88% Si)	1.45	1.45
Carbonato de manganês (47.79% Mn)	0.63	0.63
Carbonato cúprico (57.47% Cu)	0.30	0.30
Sulfato de cromo potássio 12 H_2O (10.42% Cr)	0.275	0.275
Ácido bórico (17.5% B), Mg	81.5	81.5
Fluoreto de sódio (45.24% F), Mg	63.5	63.5
Carbonato de níquel (45% Ni), Mg	31.8	31.8
Cloreto de lítio (16.38% Li), Mg	17.4	17.4
Selenato de sódio anidro (41.79% Se), Mg	10.25	10.25
Iodato de potássio (59.3% I), Mg	10.0	10.0
Paramolibdato de Amônio 4 H_2O (54.34% Mo), Mg	7.95	7.95
Vanadato de amônio (43.55% V), Mg	6.6	6.6
Sacarose	209.806	209.806

As dietas foram formuladas por empresa[†] especializada e foram quimicamente analisadas com os métodos usuais para determinação da composição centesimal. Para acompanhamento do crescimento e desenvolvimento do animal, foi controlada diariamente a ingestão alimentar e, para avaliação da adaptação dos animais à dieta e às condições nutricionais, o peso corporal foi aferido semanalmente.

[†] Pragsolucões biociência, Jau, BR

DESENHO DO ESTUDO

Após um período de cinco dias, para adequação dos animais ao ambiente do biotério, todos foram submetidos à cirurgia de instalação dos implantes na tíbia direita. Durante o período de 60 dias, necessários à osseointegração dos implantes, os animais receberam dieta com concentração adequada de magnésio. Decorrido o período de osseointegração dos implantes, os animais do grupo Mg tiveram acesso à dieta com redução do Mg com o objetivo de instalar a referida deficiência, enquanto os animais do grupo controle permaneceram com a dieta padrão. Após um período de 60 dias do inicio da dieta, todos os animais receberam ligadura no primeiro molar inferior esquerdo para indução da doença periodontal, ficando o lado contralateral como controle. Após 150 dias do início do experimento, todos os animais foram sacrificados (Figura 1).

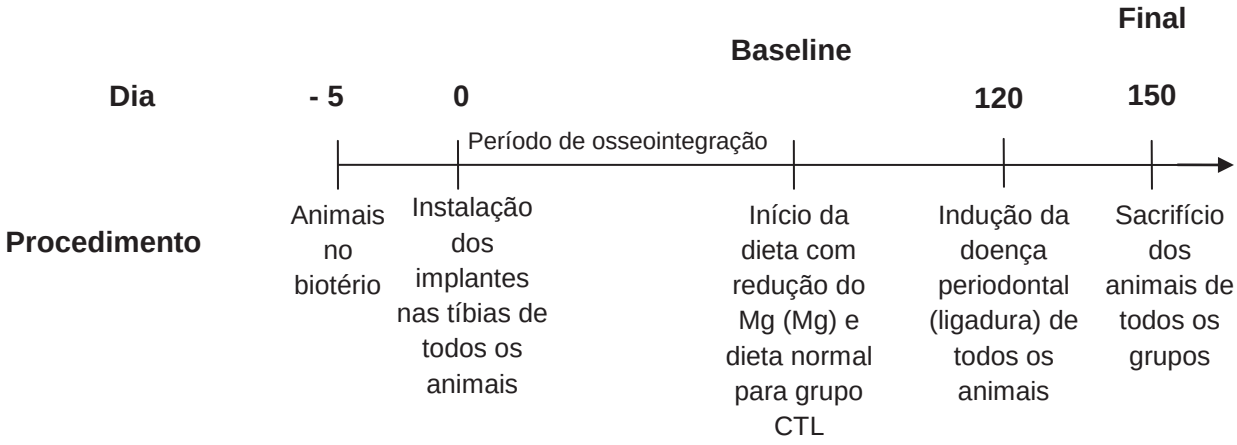

FIGURA 1 – Desenho Experimental

CIRURGIA PARA INSTALAÇÃO DOS IMPLANTES

Os animais foram anestesiados com uma combinação de cloridrato de ketamina[‡] numa concentração de 0,08ml/100g de massa corpórea e cloridrato de xilazina 2% [§] na concentração de 0,04ml/100g. Posteriormente, foram submetidos à tricotomia da região interna das pernas direita e esquerda e foi realizada a antissepsia com gaze estéril embebida em solução de iodopovidona.

Uma incisão de aproximadamente 10 mm foi realizada, em planos, na região a ser operada, mais precisamente sobre a metáfise tibial. Após uma dissecção delicada, o tecido ósseo foi submetido à osteotomia, realizada por meio de uma seqüência progressiva de fresas (fresa lança; fresa espiral de 2.0 mm) para acomodar um implante de titânio[**] de superfície porosa com 4 mm de comprimento por 2.2 mm de espessura. Todas as perfurações foram realizadas com motor elétrico[††], ajustado a 1200 rpm, sob abundante irrigação com solução salina estéril. O implante foi instalado com a ajuda de uma chave digital[‡‡]. Todo ferimento foi suturado em planos, internamente com fio reabsorvível[§§] e externamente com fio de seda[***].

[‡] Ketamina Agener – Agener União

[§] Rompum - Bayer

[**] Conexão Sistemas de Prótese

[††] BLM 600 - Driller

[‡‡] Chave Hexagonal – Conexão Sistemas de Prótese

[§§] Vicryl 5-0 – Johnson & Johnson

[***] Seda 4-0 - Johnson & Johnson

Os animais receberam, em dose única, penicilina associada à estreptomicina[†††] na dosagem 0,1 ml/kg de peso e 5mg/kg de dexametasona intramuscular[‡‡‡].

INDUÇÃO DA DOENÇA PERIODONTAL

No presente estudo, o modelo de indução de doença periodontal utilizado foi por ligadura. Inicialmente os animais foram anestesiados com uma combinação de cloridrato de ketamina[§§§] numa concentração de 0,08ml/100g de massa corpórea e cloridrato de xilazina 2% [****] na concentração de 0,04ml/100g. Posteriormente, os animais foram posicionados em mesa operatória apropriada, para permitir adequada abertura bucal, facilitando o acesso aos molares inferiores. Com o auxílio de instrumental adequado, foi introduzido um fio de algodão n°30 ao redor dos primeiros molares inferiores do lado esquerdo. Foi realizado o controle e a manutenção da ligadura durante 30 dias.

SACRIFÍCIO DOS ANIMAIS

Os animais foram sacrificados, no período previamente relatado, com aprofundamento de anestesia.

[†††] Pentabiótico - Wyeth-Whitehall Ltda

[‡‡‡] Dextar – Agener União

[§§§] Ketamina Agener – Agener União

[****] Rompum - Bayer

No período de 24 horas anterior ao sacrifício, os animais foram colocados em gaiolas metabólicas para a coleta da urina de 24 horas. A amostra coletada foi congelada a -80ºC.

No momento do sacrifício, uma amostra de sangue foi coletada por punção da artéria caudal. A amostra de sangue obtida foi deixada para coagular por 30 minutos, e então centrifugada por 10 minutos. O soro separado do coágulo foi congelado a –80^{o}C.

No sacrifício foram removidos as tíbias, os fêmures, coluna lombar, a mandíbula e os tecidos gengivais ao redor do primeiro molar inferior, direito e esquerdo.

ANÁLISES LABORATORIAIS

As concentrações séricas de osteocalcina (OCN)[††††] e paratormônio (PTH[‡‡‡‡]) foram avaliadas por meio de método ELISA, utilizando kits comerciais.

Foram avaliadas também no soro as concentrações de Mg e Ca por teste colorimétrico[§§§§].

Na urina foi determinada a concentração de deoxipiridinolina (DPD), um marcador de reabsorção óssea. Para isso, logo após a coleta da urina foi medida a concentração de creatinina urinária. O restante da amostra foi congelado e a concentração de DPD foi mensurada pelo

[††††] Biomedical Technologies Inc, Stoughton, USA

[‡‡‡‡] Scantibodies Laboratory Inc, Santee, USA

[§§§§] Labtest Diagnóstica, BRA

método ELISA[*****], corrigida pela concentração de creatinina urinária (Nm.DPD/nMCr).

ANÁLISE DA DENSIDADE ÓSSEA

Foi realizada análise da densidade mineral óssea (BMD) da coluna lombar e do fêmur esquerdo para a constatação da perda de massa óssea esqueletal. Para isso foi utilizado um densitômetro[†††††], sendo a análise da densitometria óssea realizada por Dual-energy X-ray Absorptiometry (DXA), empregando-se o *software* "*Small Animal*", fornecido pelo fabricante do aparelho, no modo "*High Resolution*".

Para calibração do aparelho, foi realizado um teste, o qual consiste em mensurar a densidade de um bloco padrão composto por três camadas sintéticas, de constituição semelhante ao osso, com área e conteúdo mineral conhecidos.

A delimitação das regiões analisadas foi realizada por dispositivos existentes no programa do aparelho, selecionando sempre o mesmo espaço a fim de padronizar as medidas de todas as sub-regiões.

Foram efetuadas medidas de BMD global e de três sub-regiões, assim determinadas (Figuras 2 e 3)

[*****] Metra biosystems, Palo Alto,CA

[†††††] QDR 2000 Hologic

Coluna Vertebral: L2) vértebra lombar 2

L3) vértebra lombar 3

L4) vértebra lombar 4

Fêmur: R1) Epífise proximal

R2) Epífise distal

R3) Diáfise

A precisão da DXA na determinação da BMD foi avaliada pela mensuração do coeficiente de variação, expresso como uma porcentagem da média[18]. Para isso, foram realizadas cinco medidas consecutivas de cada região anatômica de uma mesma amostra.

Todas as medidas foram realizadas no Laboratório de Metabolismo Ósseo da Disciplina de Reumatologia da Faculdade de Medicina de São Paulo-USP.

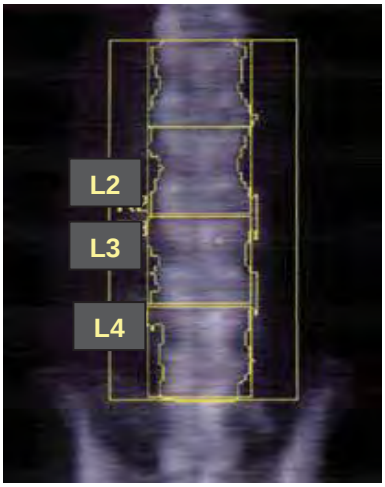

FIGURA 2- Avaliação Global e das sub-regiões da coluna lombar.

FIGURA 3- Avaliação Global e das sub-regiões do fêmur.

ANÁLISE HISTOLÓGICA DO FÊMUR

O fêmur de cada animal foi dissecado, descalcificado e seccionado, mantendo a região da epífise distal. Cada fragmento foi incluído, no sentido da superfície de corte, em bloco de parafina. Os

blocos obtidos foram seccionados em cortes seriados de 5 µm de espessura e as lâminas foram coradas com Alcian Blue (AB) e picrosirius[16] para avaliação do tecido cartilaginoso e ósseo.

Para coloração de glicoconjugados, os cortes histológicos foram imersos em solução de 1% AB 8GX dissolvido em ácido acético aquoso 3% (pH 2.5) por 40 minutos, em temperatura ambiente. Foi realizada lavagem em água corrente e os cortes foram imersos em solução de picrosirius 0,1% por 1 hora em temperatura ambiente. Foi realizada lavagem em água corrente novamente, e então foram coradas com Hematoxilina de Harris por 3-5 minutos. Após lavagem em água corrente os cortes passaram por processamento de desidratação e montagem das lâminas.

As imagens foram obtidas em 3 regiões das metáfises femorais, previamente definidas como corno direito, central e esquerdo, com auxílio do microscópio[‡‡‡‡‡](Figura 4). As imagens foram analisadas com auxilio de um software analisador de imagem[§§§§§]. Foi realizada uma avaliação por grade histológica, onde foram contados um total de 164 interseções em cada imagem. Os pontos de intersecção foram definidos como: condrócito em repouso, camada seriada, camada hipertrófica, camada calcificada, tecido ósseo, tecido cartilaginoso, medula óssea e outros (Figura 5).

[‡‡‡‡‡] Olympus BX-51 light microscope

[§§§§§] Image Pro-Express 6.0; Olympus, Tokyo, Japan

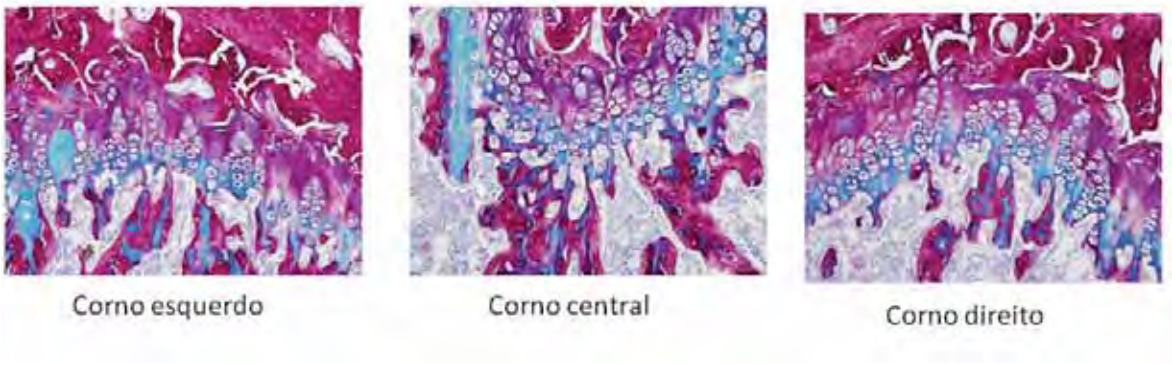

FIGURA 4 – Imagem representativa das regiões de interesse do fêmur: corno esquerdo, central e direito (20x).

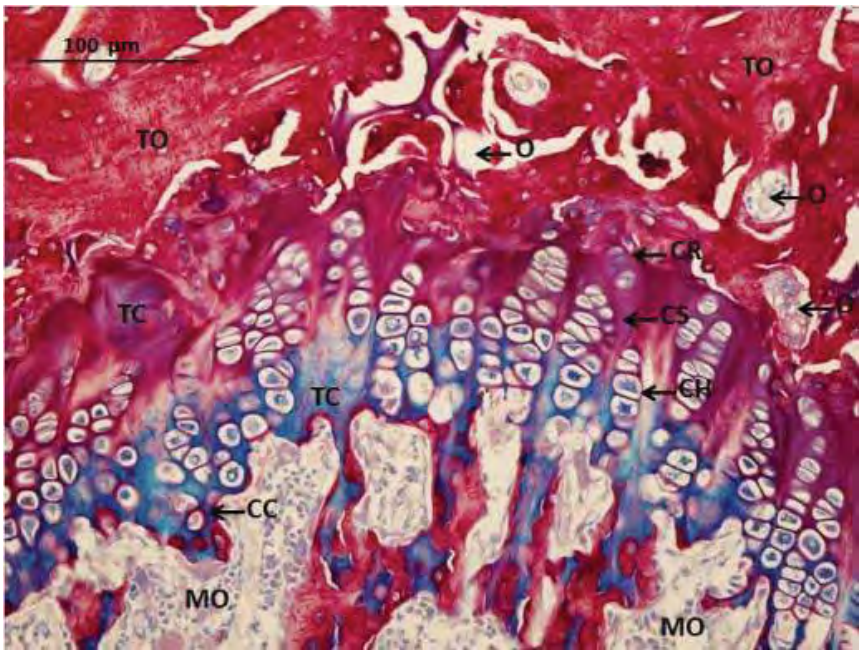

FIGURA 5 – Figura ilustrativa dos parâmetros estudados no corno esquerdo do fêmur. Legenda: CR – condrócito em repouso; CS - camada seriada; CH – camada hipertrófica; CC – Camada calcificada; TC – tecido cartilaginoso; TO – tecido ósseo; MO – medula óssea; O – outros.

ANÁLISE DA DENSIDADE ÓSSEA RADIOGRÁFICA

As imagens radiográficas dos implantes na tíbia foram obtidas por meio de um sistema de imagem digital direta - CDR (Computed Dental Radiography for Microsoft Windows) [******], o qual utiliza um sensor eletrônico em substituição ao filme radiográfico. Os implantes foram posicionados em um dispositivo com seu longo eixo perpendicular ao feixe central de raios-X e paralelo ao sensor, numa distância foco-objeto de 40 cm. O sensor foi exposto aos raios-X numa potência de 70KVp e 10mA, com tempo de exposição de 15 pulsos/segundo.

A resolução de imagem foi de 635 ppi (pixels per inch), o tamanho da imagem de 900x641 dpi e o tamanho dos pixels de 40 microns. As imagens foram armazenadas em formato TIFF (Tagged Image File Format) sem compressão de imagem (8 bits com resolução de 600 dpi).

As imagens foram exportadas do programa Schick® e importadas para um *software* analisador de imagens[**], em um computador com um processador de 1700 MHz[***], 512k, 256 DDR MB e sistema operacional Windows XP[****] em uma tela plana S-VGA de 15 polegadas (1024 x 768 pixel resolution). A densidade óssea radiográfica foi avaliada

[******] Shick Technologies, Inc

[**] Adobe® Photoshop® 6.0

[***] Athlon XP, AMD, USA

[****] Microsoft, USA

utilizando-se a análise dos níveis de cinza da radiografia (histograma) em uma área de 5x5 pixels nas regiões de interesse: corticais, superior e inferior, e região medular, em ambos os lados do implante (Figura 6).

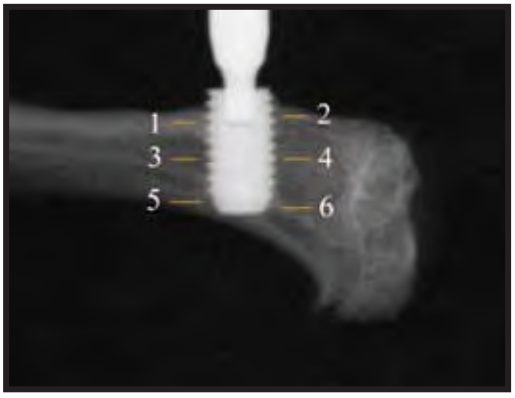

FIGURA 6- Regiões de interesse para análise da densidade óssea radiográfica (1 e 2 - cortical superior; 3 e 4 - medular; 5 e 6 - cortical inferior).

TORQUE DE REMOÇÃO

No momento do sacrifício, a tíbia esquerda foi removida e estabilizada em uma pequena morsa. Uma chave hexagonal foi conectada tanto no implante como no torquímetro e foi realizado um movimento anti-horário com o objetivo de desrosquear o implante. O pico máximo de força necessária para movimentar o implante foi registrado como o valor do torque de remoção.

ANÁLISE HISTOMORFOMÉTRICA DA TÍBIA

Após o processo de fixação e posterior lavagem, a tíbia contendo o implante, foi desidratada em solução de álcool etílico em diversas concentrações.

A infiltração plástica foi realizada com misturas de glicolmetacrilato (Technovit 7200 VLC) e álcool etílico, seguindo variações gradativas, finalizando com duas infiltrações de glicolmetacrilato puro. Após a infiltração plástica, os espécimes foram incluídos em resina e polimerizados.

O bloco foi montado em lâmina acrílica com o auxilio da resina Technovit 4000[††††††]. Por meio da utilização de um sistema de corte[‡‡‡‡‡‡], foi realizado o corte preliminar e obtida uma secção espessa (300 - 500 μm). Esta secção foi então submetida a um sistema de micro-desgaste, que resultou em uma secção de aproximadamente 30 a 50 μm de espessura.

Para histometria e histomorfometria dos parâmetros estáticos as lâminas foram coradas com azul de toluidina a 10%. Os parâmetros histomorfométricos estáticos, como superfície de reabsorção (%) e superfície osteoclástica (%), foram analisados com auxílio de um software[§§§§§§]. Quanto aos parâmetros histométricos, foram analisados a extensão linear de tecido ósseo em contato direto com a superfície do

[††††††] Kulzer, Wehrheim, Alemanha

[‡‡‡‡‡‡] Exakt Cutting System, Alemanha

[§§§§§§] Osteomeasure - Osteometrics Inc.

implante e a área entre as roscas ocupada por tecido ósseo, ambos expressos em percentual. As mensurações foram realizadas empregando-se um software analisador de imagens[*******] em toda a extensão do implante. Os valores para a extensão linear de tecido ósseo em contato direto com a superfície do implante e para a área entre as roscas ocupadas por tecido ósseo foram obtidos em pixels e pixels ao quadrado, respectivamente, convertendo-se posteriormente para µm e μm^2.

ANÁLISE DA DENSIDADE RADIOGRÁFICA DA MANDÍBULA

Foram selecionadas aleatoriamente 10 mandíbulas de cada subgrupo, do lado sem indução de doença periodontal, para avaliação da densidade radiográfica.

As imagens radiográficas das hemimandíbulas foram obtidas por meio de um sistema de imagem digital direta - CDR (Computed Dental Radiography for Microsoft Windows)[†††††††], o qual utiliza um sensor eletrônico em substituição ao filme radiográfico. O sensor foi exposto à tomada radiográfica a uma potência de 70KVp e 10mA, com tempo de exposição de 15 pulsos/segundo, com uma distância foco-sensor padronizada em 70cm. Durante as tomadas radiográficas uma escala de alumínio, composta de 8 degraus, com 1mm de diferença na altura entre

[*******] Imagetool

[†††††††] Shick Technologies, Inc.

os degraus, foi posicionada sobre o sensor e a avaliação da densidade foi comparada com a densidade da escala (Eq. Al.).

As imagens foram exportadas do programa Schick® e importadas para um *software* analisador de imagens‡‡‡‡‡‡‡, em um computador com um processador de 1700 MHz§§§§§§§, 512k, 256 DDR MB e sistema operacional Windows XP******** em uma tela plana S-VGA de 15 polegadas (1024 x 768 pixel resolution). A densidade óssea radiográfica foi avaliada utilizando-se a análise dos níveis de cinza da radiografia (histograma) em uma área de 5x5 pixels nas regiões de interesse: área 1 – abaixo do segundo molar; área 2 – ângulo da mandíbula e área 3 – mesial ao primeiro molar, conforme mostra a figura 7.

A densidade radiográfica foi calculada separadamente para cada radiografia, onde foram obtidos valores de níveis de cinza para cada degrau da escala de alumínio. Com esses valores e os valores já conhecidos, em milímetros, da altura de cada degrau foi gerado um gráfico de regressão linear. A equação gerada por esse gráfico serviu de parâmetro para a transformação dos níveis de cinza, das regiões de interesse, em milímetros equivalentes de alumínio (Eq.Al.). A conversão entre valores expressos em níveis de cinza para Eq.Al. permitiu uma comparação padronizada entre os dados de densidade das diferentes radiografias, uma vez que corrige as variações da técnica.

‡‡‡‡‡‡‡ Adobe® Photoshop® 6.0

§§§§§§§ Athlon XP, AMD, USA

******** Microsoft, USA

Para cada hemimandíbula foram realizadas 2 medidas, por um mesmo examinador cego e calibrado.

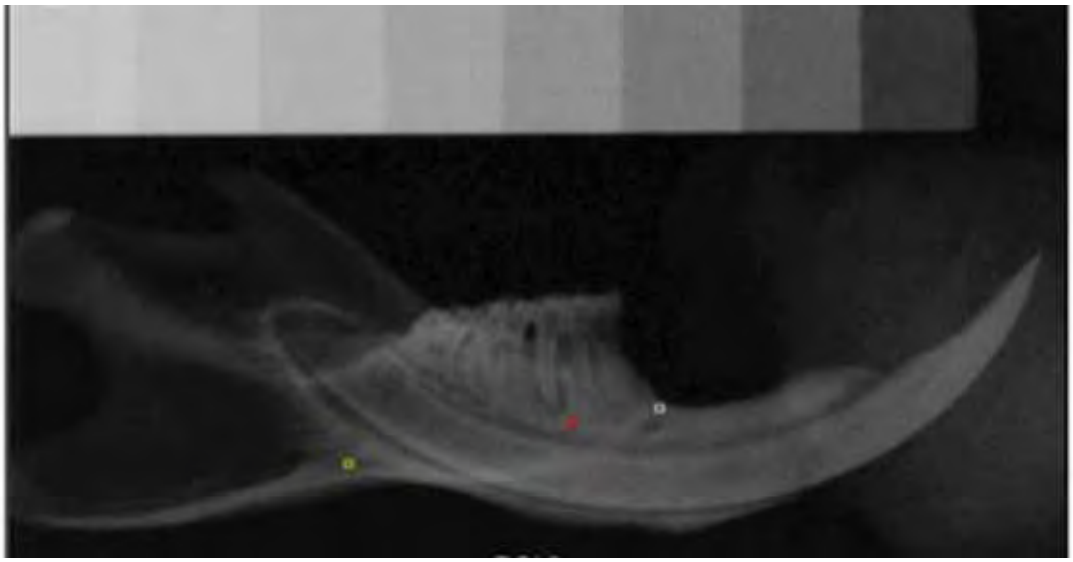

Figura 7 – Regiões de interesse análise da densidade óssea (- área 1; - área 2 e - área 3).

ANÁLISE MACROSCÓPICA DA PERDA ÓSSEA PERIODONTAL

As mandíbulas, de ambos os lados, foram cuidadosamente dissecadas e colocadas em solução de H_2O_2 por 24 horas. Posteriormente, todas as peças foram limpas com escova bitufo para que todo o tecido mole fosse removido. As mandíbulas foram coradas com solução de azul de toluidina a 10% e foram analisadas: a) perda óssea vertical na face vestibular, correspondendo à distância da junção cemento-esmalte à crista óssea, em 3 pontos (mesial, mediana e distal do primeiro molar inferior), de ambos os lados, b) Área de perda óssea na

face vestibular do primeiro molar inferior, de ambos os lados da mandíbula, conforme mostra a figura 8.

As mensurações foram realizadas utilizando-se um microscópio óptico Leica MZ6, com objetiva para aumento de 4.0/10 vezes, pelo o qual as imagens foram selecionadas e enviadas para um microcomputador acoplado ao microscópio óptico. A determinação dos valores foi realizada empregando-se um *software* analisador de imagens[††††††††].

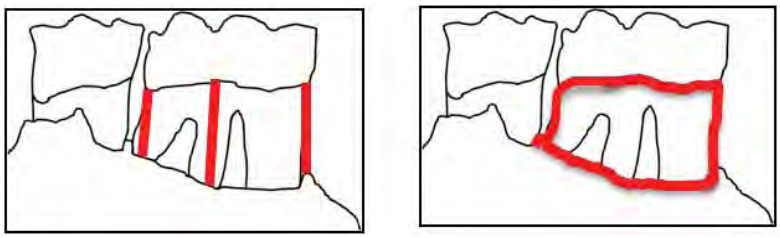

Figura 8 – Regiões de interesse para análise da perda óssea linear (raízes mesial, medial e distal) e de área, nos primeiros molares inferiores.

EXTRAÇÃO DE RNA TOTAL, TRANSCRIÇÃO REVERSA E PCR SEMI-QUANTITATIVO

O tecido gengival ao redor do primeiro molar inferior, de ambos os lados, de 5 animais de cada grupo foi conservado em trizol e congelado a -80°C, para análise da expressão de mRNA das citocinas Ligante do Receptor-Ativador do Fator Nuclear kappa β (RANKL), Osteoprotegerina (OPG) e IL6.

[††††††††] ImageJ – 1.40g/Java 1.6.0

Para o isolamento e purificação de RNA foi seguido o protocolo do fornecedor‡‡‡‡‡‡‡‡. No tecido macerado, para melhor isolamento de RNA, foram acrescentados 0,2 mL de clorofórmio para cada mL de Trizol (separação de fases). Os tubos foram agitados vigorosamente por 30 segundos e incubados à temperatura ambiente por 2 minutos, sendo, em seguida, centrifugados a 13.000 RPM a 4^{o}C por 15 minutos. A fase incolor superior foi cuidadosamente transferida para um novo tubo ao qual foi, então, acrescentado 0,5 mL de isopropanol (precipitação do RNA). Após agitação suave por inversão dos tubos, estes foram incubados por 10 minutos à temperatura ambiente e centrifugados por 10 minutos a 4^{o}C e 13.000 RPM. Após remoção por aspiração do sobrenadante, foi acrescentado 0,5 mL de etanol a 75% (lavagem do RNA), os tubos foram submetidos à breve agitação (5 segundos) em vortex e novamente centrifugados por 5 minutos a 4^{o}C e 9.500 RPM. O etanol foi removido cuidadosamente por aspiração e o RNA eluído em 10 a 40 µL (segundo o tamanho do pellet de RNA obtido) de tampão Tris-EDTA (TE, pH 7.5).

Estas amostras de RNA foram diluídas em tampão TE (1:100 a 1:1000), segundo o tamanho do pellet, para um volume final de 100 µL. A quantidade e pureza do RNA foram determinadas em espectrofotômetro de luz UV por meio da avaliação das absorbâncias a

‡‡‡‡‡‡‡‡ Invitrogen Corp., Carlsbad, USA

260 nm e da relação entre as absorbâncias a 260/280 nm, respectivamente.

A síntese de cDNA foi realizada subseqüentemente utilizando 700ng de RNA total e 200 unidades da enzima de transcriptase reversa na presença de Oligo-dT (12-18) primers, dNTPs e $MgCl_2$, segundo as instruções do fabricante[§§§§§§§§].

Os pares de primers específicos para os genes-alvo OPG, RANKL e IL6[*********] estão apresentados na Tabela 3. Como controle endógeno do RT-PCR foi utilizado a expressão de um gene constitutivo, neste caso beta actina (β-actina), cuja expressão não se altera com a deficiência de magnésio. Deve-se ressaltar que embora as sequências de pares de primers específicas para os genes-alvo de ratos tenham sido publicadas na literatura, as condições da reação de PCR (incluindo concentração de primers, temperatura de anelamento e número de ciclos) foram otimizadas em experimentos-piloto.

[§§§§§§§§] Invitrogen Corp., Carlsbad, USA

[*********] Invitrogen Corp., Carlsbad, USA

Tabela 3 – Seqüência dos primers e condições iniciais da reação de PCR (Ratos).

Gene	Primers (5' – 3') Sense (S) e antisense (AS)	Acession#	Ta (^{0}C)	Amplicon	Ciclos
B-actina	TCTCAGCTGTGGTGGTGAAG– S TGTCACCAACTGGGACGATA– AS	NM031144	57	437 bp	30
RANKL	ACGCAGATTTGCAGGACTCGAC – S TTCGTGCTCCCTCCTTTCATC – AS	F019048	60	493 bp	36
OPG	TCCTGGCACCTACCTAAAACAGCA – S CTACACTCTCGGCATTCACTTTGG - AS	U94330	57	578 bp	36
IL-6	CCGGAGAGGAGACTTCACAG – S GAGCATTGGAAGTTGGGGTA - AS	NM012589	56	428 bp	30

A reação de PCR foi realizada num volume total de 25 µL, utilizando 2 µL do produto da reação de transcriptase reversa na presença de 100 pmol/µL de primers de cada gene (50 pmol/µL de cada primer, *sense* e *antisense*). Os produtos da reação de PCR foram resolvidos por meio de eletroforese em gel de agarose a 1.5% e corados com brometo de etídeo (0.5 µg/mL). Para documentação e análise, foram obtidas imagens digitalizadas destes géis, as quais foram submetidas à análise densitométrica. A expressão dos genes-alvo foi normalizada para a expressão do gene constitutivo (β-actina) e expressas como *fold change* em relação ao controle negativo.

ANÁLISE HISTOLÓGICA DA MANDÍBULA

Foram selecionadas aleatoriamente 5 hemimandibulas do lado esquerdo, que foram fixadas em formol 4% por 48 horas. Essas espécimes foram descalcificadas por 28 dias em 10% EDTA (pH 6.9), trocados a cada 24 horas. Após o processo de descalcificação, as amostras foram desidratadas em concentrações graduais de etanol e incluídas em parafina. Secções seriadas de 5μm de espessura foram obtidas no plano sagital. Os cortes foram corados com hematoxilina e eosina (HE), e foram então, obtidas imagens com o auxilio de microscópio[†††††††††]. As imagens foram analisadas por um examinador treinado e cego para o experimento. Para a análise das imagens foi utilizado um microsoft analisador de imagem[‡‡‡‡‡‡‡‡‡]. Foi realizada uma análise descritiva por um examinador treinado e cego para o experimento.

Foi realizada também a avaliação da quantidade de osteoclasto na mandíbula. Para isso, os cortes foram submetidos à coloração específica para osteoclastos (TRACP). Para coloração de TRACP foi utilizada as amostras foram incubadas em 0.1M Tris HCl por 30 minutos em 37^{o}C. Foi então preparada solução contendo PVA, $MgCl_2$, $NaNO_2$ e KNa tartarato. Essa solução foi colocadas nas amostras que foram então incubadas por mais 2 horas em 37^{o}C. As amostras foram lavadas duas vezes em água MiliQ na temperatura de 70^{o}C. Após a coloração de TRACP, foi realizada a coloração de hematoxilina, e então

[†††††††††] Olympus BX-51 light microscope

[‡‡‡‡‡‡‡‡‡] Image Pro-Express 6.0; Olympus, Tokyo, Japan

finalizadas por processamento histológico de rotina. As imagens foram obtidas em 2 regiões da mandíbula, região de furca do 1º molar inferior e do 2º molar inferior, com auxílio do microscópio[§§§§§§§§§]. As imagens foram analisadas por um software analisador de imagem[**********]. Foi realizada a contagem de osteoclastos e avaliado o número de osteoclasto por área de tecido ósseo.

ESTUDO IN VITRO

ANIMAIS

Para os experimentos in vitro, foram utilizados camundongos C57BL/6 com 6 semanas de idade. Os animais foram mantidos por uma semana no biotério da Vrije Universiteit Medical Center. Este estudo foi aprovado pelo comitê de ética da VU University, Amsterdam.

CULTURA DE OSTEOCLASTOS

Células de medula óssea foram obtidas de fêmures e mandíbulas dissecados de animais C57BL/6 com 6 semanas de idade. As células foram isoladas conforme de Vries et al. (2005)[21], resuspensas, filtradas em um filtro para células de 100 um de espessura (Falcon/ Becton Dickinson, Franklin Lakes, NJ, USA) e mantidas em gelo em meio de cultura livre de Mg[††††††††††] (MEM alpha without nucleotides, with L-

[§§§§§§§§§] Olympus BX-51 light microscope

[**********] Image Pro-Express 6.0; Olympus, Tokyo, Japan

[††††††††††] Promocell, Heidelberg, Germany

glutamine) contendo 5% de soro‡‡‡‡‡‡‡‡‡‡, 100U/ml de penicilina, 100 µg/ml estreptomocina e 250 ng/ml amphotericin B§§§§§§§§§§.

As células da medula foram incubadas em placas de 96 poços********** em uma densidade de 1.0 x 10^5 celulas por poço em 150 ul de meio de cultura contendo 30ng/ml de fator estimulador de colônias de macrófagos (M-CSF)†††††††††† e 20 ng/mL de RANKL‡‡‡‡‡‡‡‡‡‡. As células foram incubadas em meios de cultura com diferentes concentrações de magnésio (100- quantidade adequada de Mg, ou seja, 0.8mM de Mg; 50% da quantidade de Mg, ou seja, 0.4mM de Mg; 10% da quantidade adequada de Mg, ou seja, 0.08 mM de Mg e 0%, ou seja, meio sem Mg)§§§§§§§§§§. Essas concentrações foram feitas por meio da mistura dos meios de cultura livre de Mg com o meio de cultura alpha MEM padrão.

As células foram incubadas sobre plástico e sobre cortes de tecido ósseo cortical bovino na expessura de 650um. O meio de cultura foi trocado a cada 3 dias.

AVALIAÇÃO DA FORMAÇÃO DE OSTEOCLASTOS

As células do fêmur e mandíbula foram cultivadas em plástico e sobre tecido ósseo por 6 dias. Após esse período, foi realizada

‡‡‡‡‡‡‡‡‡‡ HyClone, Logan, UT, USA

§§§§§§§§§§ Antibiotic antimyotic solution; Sigma-Aldrich, St. Louis, MO, USA

********** Costar, Cambridge, MA, USA

†††††††††† R&D Systems, Minneapolis, MN, USA

‡‡‡‡‡‡‡‡‡‡ RANKL-TEC, R&D Systems, Minneapolis, MN, USA

§§§§§§§§§§ Promocell, Heidelberg, Germany

lavagem dos poços com PBS e as células foram fixadas com formaldeído 4%, mantidas a 4C. A diferenciação celular foi avaliada pela coloração de TRACP, realizada por meio de kit comercial, conforme as instruções do fabricante[**********]. Para o núcleo das células foi realizada coloração especifica com DAPI (diamidino-2-phenylindole dihydrochloride). Foi verificada a quantidade de células multinucleadas positivas para TRACP ($TRACP^+$-MNC) para a avaliação da formação de osteclastos. A contagem foi dividida em 3 grupos pela quantidade de núcleos: células contendo 3-5, 6-10 e mais que 10 núcleos.

ANÁLISE DA CONCENTRAÇÃO DE CÁLCIO E MAGNÉSIO

Após 6 dias de incubação em plástico e em tecido ósseo, o sobrenadante dos 4 grupos com diferentes concentrações de Mg foram coletados e mantido a -20C, para posterior avaliação da concentração de Ca e Mg. O meio de cultura sem as células foi incubado nas mesmas condições. A concentração de Ca e Mg foi analisada por espectrometria de absorção atômica[††††††††††††].

ANÁLISE DE PROLIFERAÇÃO CELULAR

A proliferação celular foi avaliada pela quantidade de DNA. As células da medula óssea derivadas do fêmur de 6 animais foram

[**********] Leukocyte acid phosphatase kit, Sigma-Aldrich, Saint Louis, MO, USA

[††††††††††††] Analyst100; Perkin-Elmer Corporation, Norwalk, CT, USA

lavadas em PBS e coletadas no dia 0, após 3 e 6 dias de incubação no plástico, nas 4 diferentes concentrações de Mg na presença de M-CSF e RANKL. A quantificação do DNA foi realizada por meio de kit comercial‡‡‡‡‡‡‡‡‡‡‡‡ e mensurada pelo leitor Synergy HT§§§§§§§§§§§§, conforme indicação do fabricante.

ANÁLISE DA VIABILIDADE CELULAR

O sobrenadante das células da medula óssea derivadas do fêmur de 6 animais foram coletadas no dia 0 e após 3 dias de incubação no plástico, nas 4 diferentes concentrações de Mg na presença de M-CSF e RANKL ao meio de cultura. Alem disso, 3 dias de incubação, foi adicionado, triton na concentração de 0,5% . O triton causa danos a parede celular, o que o torna um controle positivo A citotoxidade celular foi avaliada por meio de kit comercial e mensurada pelo leitor Synergy HT**********, conforme a indicação do fabricante

PCR EM TEMPO-REAL

Para a analise de PCR em tempo real (qPCR), foram coletadas amostras de medula óssea de e camundongos apos serem cultivadas por 3 dias em meio contendo M-CSF e RANKL. O isolamento do RNA e o qPCR foi realizado como descrito anteriormente[22]. Os primers

‡‡‡‡‡‡‡‡‡‡‡‡ CyQuant cell proliferation assay kit, Invitrogen Corporation, Carlsbad, CA, USA

§§§§§§§§§§§ BioTek Instruments; Winooski, VT; USA

********** BioTek Instruments; Winooski, VT; USA

foram desenhados com o auxilio do software Primer Express 2.0 software[††††††††††††] (Tabela 4). No intuito de evitar a amplificação genômica do DNA, cada amplicon foi expandido em pelo menos 1 intron. Para avaliar a eficiência das reações de PCR, em uma das amostras foi gerada uma curva padrão para todos os genes estudados. As reações de PCR com diferentes amplificações tiveram eficiência iguais. Como controle endógeno do qPCR foi utilizado a expressão de um gene constitutivo, PBGD, cuja expressão não se altera com a deficiência de magnésio. As amostras foram testadas para TRPM-7 (Transient Receptor Potential-Melastin-like 7), MRS2, c-fos, RANK, c-fms, DC STAMP (dendritic cell-specific transmembrane protein), m-calpain, μ-calpain, IL1-β and TNF-α. Todas as amostras foram normalizadas para a expressão de PBGD pelo cálculo ΔCt ($Ct_{gene\ of\ interest}$ - Ct_{PBGD}), e a expressão genes-alvos foi expressa como $2^{-(\Delta Ct)}$.

[††††††††††††] Applied Biosystems, Foster City, CA, USA

Tabela 4 – Sequência de primers utilizada no estudo.

Primer	Sequence	Amplicon length (pb)	Accession #
c-fms	GAAACGCGACCTTCAAAAACA GGCCGGATCTTTGACATACAA	100	ENSMUSG00000024621
c-fos	TCACCCTGCCCCTTCTCA CTGATGCTCTTGACTGGC TCC	64	ENSMUSG00000021250
DC STAMP	TGTATCGGCTCATCTCCTCCAT GACTCCTTGGGTTCCTTGCTT	100	ENSMUSG00000022303
IL-1β	GGACCCATATGAGCTGAAAGCT TGTCGTTGCTTGGTTCTCCTT	100	ENSMUSG00000027398
MRS2	GGGTCCTGATTGACGATTCG TCACATTGCGATGGCTGTCT	62	ENSMUSG00000021339
TNF-α	GCCACCACGCTCTTCTGTCT GTCTGGGCCATAGAACTGATGAG	100	ENSMUSG00000024401
TRPM-7	CAACCGGAGCTTGGATTTTAAC TGAGGGCATCACCAACATGT	68	ENSMUSG00000027365
RANK	TGGGCTTCTTCTCAGATGTCTTT TGCAGTTGGTCCAAGGTTTG	59	ENSMUSG00000026321
PBGD	AGTGATGAAAGATGGGCAACT TCTGGACCATCTTCTTGCTGA	122	ENSMUSG00000032126

ANÁLISE DA ATIVIDADE DE TRACP

O sobrenadante das células da medula óssea derivadas do fêmur de 6 animais foram coletadas após 6 dias de incubação no plástico, nas 4 diferentes concentrações de Mg na presença de M-CSF e RANKL ao meio de cultura. A atividade de TRACP foi mensurada em placas de 96 poços contendo *p*NPP como substrato em 5µl de sobrenadante com a concentração final de: 10 mM *p*NPP, 1 mM ácido ascórbico, 0.1 mM $Fe(NH_4)_2(SO_4)_{2,}$ 0.1 M NaAc, 0.15 M KCl, 10 mM tartarato dissódico, 0.1% (v/v) Triton X-100. The *p*-nitrofenol liberado apos 30min de incubação a 37 °C foi convertido em *p*-nitrofenolato com a adição de 100µl of 0.3 M NaOH. A absorbância foi avaliada em 405 nm no leitor de placas Synergy HT‡‡‡‡‡‡‡‡‡‡‡‡‡. 1 unidade de TRACP corresponde a 1 µmol de *p*-nitrofenol formado por minuto.

ANÁLISE DA REABSORÇÃO ÓSSEA

As células de 6 camundongos foram cultivados nos meios de cultura com diferentes concentrações de Mg na presença de M-CSF e RANKL, sobre o tecido ósseo por um período de 8 dias. Após esse período, as células foram removidas do tecido ósseo com solução de 0,1 M de NaOH. Foram então realizadas lavagem com água destilada e o tecido ósseo foi incubado em solução de alumínio saturado ($KAl(SO_4)_2.12(H_2O)$. Após incubação, foi realizada nova lavagem com

‡‡‡‡‡‡‡‡‡‡‡‡‡ BioTek Instruments; Winooski, VT; USA

água destilada e foi então realizada a coloração com azul de coomassie. Foram obtidas 4 fotografias representativas de cada tecido ósseo, sendo mensurada a área de reabsorção com o auxilio do programa analisador de imagem Image Pro-Express 6.3§§§§§§§§§§§§. Os tecidos ósseos foram analisados em triplicata para cada animal e a área de reabsorção foi calculada pela porcentagem da superfície de osso reabsorvido.

§§§§§§§§§§§§ Olympus, Tokyo, Japan

ANEXOS

A- Comitê de Ética

B- Documentos Comprobatórios

A-

unesp

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara
FACULDADE DE ODONTOLOGIA

DECLARAÇÃO

Em atendimento ao pedido verbal feito nesta mesma data, **DECLARO**, para fins de apresentação junto à FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo com a finalidade de comprovar vínculo ao projeto para solicitação de bolsa de doutorado, que o Comitê de Ética na Experimentação Animal desta mesma Faculdade recebeu o projeto **"Influência da deficiência de magnésio na dieta sobre a densidade óssea e a osseointegração de implantes. Estudo em ratos"**, apresentado pelos pesquisadores: Profª Drª Elaine Maria Sgavioli Massucato, Profª Drª Silvana Regina Perez Orrico, Prof. Dr. Élcio Marcantonio Junior, Profª Drª Thaís Borges César, Drª Gabriela Giro, Ricardo Andrés Landázuri Del Barrio e Marina Montosa Belluci .O referido projeto deu entrada neste Comitê e recebeu autorização para realização da pesquisa.

Araraquara, 30 de setembro de 2008.

Profª Drª ELENY BALDUCCI ROSLINDO
Coordenadora

B-

Ms. Ref. No.: BONE-D-12-00247
Title: Magnesium deficiency results in an increased formation of osteoclasts
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Dear Marina,

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CLINICAL ORAL IMPLANTS RESEARCH

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Effects of magnesium intake deficiency on bone metabolism and bone tissue around osseointegrated implants

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Key words: bone markers, dental implants, magnesium deficiency, rat

Abstract

Objectives: This study evaluated the effect of magnesium dietary deficiency on bone metabolism and bone tissue around implants with established osseointegration.

Materials and methods: For this, 30 rats received an implant in the right tibial metaphysis. After 60 days for healing of the implants, the animals were divided into groups according to the diet received. Control group (CTL) received a standard diet with adequate magnesium content, while test group (Mg) received the same diet except for a 90% reduction of magnesium. The animals were sacrificed after 90 days for evaluation of calcium, magnesium, osteocalcin and parathyroid hormone (PTH) serum levels and the deoxypyridinoline (DPD) level in the urine. The effect of magnesium deficiency on skeletal bone tissue was evaluated by densitometry of the lumbar vertebras, while the effect of bone tissue around titanium implants was evaluated by radiographic measurement of cortical bone thickness and bone density. The effect on biomechanical characteristics was verified by implant removal torque testing.

Results: Magnesium dietary deficiency resulted in a decrease of the magnesium serum level and an increase of PTH and DPD levels ($P < 0.05$). The Mg group also presented a loss of systemic bone mass, decreased cortical bone thickness and lower values of removal torque of the implants ($P < 0.01$).

Conclusions: The present study concluded that magnesium-deficient diet had a negative influence on bone metabolism as well as on the bone tissue around the implants.

Magnesium is one of the most abundant minerals in the body and is essential for several enzymes and cell functions, acting as an important modifier of the inflammatory and immune response (Maguire & Cowan 2002). It also plays a relevant role on bone tissue and mineral homeostasis and may directly affect the function of bone cells and the hydroxyapatite crystal growth (Cheraskin et al. 1990).

It is estimated that from 2.5 to 15% of the world population suffers from some form of hypomagnesemia (Sahbagh et al. 2008). Magnesium dietary deficiency may be common in industrialized countries, as reported in the United States (Marx & Nesmith 1997; Ford & Mokdad 2003) and European countries (Schimatschek & Rempis 2001; Tarver et al. 2006). Although it is a mineral found in many foods, its absorption requires ideal conditions and may be easily inhibited by several factors. Even after being absorbed in the body, several substances contribute to an increased kidney excretion of magnesium such as excessive alcohol intake, diuretics, coffee, tea, salt, phosphoric acid and sugar, all common in diets nowadays (Johnson 2001). Some manifestations may be related to a deficiency such as hypertension, vascular function alteration, insulin resistance and/or altered insulin secretion (Evangelopoulos et al. 2008).

Some epidemiological studies show a positive correlation between a magnesium-deficient diet and an increase in loss of bone mass and/or a decrease in bone density, which suggest that this mineral deficiency may be a risk factor for osteoporosis (New et al. 1997; Tucker et al. 1999; Wang et al. 1996). Animal studies with different levels of deficiency showed bone loss, characterized by a decrease of trabecular bone volume, followed by an increase in the release of pro-inflammatory cytokines and alteration in secretion and action of the parathyroid hormone (PTH), contributing to the decrease in bone formation (Rude et al. 2004, 2005, 2006). For a prolonged period (12 months), a magnesium

Autorizo a reprodução deste trabalho.

Araraquara, 16 de abril de 2012.

MARINA MONTOSA BELLUCI