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Tese de Doutorado

INFLUÊNCIA DA SUPLEMENTAÇÃO DE CÁLCIO SOBRE OS NÍVEIS E OS INDICADORES DA EXPOSIÇÃO AO CHUMBO EM MULHERES NA PÓS-MENOPAUSA

Marla Hahn Veroneze

Santa Maria, RS, Brasil

2008

INFLUÊNCIA DA SUPLEMENTAÇÃO DE CÁLCIO SOBRE OS NÍVEIS E OS INDICADORES DA EXPOSIÇÃO AO CHUMBO EM MULHERES NA PÓS-MENOPAUSA

por

Marla Hahn Veroneze

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica

Orientadora: Profª. Dra. Tatiana Emanuelli

Santa Maria, RS, Brasil

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Universidade Federal de Santa Maria
Centro de Ciências Naturais e Exatas
Programa de Pós-Graduação em Ciências Biológicas:
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elaborada por
Marla Hahn Veroneze

como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica

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Santa Maria, 07 de Janeiro de 2008.

DEDICATÓRIA

Dedico este trabalho a meu filho Enzo,

pelas muitas horas que não pude estar contigo,

e ao meu marido Edson, que com grande

sabedoria soube muitas vezes ser pai e mãe,

sem ter deixado de ser esposo.

Amo muito vocês.

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

Influência da suplementação de cálcio sobre os níveis de chumbo e indicadores da exposição ao chumbo em mulheres na pós-menopausa

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Data e local da defesa: Santa Maria, 07 de Janeiro de 2008.
Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

A acelerada perda óssea que ocorre durante a menopausa e torna-se mais acentuada no período pós-menopáusico, mediada pelo término na produção de estrogênio, pode constituir uma ameaça para mulheres nessa fase da vida, no que diz respeito à toxicidade do chumbo. Aproximadamente 95% do chumbo acumulado no organismo está depositado nos ossos e com a desmineralização óssea o metal passa para a corrente sanguínea, constituindo um risco potencial. Por outro lado, uma adequada suplementação de cálcio parece reduzir a absorção gastrintestinal de chumbo. No entanto, estudos realizados nos Estados Unidos revelaram níveis relativamente elevados de chumbo em suplementos de cálcio. No presente estudo, nós avaliamos o conteúdo de chumbo nos suplementos de cálcio disponíveis no Brasil. Investigamos também os efeitos da suplementação de cálcio e da doença óssea sobre os níveis sanguíneos de chumbo, a atividade da enzima δ-aminolevulinato desidratase (δ-ALAD), índice de reativação da δ-ALAD e a atividade de enzimas antioxidantes em mulheres pós-menopáusicas não expostas ocupacionalmente ao chumbo. Foi realizado um estudo transversal e outro prospectivo, onde em ambos os estudos foram avaliados os parâmetros bioquímicos; sendo que no estudo prospectivo esses parâmetros foram avaliados antes e após três meses de suplementação de cálcio. Um total de 11 produtos à base de cálcio foram selecionados e o conteúdo de chumbo e cálcio foi determinado por espectrometria de absorção atômica com forno de grafite e de chama, respectivamente. Os níveis de chumbo sanguíneo foram medidos por espectrometria de massa com plasma acoplado indutivamente e a densidade mineral óssea (DMO) foi determinada na lombar (DMO L1-L4) e no colo do fêmur (DMO fêmur) por absorciometria de duplo feixe de raios-X. A atividade da δ-ALAD e das enzimas antioxidantes foram determinados em sangue total usando métodos espectrofotométricos. O índice de reativação da δ-ALAD foi determinado pela medida da atividade da enzima em presença de 3

mM de $ZnCl_2$ e 10 mM de DL-ditiotreitol. A menor quantidade de chumbo por grama de cálcio foi encontrada nos suplementos de cálcio à base de ossos (< que o limite de detecção) e a maior quantidade de chumbo por grama de cálcio foi encontrada na dolomita (2,3±1,2 $\mu g.g^{-1}$ de cálcio medido). Nenhuma diferença foi observada na atividade da δ-ALAD ou no índice de reativação da δ-ALAD entre as mulheres pós-menopáusicas com e sem doença óssea, em ambos os estudos, transversal e prospectivo. No estudo prospectivo, três meses de suplementação de cálcio aumentou os níveis de chumbo sanguíneo no grupo com osteopenia (4,6 µg/dL) quando comparado ao grupo controle (3,7 µg/dL) e diminuiu a atividade da fosfatase alcalina em todos os grupos: controle (66,2 vs. 71,9 U/L antes do início do tratamento) osteopenia (67,1 vs. 71,1 U/L) e osteoporose (83,9 vs. 86,1 U/L). A atividade da catalase (CAT) e da superóxido dismutase (SOD) não foram diferentes entre as mulheres pós-menopáusicas com e sem doença óssea, em ambos os estudos, transversal e prospectivo. No entanto, a atividade da glutationa peroxidase (GPx) foi significativamente maior no grupo osteopenia (23,32 µmol NADPH/g Hb/min) quando comparado ao grupo controle (18,56 µmol NADPH/g Hb/min) no estudo transversal. Esse resultado foi interpretado como uma resposta defensiva contra a produção excessiva de espécies reativas de oxigênio nas mulheres com osteopenia. Os resultados do estudo transversal indicaram que a reabsorção óssea associada com osteopenia/osteoporose não representa um risco de toxicidade do chumbo em mulheres na pós-menopausa expostas a baixos níveis de chumbo. No entanto, os resultados do estudo prospectivo sugerem que três meses de suplementação de cálcio contribuíram para um pequeno, mas significativo aumento nos níveis sanguíneos de chumbo em mulheres na pós-menopausa com doença óssea. Embora os níveis de chumbo encontrados nos suplementos de cálcio tenham ficado abaixo dos limites estabelecidos nos Estados Unidos, faz-se necessário a regulamentação, através de uma legislação específica, dos níveis permitidos de chumbo em suplementos de cálcio no Brasil. Também seria importante um programa de monitoramento de tais níveis, pois através dos nossos resultados constatamos que os suplementos de cálcio atuam como uma pequena fonte de chumbo, mas que poderá vir a causar efeitos deletérios, principalmente em mulheres na pós-menopausa.

Palavras-chave: pós-menopausa; chumbo; osteopenia; osteoporose; δ-ALAD; glutationa peroxidase, catalase, superóxido dismutase.

ABSTRACT

Thesis of Doctor's Degree
Graduate Program on Biological Sciences: Toxicological Biochemistry
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Influence of calcium supplementation on blood lead levels and markers of lead exposure in posmenopausal women

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Date and place of the defense: Santa Maria, January 07, 2008.

The accelerated bone loss that occurs during menopause and becomes more prone in the postmenopausal period is mediated by the ending of estrogen production. This bone loss can be a threat for women in this period of life concerning to the lead toxicity. Around 95% of the lead accumulated in the body is stored in the bones and may be mobilized to the bloodstream during bone demineralization, posing a potential risk. On the other hand, an adequate calcium supplementation seems to reduce gastrointestinal lead absorption. However, studies carried out in the United States revealed relatively high lead levels in calcium supplements. In the present study, we evaluated the content of lead in calcium supplements available in Brazil. We also investigated the effect of calcium supplementation and bone diseases on blood lead levels, δ-aminolevulinic acid dehydratase (δ-ALAD) activity, δ-ALAD reactivation index and antioxidant enzymes activities in postmenopausal women non-occupationally exposed to lead. Two studies were conducted, one with a cross-sectional design and another with a prospective design. Biochemical parameters were evaluated in both studies. In the prospective study these parameters were evaluated before and after three months of calcium supplementation. A total of 11 calcium-based products were selected and their lead and calcium content were determined by graphite furnace and flame atomic absorption spectrometry, respectively. Blood lead was assessed by inductively coupled plasma mass spectrometry and bone mineral density (BMD) was evaluated at the lumbar spine (BMD L1-L4) and femoral neck (BMD femur) by dual energy X-ray absorptiometry. δ-ALAD activity and antioxidant enzymes activities were determined in whole blood using spectrophotometric methods. δ-ALAD reactivation index was determined by measuring enzyme activity in the presence of 3 mM $ZnCl_2$ and 10 mM DL-dithiothreitol. The lowest lead content per gram of calcium was found in bonemeal (< limit of quantification) and the highest lead content per gram of calcium was found in dolomite (2.3±1.2 μg. g^{-1} of measured calcium). No differences were observed in δ-ALAD activity or δ-ALAD reactivation index between postmenopausal women with and without bone diseases, both in the cross-sectional and in the prospective study. In the prospective study, three months of calcium supplementation increased blood lead levels in osteopenia (4.6 μg/dL) when compared with control group (3.7

µg/dL) and decreased alkaline phosphatase activity in all groups: control (66.2 vs. 71.9 U/L before the beginning of the treatment) osteopenia (67.1 vs. 71.1 U/L) and osteoporosis (83.9 vs. 86.1 U/L). Catalase (CAT) and superoxide dismutase (SOD) activities were not different between postmenopausal women with and without bone diseases, both in the cross-sectional and in the prospective study. However, gluthatione peroxidase (GPx) activity was significantly higher in osteopenia group (23.32 µmol NADPH/g Hb/min) as compared to control group (18.56 µmol NADPH/g Hb/min) in the cross-sectional study. This finding was interpreted as a defense response to counteract the overproduction of reactive oxygen species in women with osteopenia. Results of the cross-sectional study indicated that bone resorption associated to osteopenia/osteoporosis does not pose a risk of lead toxicity in postmenopausal women exposed to background lead levels. However, results of the prospective suggest that three months of calcium supplementation contributed to a small, but significant increase of blood lead levels in postmenopausal women with bone disease. Although lead levels found in calcium supplements were below the limits established in the United States, it is important to regulate the allowed lead levels in calcium supplements in Brazil through of a specific legislation. A monitoring program of lead levels would also be important, because our results revealed that calcium supplements are a small lead source. Despite being a low lead source, it could cause deleterious effects, mainly in women in the postmenopausal.

Keywords: postmenopausal; lead; osteopenia; osteoporosis; δ-ALAD; glutathione peroxidase; catalase; superoxide dismutase.

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δ-ALAD – Delta-aminolevulinato desidratase
ALA – Ácido delta-aminolevulínico
ALP – Fosfatase alcalina
SOD – Superóxido dismutase
GPx – Glutationa peroxidase
CAT – Catalase
ICP-MS – Espectrometria de massas com plasma acoplado indutivamente
DEXA – Absorciometria de duplo feixe de raios-X
DMO – Densidade mineral óssea

SUMÁRIO

APRESENTAÇÃO

Os resultados que fazem parte desta tese são apresentados sob a forma de artigos e manuscritos, os quais se encontram no item ARTIGOS CIENTÍFICOS E MANUSCRITOS. As seções Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e manuscritos e representam na íntegra este estudo.

Os itens DISCUSSÃO E CONCLUSÃO, dispostos após os artigos, contém interpretações e comentários gerais referentes ao presente estudo e relacionados aos artigos científicos deste trabalho.

As REFERÊNCIAS BIBLIOGRÁFICAS são relacionadas às citações que aparecem nos itens INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA e DISCUSSÃO desta tese.

1 INTRODUÇÃO

A exposição ao chumbo pode provocar anemia, insuficiência renal, fraqueza neuromuscular, doenças cardiovasculares e alterações hematológicas (SARYAN & ZENZ, 1994; WHO, 1995; ATSDR, 1999).

Os efeitos do chumbo sobre o sistema hematológico estão relacionados à ação inibitória deste metal sobre a via de biossíntese do heme, o grupo prostético da hemoglobina. Nesta rota o chumbo inibe a atividade de enzimas como a δ-aminolevulinato desidratase (δ-ALAD) e a ferroquelatase (WILDT *et al*., 1987; ROCHA *et al*., 1995; 2001; GOULART *et al*., 2001). Foi demonstrado que a δ-ALAD é a principal proteína responsável pela ligação de chumbo nos eritrócitos (BERGDAHL *et al*., 1997). Além disso, a atividade da δ-ALAD eritrocitária é geralmente aceita como indicador bioquímico da intoxicação aguda ou crônica por chumbo (LOBIN & GORMAN, 1986). Em humanos, a δ-ALAD eritrocitária é inibida a partir de concentrações sanguíneas de chumbo acima de 5 $\mu g.dL^{-1}$ (CAMPAGNA *et al*., 1999).

A inibição da δ-ALAD provoca acúmulo de ácido delta-aminolevulínico (ALA) e aumento na quantidade de protoporfirina IX (COSTA *et al*., 1997), efeitos que têm sido implicados na toxicidade do chumbo. Tem sido demonstrado experimentalmente, que o ALA acumulado pode sofrer autoxidação provocando danos oxidativos no sistema nervoso central (DEMASI *et al*., 1996; EMANUELLI *et al*., 2001, 2003) e no DNA (FRAGA *et al*., 1994; ONUKI *et al*., 1994). Além disso, existem evidências de que este efeito pró-oxidante do ALA pode estar relacionado a alterações na atividade das enzimas antioxidantes eritrocitárias superóxido dismutase e glutationa peroxidase, e a aumentos na metemoglobina em humanos expostos ao chumbo (MEDEIROS *et al*., 1982; MONTEIRO *et al*., 1985; HERMES-LIMA *et al*., 1991; BECHARA *et al*., 1993; COSTA *et al*., 1997).

O chumbo é quimicamente semelhante ao cálcio, podendo mimetizar a ação deste em muitos processos fisiológicos (GODWIN *et al*., 2001). O chumbo pode substituir o cálcio no organismo afetando a neurotransmissão (SILBERGELD &

ADLER, 1978; GOYER, 1988). Pode também substituir o cálcio, nos ossos, depositando-se neste tecido (POUNDS *et al*., 1991). Mais de 95% do chumbo acumulado no organismo está localizado nos ossos, onde o metal apresenta uma meia vida de aproximadamente 27 anos (WHO, 1995). Assim, o chumbo depositado, pode ser mobilizado em casos de desmineralização óssea, tais como deficiência de cálcio na dieta, gestação, lactação (GULSON *et al*., 1997, 1998 a,b) e menopausa (SILBERGELD *et al*., 1988; SILBERGELD, 1991; SYMANSKI & HERTZ-PICCIOTTO, 1995; BAECKLUND *et al*., 1999; HERNANDEZ-AVILA *et al*., 2000), mesmo longo tempo após o término da exposição externa a este metal. Por outro lado, foi demonstrado que a ingestão de leite ou a suplementação com cálcio parece reduzir a absorção gastrintestinal de chumbo e limitar os níveis sanguíneos de chumbo durante a gestação, a lactação (FARIAS *et al*., 1996; HERNANDEZ-AVILA *et al*., 1997; PIRES *et al*., 2002; DONANGELO *et al*., 2002), e também na fase pós-menopausa (WEYERMANN & BRENNER, 1998).

OISHI *et al*. (1996) demonstraram que as mulheres são mais sensíveis a alterações induzidas pelo chumbo no metabolismo das porfirinas, do que os homens. Assim, a acelerada perda óssea que pode ocorrer no climatério, tanto na menopausa quanto no período pós-menopausa, mediada pela redução na produção de estrogênio, pode constituir uma ameaça para mulheres mais idosas, no que diz respeito a toxicidade do chumbo. Além disso, o chumbo pode se constituir em um risco potencial para a osteoporose, tanto pela indução de disfunção renal e inibição da ativação da 1,25-dihidroxivitamina D, quanto por alterar a responsividade das células ósseas (osteoblastos e osteoclastos) à regulação hormonal (POUNDS *et al*., 1991; SILBERGELD *et al*., 1999).

Os suplementos de cálcio são geralmente prescritos na prevenção e tratamento da osteoporose, no climatério, especialmente após a menopausa (BAYLINK *et al*., 1999), sendo utilizados como adjuvantes no tratamento da osteopenia para prevenção de fraturas. No entanto, um trabalho realizado nos Estados Unidos revelou a ocorrência de alterações neurológicas em pacientes que estavam tomando suplementos de cálcio e apresentavam níveis relativamente elevados de chumbo no cabelo (ROBERTS, 1983). A preocupação com a contaminação foi reforçada por outros trabalhos que também revelaram a presença de níveis preocupantes de chumbo em suplementos de cálcio (BOURGOIN *et al*., 1993; WHITING, 1994; ROSS

et al., 2000; SCELFO & FLEGAL, 2000). Assim, apesar da suplementação com cálcio poder reduzir a absorção e a toxicidade do chumbo, a presença de chumbo como contaminante nos suplementos de cálcio poderia torná-los fatores de risco para a exposição ao chumbo.

Não foram encontrados trabalhos avaliando os níveis de chumbo em suplementos comercializados no Brasil. Além disso, não existe, no Brasil, uma legislação específica regulamentando os níveis de chumbo em suplementos de cálcio. A Farmacopéia Brasileira estabelece que o teor máximo de metais pesados em carbonato de cálcio utilizado em insumos farmacêuticos e medicamentos deve ser de 0,002%.

Considerando a toxicidade do chumbo e o fato de não se saber qual a quantidade de cálcio necessária para prevenir os efeitos do chumbo e tampouco o quanto de chumbo se ingere ao consumir suplementos de cálcio é que percebemos o quanto esse assunto é importante e se faz necessário sua investigação para saúde pública. Assim, o presente estudo teve por objetivo avaliar a influência da suplementação de cálcio sobre os níveis sanguíneos de chumbo, a atividade da δ-ALAD e indicadores de estresse oxidativo em mulheres na pós-menopausa. Nessa perspectiva, os objetivos específicos foram:

- ➔ Quantificar o chumbo presente nos suplementos de cálcio mais utilizados por mulheres na pós-menopausa no Brasil.
- ➔ Investigar em mulheres na pós-menopausa não expostas ocupacionalmente ao chumbo:
  - ➔ O efeito da suplementação de cálcio e de doenças ósseas sobre a atividade das enzimas antioxidantes.
  - ➔ O efeito de doenças ósseas sobre os níveis sanguíneos de chumbo, atividade da δ-ALAD e reativação da δ-ALAD.
  - ➔ O efeito da suplementação de cálcio durante três meses e de doenças ósseas sobre os níveis sanguíneos de chumbo, atividade da δ-ALAD e enzimas antioxidantes.

2 REVISÃO BIBLIOGRÁFICA

2.1 A menopausa

A menopausa é definida como o último período menstrual, sendo completamente estabelecida após um ano ou mais de amenorréia (MIQUEL *et al*., 2006). O envelhecimento reprodutivo ocorre rapidamente após a terceira década de vida, e a fecundidade é extremamente baixa antes da menopausa, sinalizando que o envelhecimento reprodutivo precede a menopausa em 5 a 10 anos. O corpo sofre mudanças e a mulher passa a apresentar sinais e sintomas característicos da diminuição na produção de estrogênios, como ondas de calor, disfunções urogenitais, desenvolvimento de doenças cardiovasculares, osteoporose, Alzheimer e depressão (SOWERS, 1998; SILBERGELD & FLAWS, 1999).

Os receptores de estrogênio são abundantes em todo o corpo e por isso a ausência de estrogênio exerce potencial influência praticamente em todos os sistemas. No sistema nervoso central, o estrogênio é importante para o fluxo sanguíneo, a atividade sináptica, o crescimento neuronal, a sobrevida dos neurônios colinérgicos, assim como para a cognição, e sua deficiência resulta em alterações sintomáticas e fisiológicas (CECIL & RUSSELL, 2005). No sistema cardiovascular a alteração mais significativa decorrente da ausência de estrogênio é a elevação dos níveis plasmáticos de colesterol total, a qual é devido ao aumento nos níveis do colesterol ligado à lipoproteína de baixa densidade (LDL-C) (O'KEEFE et al., 1995).

A deficiência de estrogênio exerce um efeito negativo sobre o colágeno, o qual é importante para os ossos, pele e outros órgãos, como a pelve e o sistema urinário, podendo dessa forma levar a atrofia da mucosa vaginal e uretral, e conseqüentemente à incontinência urinária (CECIL & RUSSELL, 2005). Por fim, a descoberta dos receptores de estrogênio nos osteoblastos sugere que sua deficiência pode alterar

diretamente a formação óssea (MANO *et al*., 1996; MITRA *et al*., 2006). Pode-se dizer que a causa principal da osteoporose é a osteogênese deficiente e por conseqüência a reabsorção óssea aumentada que ultrapassa os limites causando desequilíbrio no metabolismo ósseo (TURNER *et al*., 1994).

2.2 O tecido ósseo

O osso é uma forma especializada de tecido conjuntivo, que é constituído de células e de matriz extracelular, contendo componentes minerais e orgânicos. A mineralização óssea produz um tecido rígido, o qual é composto fundamentalmente por cálcio e fosfato, enquanto que a matriz orgânica é principalmente formada por colágeno do tipo I (RIGGS & HARTMANN, 2003; RIGGS, 2003).

O tecido ósseo é metabolicamente ativo, sofrendo um processo contínuo de renovação e remodelamento. Por esta razão as células ósseas são responsáveis pela regulação e distribuição dos componentes inorgânicos, pela homeostasia mineral e esquelética, que é a contínua formação e reabsorção da matriz do osso (BURTIS & ASHWOOD, 1996).

Os elementos celulares do osso são os osteoblastos, osteócitos, células de revestimento ósseo e osteoclastos. As células de revestimento ou osteoprogenitoras são as precursoras dos osteoblastos, que são células secretoras de colágeno e de substância fundamental. O osteócito representa um osteoblasto transformado, ou seja, maduro, que ficou aprisionado na matriz óssea, sendo metabolicamente menos ativo e não tendo função conhecida. Os osteoclastos, por sua vez, são originários das células hematopoiéticas. Além das células, temos um tecido pré-ósseo ou ósseo não-calcificado, que é constituído de substância fundamental, denominado osteóide (DOWNEY & SIEGEL, 2006).

A localização dessas células no osso também é diferente, enquanto os osteócitos estão localizados no interior do osso, as demais células encontram-se ao longo da superfície óssea (DOWNEY & SIEGEL, 2006) como mostra a Figura 1.

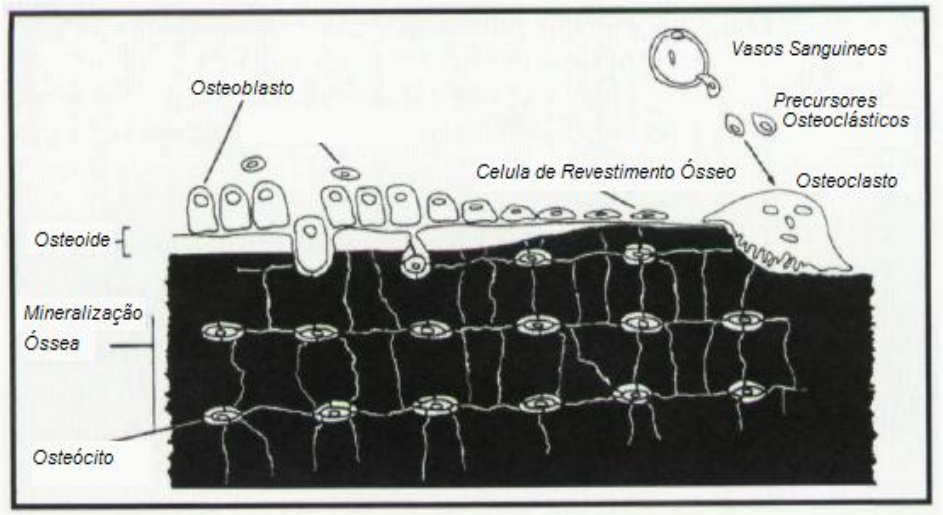

Figura 1 – A origem e localização das células ósseas (DOWNEY & SIEGEL, 2006).

Os osteoblastos sintetizam a matriz óssea, sendo responsáveis pela renovação ou pela formação do tecido ósseo, já os osteoclastos possuem enzimas que desmineralizam e digerem a matriz óssea, fazendo assim a reabsorção óssea (DOWNEY & SIEGEL, 2006).

Os dois principais tipos de tecido ósseo são o trabecular, uma estrutura de aspecto esponjoso; e o cortical, mais sólido e formado por lamelas ósseas. Eles diferem quanto à distribuição espacial das células, densidade da matriz mineralizada, distribuição dos vasos sanguíneos e área ocupada pela medula óssea. Em função de sua maior superfície, o osso trabecular é metabolicamente mais ativo que o cortical, mas em ambos os osteoblastos e osteoclastos movem-se sobre a superfície, sendo que os osteoblastos podem tornar-se embebidos na matriz, dando origem aos osteócitos (RIGGS & HARTMANN, 2003; RIGGS, 2003).

2.3 Homeostase esquelética

O cálcio participa da contração muscular, do tecido ósseo, da secreção de hormônios e da coagulação sanguínea. Sua homeostase é modulada por mecanismos regulatórios complexos envolvendo fatores locais e sistêmicos que atuam sobre as células ósseas. O paratormônio (PTH), que é produzido e liberado pelas glândulas paratireóides, é o principal regulador do metabolismo do cálcio, sendo liberado sempre que o nível plasmático de cálcio cair abaixo dos limites normais e sua célula-alvo no tecido ósseo é o osteoclasto, aumentando assim a atividade reabsortiva. O estrogênio atua diretamente sobre os osteoblastos, ativando-os. Já o hormônio 1,25-diidroxicolecalciferol (1,25-DHCC), formado a partir da vitamina D (colecalciferol) estimula a absorção intestinal de cálcio e PO_4 e o aumento da reabsorção óssea pelos osteoclastos (BERGLUND *et al*., 2000).

Além disso, temos o efeito do hormônio calcitonina, um peptídeo com 32 aminoácidos secretado pelas células parafoliculares ou C da tireóide, que parece atuar contrabalançando a ação do PTH, sendo sua secreção mediada principalmente pelo aumento de cálcio no sangue (BURTIS & ASHWOOD, 1996).

O cálcio extracelular é o responsável pela manutenção do cálcio intracelular, pela mineralização óssea, coagulação do sangue, manutenção do potencial de membrana, contração muscular e pela ação do cálcio como segundo mensageiro nas atividades enzimáticas e na secreção hormonal (AURBACH *et al*.,1992). Encontra-se no plasma, estando aproximadamente 50% na forma livre (ionizado), 40% ligado a proteínas plasmáticas, sendo que destes 80% está ligado à albumina e os 20 % restantes a globulinas, e 10% complexado, com bicarbonato, lactato, fosfato e citrato. O cálcio ligado ao PO_4 não sofre reabsorção tubular renal (MOTTA, 2003).

O cálcio ionizado é biologicamente ativo, firmemente regulado por hormônios e origina-se tanto da absorção no intestino delgado quanto da reabsorção dos ossos, sendo mantido sempre sob um rígido controle homeostático. O cálcio do esqueleto é o principal local de armazenamento e mobilização de cálcio para o “pool” extracelular e intracelular (COHEN & ROE, 2000).

2.3.1 Marcadores do metabolismo ósseo

Os marcadores do metabolismo ósseo servem como parâmetros de avaliação da homeostase esquelética. Durante o período da vida adulta, a atividade metabólica óssea, e conseqüentemente os níveis dos marcadores, tendem a ser mais baixos que os observados na infância e na adolescência (RAUCH *et al*., 1994). Durante a gravidez e a lactação, o metabolismo ósseo torna-se mais acelerado, resultando em aumento dos níveis dos marcadores de formação e reabsorção (SOWERS *et al*., 1995). Após a menopausa, os marcadores também tendem a se elevar, sendo que os marcadores de reabsorção apresentam-se mais elevados do que os de formação (KUSHIDA *et al*., 1995).

A fosfatase alcalina e a osteocalcina são os principais marcadores de formação óssea, que refletem a atividade osteoblástica em diferentes estágios de diferenciação desta célula. A fase de produção da matriz colágena precede a mineralização e coincide com uma maior produção de fosfatase alcalina, enquanto que a mineralização coincide com uma maior produção de osteocalcina (STEIN & LIAN, 1993). A osteocalcina é a principal proteína não-colágena do osso e é sintetizada pelos osteoblastos maduros, que a depositam em quantidades significativas na matriz óssea. No processo de reabsorção a matriz óssea é totalmente destruída (POWER & FOTTRELL, 1991). A fosfatase alcalina é encontrada em muitos tecidos, sendo que em condições normais as duas formas predominantes na circulação, são a hepática e a óssea (>90%), estando em quantidades equivalentes e localizando-se na superfície externa da célula onde exerce sua atividade (VIEIRA, 1999).

A hidroxiprolina, o N-telopeptídeo (NTx) de ligação cruzada do colágeno tipo I, e o cálcio urinário total, são os principais marcadores de reabsorção óssea (VIEIRA, 1999).

O uso dos marcadores bioquímicos se estende, teoricamente, a qualquer condição que leve a uma alteração do metabolismo ósseo, com aumento ou diminuição da remodelação óssea. Dentre esses marcadores podemos destacar como sendo os métodos de análise mais difundidos a dosagem de fosfatase alcalina e o

cálcio total urinário, sendo que este último reflete além da reabsorção óssea, a absorção intestinal e a filtração e reabsorção tubular renal (MOTTA, 2003).

2.4 Doença óssea

A constante de formação e reabsorção óssea, também chamada “turnover ósseo”, está em equilíbrio durante um curto período da fase adulta, em média dos 20 até os 40 anos, onde com uma dieta rica em cálcio, exercícios físicos diários, exposição constante ao sol e fatores genéticos favoráveis, se obtém uma boa formação do pico de densidade mineral óssea (CECIL & RUSSELL, 2005). O pico será determinante para o próximo período, onde a reabsorção óssea passa a exceder a formação óssea. Isso ocorre durante e após a menopausa, devido ao término da produção de estrogênio (BERGLUND *et al*., 2000). A densidade mineral óssea (DMO) está relacionada à resistência óssea, a qual está diretamente relacionada à formação de um alto pico de densidade óssea (BAYLINK *et al*., 1999).

Os fatores genéticos são relevantes na patogênese da osteoporose, pois são considerados os determinantes da massa óssea (RALSTON, 1997). Outros fatores, quando associados, também podem contribuir para a instalação da doença, como a falta de exercício físico, o tempo de menopausa e a multiparidade (COHEN & ROE, 2000; REGINSTER, 2004).

Atualmente a absorciometria com raios X de duplo feixe (DEXA) continua sendo o melhor método de detecção da DMO, apresentando alta precisão, reprodutibilidade e baixa exposição à radiação (CECIL & RUSSELL, 2005), permitindo avaliar a mineralização dos ossos, diagnosticar a doença óssea, e assim monitorar o tratamento (BURTIS & ASHWOOD, 1996). O diagnóstico do grau de dano ósseo através da densitometria óssea é feito comparando-se quantos desvios padrões a medida da DMO do paciente está distante do pico de massa óssea estimado para um adulto jovem (20 a 30 anos) e saudável, de mesmo sexo e etnia. Com isso, se estabelece o escore-T, como mostra a Tabela 1 (GASS & DAWSON-HUGHES, 2006).

Tabela 1 – Classificação segundo a Organização Mundial da Saúde para osteoporose (Adaptado de GASS & DAWSON-HUGHES, 2006).

Categorias	Densidade Mineral Óssea
Normal	Dentro de 1 DP de um adulto jovem normal
Osteopenia	Entre 1 e 2.5 DP abaixo de um jovem adulto normal
Osteoporose	>2.5 DP abaixo de um jovem adulto normal

DP: desvio padrão.

A nomenclatura da Organização Mundial da Saúde utiliza o termo ***osteopenia*** para referir-se aos indivíduos cuja densidade mineral óssea se situa entre 1 e 2,5 desvios padrões abaixo da massa óssea máxima, e o termo ***osteoporose*** para indicar os indivíduos cuja densidade mineral óssea é superior a 2,5 desvios padrões abaixo da massa óssea máxima. Em geral para cada desvio padrão de redução da densidade mineral óssea, o risco de fraturas osteoporóticas aumenta cerca de 50%, independente da técnica ou do local empregado para avaliar a densidade óssea (CECIL & RUSSELL, 2005).

Imperfeições na microestrutura do esqueleto e diminuição na massa óssea são características da osteoporose e predispõe a pessoa a um aumento no risco de fratura (GASS & DAWSON-HUGHES, 2006). A osteoporose é uma doença universal e de maior incidência em mulheres da raça branca (HICH & KERSTETTER, 2000; PINTO *et al*., 2002).

As fraturas osteoporóticas mais comuns são na região do quadril, fêmur proximal, vértebras e região do punho. Destas, as fraturas vertebrais, em episódios agudos e dolorosos, são as de maior incidência, ocorrendo por achatamento dos corpos vertebrais, predominantemente em mulheres pós-menopáusicas após 15-20 anos. Em mulheres mais idosas o achatamento vertebral é lento, gradativo e geralmente indolor. Porém, as fraturas de quadril representam as maiores complicações da osteoporose, pois obrigam a intervenção hospitalar e geralmente são causadas por quedas acidentais ou diminuição da massa óssea (PINTO *et al*., 2002).

A osteoporose hoje constitui um problema de saúde pública, mas que pode ser controlada em mulheres na pós-menopausa através de diversos fatores, como o acompanhamento médico, mudanças de estilo de vida e ingestão de cálcio (CECIL & RUSSELL, 2005).

2.5 Tratamento da doença óssea

A intervenção precoce consegue evitar o desenvolvimento da osteoporose, pois é uma doença multifatorial, que pode agir silenciosamente durante décadas, sem apresentar nenhuma sintomatologia. Já a intervenção tardia pode refrear o desenvolvimento da doença, desde que utilizando o tratamento adequado, evitando assim o risco de fraturas ou mesmo aumentado a massa óssea (CECIL & RUSSELL, 2005).

Os agentes farmacológicos diminuem a reabsorção óssea promovendo secundariamente o ganho de massa óssea ou são de efeito anabólico e produzem aumento direto na massa óssea. Atualmente os tratamentos para a osteoporose incluem a suplementação de cálcio com ou sem vitamina D e os tratamentos adicionais com estrogênios (TRH), calcitonina, bisfosfonatos, fluoretos, moduladores seletivos de estrogênio e mais recentemente o ranelato de estrôncio (Protelos®) (AKESSON, 2003; MARIE, 2006).

A suplementação de cálcio, seja acompanhada ou não da vitamina D, normalmente é prescrita. Devido à diminuição da absorção intestinal de cálcio com a idade, faz-se necessário uma suplementação adicional de cálcio, mesmo com uma dieta enriquecida com produtos a base de cálcio, como mostra a Tabela 2 (GASS & DAWSON-HUGHES, 2006).

Tabela 2 – Recomendações de suplementação de cálcio e vitamina D (Gass & Dawson-Hughes, 2006).

	Uso	Dosagem
Cálcio	Suplementação recomendada para a maioria dos homens e mulheres com idade >50 anos	Ingestão total 1,0 a 1,5 g/dia (dose ajustada de acordo com a ingestão de cálcio na dieta)
Vitamina D		50 a 70 anos: 400 UI/dia
	Suplementação recomendada para a maioria dos homens e mulheres	≥70 anos: 600 UI/dia
		Em pacientes com risco de deficiência por inadequada exposição solar: 800 UI/dia

UI: unidades internacionais.

A absorção intestinal do cálcio, mesmo não sofrendo a ação da idade já é deficiente devido à formação de fosfato e/ou oxalatos de cálcio insolúveis. A absorção líquida de cálcio pelo trato intestinal, para um adulto jovem é de aproximadamente 10 a 20% do consumo dietético (MOTTA, 2003); o que torna quase uma obrigatoriedade a suplementação de cálcio para adultos maduros.

A terapia de cálcio parece ser mais efetiva para deter a perda óssea em mulheres numa fase avançada da menopausa, mas de um modo geral é benéfica para mulheres tanto na pré quanto na pós-menopausa. Além disso, nos Estados Unidos é recomendado que jovens, entre 11 e 24 anos de idade tenham uma ingestão de 1200 a 1500 mg de cálcio/dia; e adultos do sexo masculino, entre 25 e 65 anos e mulheres entre 25 e 50 anos de idade devem consumir 1500 mg de cálcio/dia, demonstrando que a suplementação de cálcio não só é recomendada para mulheres que estão tendo uma maior perda de cálcio, mas também durante a formação do pico de densidade mineral óssea para ambos os sexos (CECIL & RUSSELL, 2005).

2.5.1 Contaminação dos suplementos de cálcio

Investigações sobre a contaminação de suplementos de cálcio tiveram início na década de sessenta, nos Estados Unidos, onde foram detectados níveis relativamente elevados de chumbo em suplementos de cálcio (CROSBY, 1977). Em 1980 esse assunto foi novamente pautado após um estudo ter evidenciado alterações neurológicas, tais como déficit cognitivo em pacientes que apresentaram níveis elevados de chumbo nos cabelos e que tinham sido submetidos a tratamento com dolomita e/ou suplementação de cálcio (ROBERTS, 1983). Na década de noventa, outro estudo veio a reafirmar esse achado (BOURGOIN *et al*., 1993); o que levou a Federação dos EUA a tomar medidas preventivas, reduzindo os níveis de exposição ao chumbo (NATIONAL RESEARCH COUNCIL, 1993). No estado da Califórnia houve uma intervenção maior, sendo proibido um consumo superior a 1,5 µg de chumbo/1g de cálcio/dia (CALIFÓRNIA ATTOENEY GENERAL´S OFFICE, 1997).

Em 2000, Ross e colaboradores fizeram uma análise do conteúdo de chumbo em alguns suplementos de cálcio utilizados nos Estados Unidos e constataram que

todas as amostras excederam 1 µg de chumbo/dia, considerando o consumo prescrito para a prevenção da osteoporose (1500 mg de cálcio/dia), mas nenhuma amostra excedeu 6 µg de chumbo/dia. Porém, sabe-se que a absorção gastrintestinal do chumbo é inversamente proporcional à quantidade de cálcio presente (Goyer, 1995), e alguns estudos demonstraram que a suplementação de cálcio foi eficiente em reduzir os níveis de chumbo sanguíneo em mulheres grávidas, cujas dietas tinham sido deficientes em cálcio (FARIAS *et al*., 1996; HERNANDEZ-AVILA *et al*., 1996).

2.6 Toxicocinética e toxicodinâmica do chumbo

O chumbo é um metal pesado que está naturalmente presente no meio ambiente, especialmente em áreas industriais, mas também em áreas urbanizadas (KALINA *et al*., 1999; BIASIOLI *et al*., 2006). Sua contaminação abrange o ar, a água e o solo. No ar, o maior volume de substâncias contendo chumbo resulta da liberação de processos industriais, tais como a produção petrolífera, de baterias, tintas, corantes, cerâmica, cabos, tubulações e de munições. Há pouco tempo atrás, as descargas automotivas também representavam uma fonte potencial de chumbo, pela sua presença como antidetonante na gasolina, o qual foi reduzido drasticamente em muitos países e proibido em outros, como no Brasil. O chumbo depositado na água e no solo é proveniente da atmosfera e das atividades antropogênicas (CRA, 2001).

Após a absorção, o chumbo pode ser encontrado no sangue, tecidos moles e mineralizados (ATSDR, 1999). Sua toxicidade resulta, principalmente, de sua interferência no funcionamento das membranas biológicas e enzimas, formando complexos estáveis com ligantes contendo nitrogênio ou oxigênio e grupamentos -SH, $-H_2PO_3$, $-NH_2$ e -OH, que funcionam como doadores de elétrons. O chumbo tem também alta afinidade por aminas e por aminoácidos simples (SARYAN & ZENZ, 1994; ATSDR, 1999). As interações bioquímicas do chumbo com os grupamentos -SH são consideradas de grande significado toxicológico, visto que, se tal interação ocorrer em uma enzima, sua atividade pode ser inibida e resultar em efeitos tóxicos (ATSDR, 1999).

A absorção do chumbo no organismo sofre influência de fatores como a concentração e o tempo de exposição ao metal (SCHIFER *et al*., 2005), fatores

endógenos, como a constituição genética, o estado de saúde e a idade (KLASSEN, 1991; PAOLIELLO & CHASIN, 2001), a dieta e o estado nutricional do organismo (MIDIO & MARTINS, 2000). Além disso, a baixa ingestão de cálcio, fósforo, ferro e proteínas podem provocar um aumento na absorção de chumbo (PAOLIELLO & CHASIN, 2001).

O chumbo é um metal tóxico não essencial, que entra no corpo humano principalmente por inalação ou ingestão. Dentre os compostos de chumbo podemos destacar os inorgânicos, que penetram no organismo principalmente pelas vias respiratória e digestiva, e após atingir a circulação associam-se aos eritrócitos (KLASSEN, 1991) e os orgânicos (tetraetila e tetrametila), os quais por serem lipossolúveis são facilmente absorvidos pela pele e também pelos pulmões e pelo trato gastrintestinal (TSALEV & ZAPRIANOV, 1985; KLAASSEN, 1991).

2.6.1 Chumbo e o metabolismo do cálcio

O chumbo é um metal pertencente à família química dos metais do grupo IVA na tabela periódica, juntamente com o carbono, germânio, silício e estanho. Seu íon estável é a espécie divalente, a qual forma o sulfeto iônico PbS ($Pb^{2+}S^{2+}$) que é a base do componente metálico do minério galena, do qual é extraído quase todo o chumbo (BAIRD, 2002).

Acredita-se que os íons Pb^{2+} e Ca^{2+}, devido a sua grande semelhança atômica e iônica (POPOVIC *et al*., 2005), ao serem absorvidos, ocupam os mesmos sítios de ligação dos transportadores presentes na mucosa intestinal (MUSHAK, 1991) e apesar destes apresentarem maior afinidade de ligação por chumbo, uma dieta rica em cálcio ajuda a diminuir a sua absorção (IPCS, 1995).

O sítio primário de armazenamento do chumbo no organismo é o tecido ósseo, que contém aproximadamente 95% do conteúdo corpóreo total desse metal em adultos (SANÍN *et al*., 1998). Sua concentração nos ossos varia com a idade e o tipo de osso. O seu armazenamento nos ossos ocorre na matriz inorgânica da superfície óssea, formada pelos cristais de hidroxiapatita [$Ca_3(PO_4)_2CaOH$]; sendo sua ligação em dois tipos de ossos, o trabecular e o cortical. No primeiro a ligação é mais fraca,

podendo ser desfeita na presença de quelantes; já no osso cortical o chumbo liga-se fortemente, resultado da sua precipitação na forma de fosfato insolúvel, o qual desloca o cálcio da matriz inorgânica, que por sua vez não é movimentado da superfície óssea pela administração de quelantes (ATSDR, 1999).

A volta do chumbo ósseo para a corrente sanguínea ocorre normalmente pela atividade osteoclástica, pela acidose e pela troca iônica. Em relação à troca iônica é importante ressaltar a íntima relação entre o Ca-Pb; pois todo fator que auxilia na fixação ou na liberação do cálcio dos ossos, de igual maneira atua em relação ao chumbo depositado. Outro fator importante na troca iônica é a influência da dieta, pois a absorção aumentada de fosfato favorece a deposição do chumbo nos ossos e a baixa concentração desse íon leva ao aumento da concentração de chumbo na corrente sanguínea e nos tecidos moles. Já a acidose provoca a solubilização da hidroxiapatita e conseqüentemente, a liberação do chumbo armazenado (SMITH *et al*., 1996).

A concentração do metal nos ossos aumenta progressivamente ao longo da exposição, enquanto que nos tecidos moles não. Assim a concentração de chumbo nos ossos reflete uma exposição antiga e nos tecidos moles, uma exposição recente (ATSDR, 1999). No caso de indivíduos pré-expostos ao chumbo, uma maior absorção de cálcio, especialmente na ausência do fosfato, também aumenta a concentração sanguínea de chumbo, porque o cálcio irá deslocar o chumbo depositado nos ossos; mas em exposição recente, quando a concentração de chumbo nos ossos não é significativa, o cálcio irá competir pelo fosfato, e o chumbo se depositará menos e ficará mais na corrente sanguínea (SMITH *et al*., 1996).

2.6.2 Chumbo e doença óssea

Muitos estudos têm relatado o aumento de chumbo na circulação de indivíduos que sofreram desmineralização óssea, como por exemplo, durante a gestação (ROTHERNBERG *et al*., 2000), a lactação (GULSON *et al*, 1998a; TELLEZ-ROJO, 2002), a menopausa (HERNANDEZ-AVILA *et al*., 2000; LATORRE *et al*.,2003) e em casos de osteoporose (VIG & HU, 2000); levando a aparição de sintomas tóxicos,

mesmo depois de cessada a exposição (SILBERGELD, 1993; SARYAN & ZENZ, 1994; ATSDR, 1999).

SILBERGELD (1991), estudando um grupo de mulheres na pós-menopausa, sugere que durante a gestação dessas mulheres houve mobilização do chumbo do osso, de modo que uma menor quantidade do metal estava disponível para a mobilização durante a desmineralização óssea, que ocorreu após a menopausa. Semelhante resultado foi encontrado por SYMANSKI & HERTZ-PICCITTO (1995), que, além disso, concluíram que mulheres com menopausa recente apresentam concentrações de chumbo sanguíneo mais elevadas do que aquelas cuja menopausa já ocorrera há mais de quatro anos.

Embora a concentração de chumbo sanguíneo seja aceita como indicador de exposição total ao metal, e de fato seja o indicador mais comumente avaliado, dados recentes sugerem que o chumbo no sangue não representa adequadamente os níveis desse elemento nos ossos e que os estoques de chumbo no osso podem influenciar de maneira independente a sua concentração no sangue (TSAIH *et al*., 1999). Existem evidências de que o chumbo no sangue pode agravar o curso da doença óssea por promover a inibição da ativação da vitamina D (SILBERGELD *et al*., 1988), inibição da diferenciação osteoblástica (KLEIN & WIREN, 1993), alteracão nos níveis circulantes dos hormônios responsáveis pela homeostase do cálcio (POTULA & KAYE, 2005) e diminuição da síntese do colágeno, com conseqüente diminuição da matriz orgânica (LONG & ROSEN, 1992), levando a uma maior redução da densidade mineral óssea.

2.6.3 Chumbo e δ-ALAD

A interferência do chumbo na biossíntese do heme é bem estabelecida e sua conseqüência mais conhecida é o aparecimento de anemia, devido a diminuição da formação desse grupamento (WHO, 1995).

O heme é uma ferroporfirina sintetizada nos eritroblastos da medula óssea. A reação inicial é a condensação da glicina com o succinil-CoA, formando o ácido delta aminolevulínico (δ-ALA), que ocorre na mitocôndria e é catalisada pela enzima δ-ALA sintase (δ-ALA-S). Duas moléculas do δ-ALA se condensam para formar o

porfobilinogênio (PBG), reação catalisada pela enzima δ-ALA desidratase (δ-ALAD). Após varias reações que se seguem, vai haver a formação do coproporfirinogênio (CPG), que é oxidado a protoporfirina, pela ação da enzima CPG oxidase. A última reação é a incorporação do ferro bivalente à protoporfirina, com formação do heme, que é catalisada pela ferroquelatase (KLAASSEN, 1991).

O chumbo interfere na atividade de três enzimas envolvidas na síntese do heme, a δ-ALAD, a COPRO oxidase e a ferroquelatase (WILDT *et al*., 1987; ROCHA *et al*., 1995; 2001; GOULART *et al*., 2001), como mostra a Figura 2 (ONUKI *et al*., 2002).

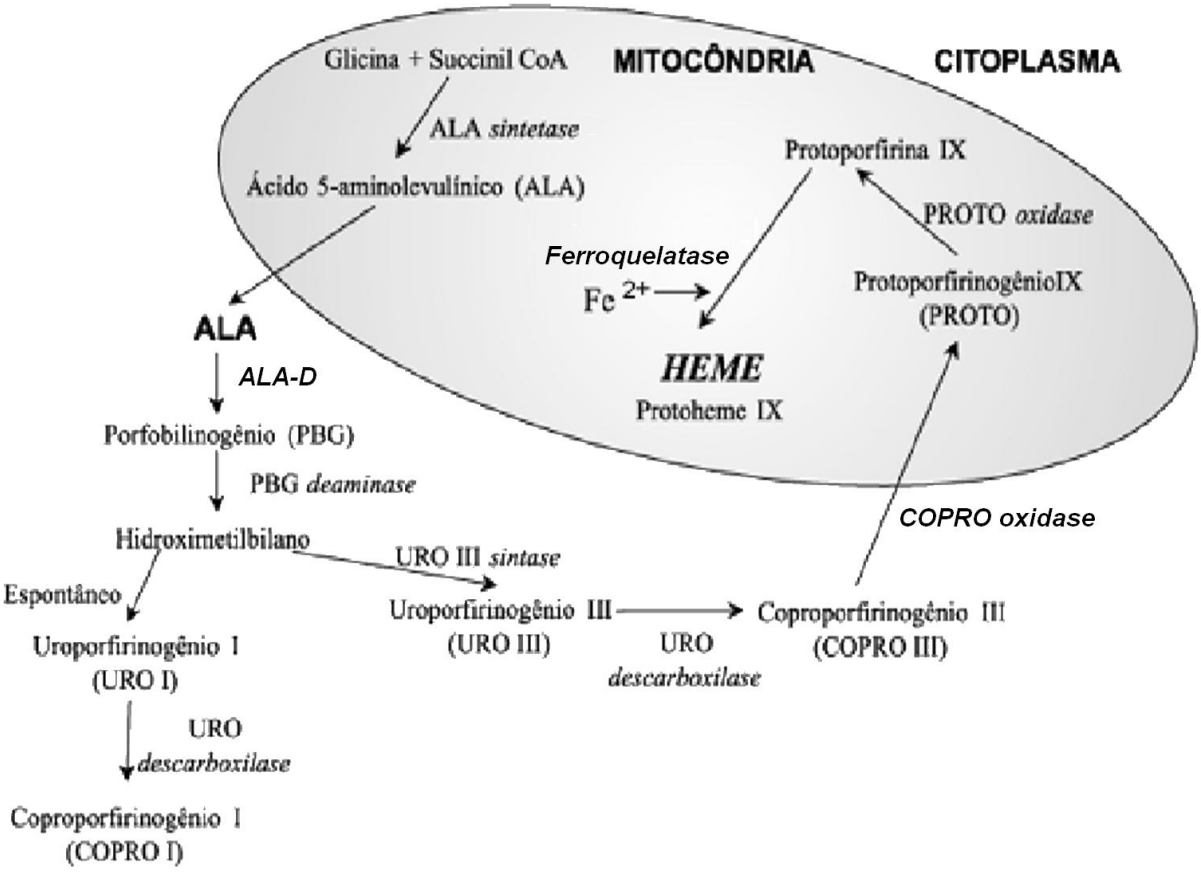

Figura 2 – Via de biossíntese do grupamento heme (ONUKI *et al*., 2002).

O mecanismo através do qual o chumbo inibe a δ-ALAD ainda não está bem estabelecido, mas o mais aceito é a competição entre o chumbo e o zinco, que é o co-fator dessa enzima, pela ligação a um grupamento sulfidrila no sítio ativo da enzima, inibindo sua ação. A inibição da atividade desta enzima é considerada um dos mais

sensíveis parâmetros biológicos para a intoxicação por chumbo (CHALEVELAKIS *et al*., 1995), em populações expostas, com concentração de chumbo sanguíneo > 10 µg/dL (WHO, 1996). Assim, a atividade da enzima δ-ALAD para populações não expostas não é considerada adequada (WETMUR, 1994; MILKOVIC-KRAUSS *et al*., 1997), mas a porcentagem de reativação, a qual baseia-se na porcentagem de δ-ALAD que tornou-se ativa após a incubação com zinco e DTT, parece ser um bom parâmetro bioquímico para populações não expostas ao chumbo (SAKAI *et al*., 1980; POLO *et al*., 1995).

A inibição da δ-ALAD resulta no acúmulo de seu substrato, o δ-ALA, que devido ao seu baixo peso molecular ultrapassa facilmente as membranas celulares elevando-se no plasma e sendo conseqüentemente crescente sua excreção na urina (CALDEIRA, 2000). O δ-ALA no plasma sofre enolização sob pH fisiológico e conseqüente oxidação catalisada por complexos de ferro gerando espécies reativas de oxigênio (ERO) (MONTEIRO *et al*., 1985), provocando danos oxidativos no DNA, peroxidação lipídica e depleção do sistema de defesa antioxidante celular (GURER & ERCAL, 2000).

2.7 Enzimas antioxidantes e estresse oxidativo

Os principais sistemas de defesa não-enzimáticos compreendem as vitaminas antioxidantes A (β-caroteno), C (ácido L-ascórbico) e E (α-tocoferol), ácido úrico, glutationa reduzida (GSH) e grupamentos sulfidrilas livres; que atuam em conjunto com as defesas enzimáticas, que são exercidas pelas enzimas antioxidantes superóxido dismutase (SOD), glutationa peroxidase (GPx) e catalase (CAT) (YU, 1994). As espécies reativas de oxigênio (EROs), que constituem o sistema pró-oxidante são formadas por um grande número de moléculas quimicamente reativas e derivadas do oxigênio, como por exemplo o ânion radical superóxido ($O_2^{\cdot -}$), peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($^{\cdot}OH$) entre outros (NORDBERG & ARNÉR, 2001).

As enzimas antioxidantes constituem o principal mecanismo de defesa antioxidante intracelular, pois protegem as células aeróbicas e demais estruturas

corporais de injúrias oxidativas causadas por EROs geradas durante o metabolismo normal (FRIDOVICH, 1978). Elas eliminam $O_2^{\cdot -}$, H_2O_2 e hidroperóxidos, que poderiam oxidar os substratos celulares, prevenindo as reações em cadeia dos radicais livres, através da diminuição na concentração disponível destes para iniciar o processo (YU, 1994).

A SOD citosólica e extracelular é uma enzima dependente de Cu^{2+} e Zn^{2+}, já a SOD mitocondrial é dependente de Mn^{2+} como co-fator, mas ambas as isoformas catalisam a dismutação dos ânions superóxido ($O_2^{\cdot -}$), produzindo peróxido de hidrogênio (H_2O_2), que pode ser reduzido por ação das enzimas CAT e GPx (AMES *et al*., 1993; NORDBERG & ARNER, 2001).

A CAT é encontrada nos hepatócitos e eritrócitos, estando presente em grandes concentrações nos peroxissomos e em baixas concentrações nas mitocôndrias (HALLIWELL & GUTTERIDGE, 1989). É responsável pela remoção do H_2O_2, o qual é desidratado enzimaticamente a H_2O e O_2 molecular (YU, 1994).

A atividade enzimática da GPx é um dos mais eficientes meios de controle dos níveis de H_2O_2 e hidroperóxidos, que surgem a partir de complexos lipídicos como o colesterol, mesmo quando os peróxidos estão presentes na membrana celular (YU, 1994). Essa enzima é dependente de GSH e age conjuntamente com a glutationa redutase (GR). A GR é responsável pela regeneração da glutationa oxidada (GSSG) em sua forma reduzida (GSH) na presença de nicotinamida adenina dinucleotídeo fosfato (NADPH), e tem por objetivo impedir a paralisação do ciclo metabólico da glutationa e da GPx (NORDBERG & ARNER, 2001).

Apesar dessas defesas antioxidantes reduzirem os riscos de lesões oxidativas por EROs, os organismos podem vivenciar situações onde a proteção é insuficiente. O desequilíbrio entre a formação e a remoção dos radicais livres no organismo, decorrente da diminuição dos antioxidantes endógenos ou do aumento da geração de EROs, gera um estado pró-oxidante que favorece a ocorrência de lesões oxidativas em macromoléculas e estruturas celulares, inclusive podendo resultar em morte celular. Este tipo de processo oxidativo é definido como estresse oxidativo, onde se estabelece uma situação de desequilíbrio entre as concentrações de espécies pró e antioxidante (HALLIWELL & GUTTERIDGE, 1991), como mostra a Figura 3 (NORDBERG & ARNÉR, 2001).

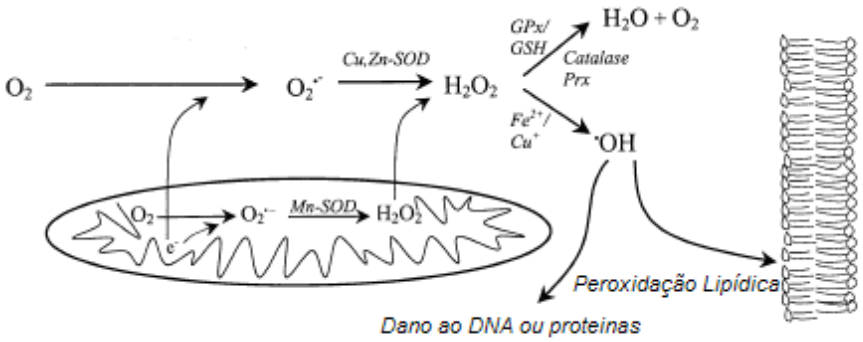

Figura 3 - Esquema simplificado não estequiométrico dos sistemas oxidante e antioxidante nas células (NORDBERG & ARNÉR, 2001).

Está bem relatado na literatura que as enzimas antioxidantes sofrem alterações em decorrência da menopausa, pelo aumento do estresse oxidativo, devido à redução da síntese do estrogênio (TREVISAN *et al*., 2001; KE *et al*., 2003; Bednarek-TUPIKOWASKA *et al*., 2004). Essas modificações no sistema de defesa antioxidante também estão evidenciadas no envelhecimento (NOHL, 1993; FINKEL & HOLBROOK, 2000; ÍNAL *et al.*, 2001; KASAPOGLU & ÖZBEN, 2001) e em outras doenças, tais como aterosclerose (ROSS, 1993; JIALAL *et al*., 2001), diabetes mellitus (CRISTENSEN & SVENDSEN, 1999) e osteoporose (BASU *et al*., 2001).

De fato, MAGGIO *et al*. (2002), constaram que os níveis plasmáticos de antioxidantes exógenos, como vitaminas C, A e E, e a atividade das enzimas antioxidantes, SOD e GPx, estavam diminuídas em mulheres osteoporóticas quando comparadas com mulheres com DMO normal. Em 2003, outro estudo desse mesmo autor veio reforçar esses dados anteriormente encontrados (MAGGIO *et al*., 2003).

Recentemente estudando-se células vasculares e ósseas que foram tratadas com H_2O_2 verificou-se que essa espécie radicalar atuou como um modulador positivo da diferenciação osteoblástica em células vasculares e como inibidor da diferenciação osteoblástica nas células ósseas. Tal fato leva a calcificação das células vasculares, ocasionando a aterosclerose e diminui a formação óssea, podendo levar à osteoporose (MODY *et al*., 2001).

Existe uma necessidade de maiores informações sobre a relação entre o estresse oxidativo e o metabolismo ósseo, bem como sobre o efeito do chumbo acumulado nos ossos ao longo da vida e a variação dos níveis sanguíneos desse

metal em decorrência da osteoporose em mulheres pós-menopáusicas não expostas ocupacionalmente a esse metal, mas que serão tratadas com suplementos de cálcio.

3 ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos e manuscritos, os quais se encontram aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se nos próprios artigos e manuscritos. As apresentações dos artigos estão baseadas na versão final de impressão (**Artigo 1 e 2**) e os manuscritos encontram-se na versão para submissão (**Manuscrito 1** e **2**).

3.1 Artigo 1

LEAD CONTENT OF DIETARY CALCIUM SUPPLEMENTS AVAILABLE IN BRAZIL

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Lead content of dietary calcium supplements available in Brazil

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Abstract
The lead and calcium content of calcium supplements available in Brazil were determined by graphite furnace and flame atomic absorption spectrometry, respectively. Samples were microwave-digested in concentrated HNO_3. Citric acid was used as a chemical modifier in the lead analysis. Supplements were classified into six categories: oyster industrialized (OI, $n=4$), oyster prepared in pharmacy (OP, $n=3$), refined industrialized (RI, $n=6$), refined prepared in pharmacy (RP, $n=3$), bone meal (B, $n=3$), and dolomite (D, $n=4$). Lead levels ($\mu g\,g^{-1}$ of measured calcium) were higher in D products (2.33), followed by OI, RP, OP, and RI products (1.46, 1.32, 1.29, 0.75), while B products had levels lower than the limit of quantification ($0.02\,\mu g\,g^{-1}$ unit weight). Daily lead intake of eight supplements exceeded the limit of California, USA ($1.5\,\mu g\,g^{-1}$ calcium), but none exceeded the federal limit of USA ($7.5\,\mu g\,g^{-1}$ calcium) or the provisional tolerable lead intake by FAO/WHO ($25\,\mu g\,kg^{-1}$ per week).

Keywords: *Lead, calcium supplements, graphite furnace atomic absorption spectrometry, bone meal, dolomite, oyster shell*

Introduction

Lead is a toxic metal that affects the central nervous system and the heme biosynthesis pathway (Wildt et al. 1987, Rocha et al. 1995, 2001, WHO 1995, Campagna et al. 1999). In addition, lead behaves like calcium in terms of compartmentalization in the body (Bhattacharyya et al. 1995) and interferes with calcium metabolism, affecting many of its biological functions (Silbergeld and Addler 1978, Simons 1994). More than 90% of the lead body burden is accumulated in bone (half-life ~ 27 years) (Rabinowitz 1991, WHO 1995). Bone lead is continuously mobilized into the bloodstream via normal bone turnover, but mobilization is increased significantly during conditions of bone demineralization. A major implication of this finding is that even low level lead exposure, over a relatively long time, may result in an increased body burden of lead, which would be releasable in toxicologically significant amounts during critical physiological states such as pregnancy, lactation, and menopause (Silbergeld et al. 1988, Gulson et al. 1998).

Calcium supplements are invariably prescribed for the prevention and treatment of bone re-sorption states, such as osteoporosis, pregnancy, and lactation (Silbergeld and Addler 1978, Simons 1994, Finkelstein 2004). However, some calcium supplements may also contain relatively high amounts of lead, because this metal is a frequent contaminant of the mined calcium carbonate and oyster shells that are used as raw materials for many calcium supplements (National Research Council 1993). Besides, an association between neurological disorders and relatively high lead levels in hair was observed in some patients who were taking either dolomite or bone meal supplements, in the USA (Roberts 1983). Therefore, there has been a growing concern that ingestion of certain calcium supplements might contribute to an increased risk of lead poisoning (Ross et al. 2000, Scelfo and Flegal 2000).

In fact, in the last few decades, various studies performed in the USA and in Canada revealed the presence of lead levels of concern in calcium

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supplements (Capar and Gould 1979, Bourgoin et al. 1993, Whiting 1994, Ross et al. 2000). Results from those studies lead to the adoption of measures to reduce lead intake from calcium supplements, including a public caution to limit the intake of calcium supplements (US FDA 1982) and the restriction of the levels of lead contamination tolerated by FDA (Food and Drug Administration; Scelfo and Flegal 2000). In the last few years, the lead content in oyster calcium supplements in the USA was significantly reduced, however the overall lead levels found in calcium supplements are still of concern (Scelfo and Flegal 2000). Scelfo and Flegal (2000) observed that two-thirds of the calcium supplements collected in California, USA, in 1996 contained lead levels higher than those allowed by the legislation of that state (1.5 µg/daily dose of 1 g calcium).

To our knowledge there is no study evaluating lead levels in calcium supplements marketed in Brazil. The Brazilian Pharmacopea (2000) establishes 0.002% as the maximum content of heavy metals (including lead) allowed in calcium carbonate used in pharmaceutical inputs and medicines. However, there is no specific legislation regulating lead levels in calcium supplements, which makes lead contamination a special concern for calcium supplements marketed in Brazil. Considering the toxicological relevance of lead, the objective of the present study was to evaluate the content of lead in calcium supplements marketed in Brazil.

Materials and methods

Samples

Samples of calcium supplements ($n = 23$) were purchased in Santa Maria (RS, Brazil), from local and national chains of pharmacies, between June 2003 and January 2004 (see Table I). A total of 11 calcium-based products were selected. To assure that samples were representative of the different products evaluated, three samples (of three different lot numbers) of each product were independently analysed, except for dolomite, dolomitex and Oscal 500 + D, which had only one sample of each manufacturer analysed due to limited availability. According to the product label, the calcium used in the supplements was either refined or from a natural source. Natural calcium sources were bone meal (ossein hydroxyapatite), dolomite ($CaMg(CO_3)_2$), and oyster shell ($CaCO_3$). Supplements containing refined and oyster shell calcium were classified into industrialized or prepared in pharmacy (products prepared in pharmacy following a medical prescription), according to the manufacture process (Table I). All dolomite and bone meal supplements evaluated were industrialized products.

Procedures

Dietary calcium supplements were ground up to particle size ≤ 80 µm, dried at 100°C for 2 h and kept in polypropylene vials before subsequent decomposition.

Sample decomposition was performed in concentrated HNO_3 in a microwave oven (Multiwave 3000, Anton Paar, Graz, Austria) operated at 600 W for 10 min, 1400 W for 10 min, and 20 min for cooling the system. All reagents used were of analytical grade (Merck, Darmstadt, Germany). Four test samples were digested for each individual sample. One of the replicates was treated with a spike of Pb (10 µg l^{-1}) before digestion to verify occasional analyte losses during the analytical procedure. Recoveries for lead spikes were between 98 and 105%, showing that no lead losses occurred during the analytical procedure.

Table I. Characteristics of dietary calcium supplements assayed.

Product number	Manufacturer (location)	Name	Active content[1]	Classification	Number of samples analysed
1	AVENTIS PHARMA Inc. (USA)	Oscal 500	Oyster calcium carbonate	OI	3
2	AVENTIS PHARMA Inc. (USA)	Oscal 500 + D	Oyster calcium carbonate	OI	1
3	Laboratório Wyeth-Whitehall Ltda. (Brazil)	Caltrate 600 + M	Calcium carbonate	RI	3
4	Novartis Biociências S.A. (Brazil)	Calcium Sandoz F	Calcium carbonate and calcium lactogluconate	RI	3
5	ASTA Medica Ltda. (Brazil)	Ossopan	Ossein hydroxyapatite	B	3
6	Rainha Indústria e Comércio Ltda. (Brazil)	Dolomitex	Calcium	D	1
7	Produtos Naturais Floss Ltda. (Brazil)	Dolomite	Calcium and magnesium	D	1
8	Zanardi Produtos Naturais (Brazil)	Dolomite	Calcium and magnesium	D	1
9	Mosteiro Devakan (Brazil)	Dolomite	Calcium and magnesium	D	1
10	Prepared in pharmacy (Brazil)	Calcium carbonate	Calcium carbonate	RP	3
11	Prepared in pharmacy (Brazil)	Oyster calcium	Oyster calcium	OP	3

[1]According to the product label. OI = oyster industrialized, OP = oyster prepared in pharmacy, RI = refined industrialized, RP = refined prepared in pharmacy, B = bonemeal, D = dolomite.

Table II. Graphite furnace heating program for lead determination in dietary calcium supplements.

Step	Temperature (°C)	Ramp (°C s^{-1})	Hold time (s)	Argon flow
Drying	130	15	40	Max.
Pyrolysis	800	100	30	Max.
AZ	800	0	6	Stop
Atomisation	1800	3000	5	Stop
Cleanout	2600	3000	4	Max.

Used modifier: citric acid (100 µg).

Lead measurements were carried out in an Analytik Jena spectrometer (Analytik Jena AG, Model AAS 5 EA, Jena, Germany) equipped with a transversely heated graphite atomizer and continuum source background correction system. The parameters for the heating program for Pb measurements (see Table II) were optimized from the pyrolysis and atomization curves for lead that were constructed using a randomly chosen sample digest. Standard addition and matrix matching methods were performed for lead determination from the analytical curves obtained with 5, 10, 15 and 20 µg l^{-1} of Pb standard solutions, with the addition of 100 µg of citric acid. With the use of 100 µg of citric acid as a chemical modifier, good linearity of the analytical curves was achieved (better than 0.999). The achieved relative and absolute limits of detection were 0.02 µg g^{-1} and 8 pg (based on 10 replicates of the blank, 3 s).

Calcium measurements were carried out using a Model Vario 6 FL atomic absorption spectrometer (Analytik Jena AG, Germany), equipped with a deuterium background corrector system. For Ca measurements an ionisation buffer (10 ± 0.2 g l^{-1} CsCl, 100 ± 0.2 g l^{-1} La in 0.2% HNO_3, Merck) was added to the sample digests.

Calculations and statistical analysis

Results were expressed as micrograms of lead per gram of homogenized specimen (µg g^{-1} unit weight), and then converted into micrograms per tablet or capsule (µg per unit) and micrograms per prescribed daily dose (according to the product label) (Table III). Average unit weight for each formulation was determined from the weight of 10 tablets. According to the product labels, the content of calcium per unit of supplement and the daily calcium dosage suggested for each brand varied widely. Therefore, daily lead intake from the supplements evaluated was also calculated based on a recommended daily calcium intake of 1000 mg. Since there were some differences between calcium content determined by AAS and the values specified in product labels (see Figure 1), daily lead intake normalized to 1000 mg calcium was calculated based either on the calcium content determined by AAS (µg g^{-1} calcium, see Table III) or on the calcium content specified in the product label. For calculations based on the content of calcium specified in the product label, the lead content of supplements (determined by AAS) was normalized to the number of units that should be ingested to attain 1000 mg calcium (µg/day; Figure 2). Differences in lead content among the different categories of calcium supplements (6) were evaluated using one-way analysis of variance (ANOVA). Interlot variability was evaluated using ANOVA (4 brands × 3 lots) with lot factor considered as repeated measure. Differences between means were considered significant when $p < 0.05$ (Duncan's test).

Results

Calcium content of calcium supplements

According to the information contained in product labels, the content of calcium per unit of supplement varied widely (42.8–600.0 mg). While it might be expected that the daily calcium dosage suggested for each brand would normalize the amount of calcium ingested, this was not the case; and the suggested daily dosage of calcium ranged from 250–1700 mg. Figure 1 shows the content of calcium found in calcium supplements by AAS analysis (as a percentage of the calcium levels specified in the product label) according to the source of calcium and the manufacture process. Results presented are of 22 calcium supplements because the result of one dolomite sample that did not specify calcium levels in the label could not be calculated. Of the 22 calcium supplements analysed, eight exhibited 98% or more than the calcium levels specified in the product label, seven exhibited 91–98% of the calcium levels specified, and only seven exhibited 80–90% of the calcium levels specified in the label (Figure 1).

Lead content of calcium supplements

Table III shows the lead content of dietary calcium supplements according to the source of calcium and the manufacture process. The lowest lead content per gram of product was found in bone-based supplements, which showed lead levels (<LOQ) significantly lower ($p < 0.05$) than those found in supplements of refined calcium prepared in pharmacy, dolomite and oyster-based products. Industrialized supplements of refined calcium showed intermediate lead levels.

Since supplement tablets vary in weight due to binders, the nature of the calcium salt and added

Table III. Lead content of dietary calcium supplements.

Categories of calcium supplements	Pb (μg g^{-1} unit weight)	Pb (μg per unit)	Number of units per day[1]	Pb (μg per prescribed daily dose[1])	Pb (μg g^{-1} of calcium[2])
Oyster industrialized ($n=4$)	$0.43^{a} \pm 0.15$ (0.28–0.60)	0.69 ± 0.24 (0.44–0.95)	1	$0.69^{b} \pm 0.24$ (0.44–0.95)	$1.46^{a,b} \pm 0.52$ (0.91–2.01)
Oyster prepared in pharmacy ($n=3$)	$0.43^{a} \pm 0.21$ (0.22–0.63)	0.22 ± 0.10 (0.11–0.32)	2	$0.43^{b,c} \pm 0.21$ (0.21–0.63)	$1.29^{a,b,c} \pm 0.63$ (0.64–1.90)
Refined industrialized ($n=6$)	$0.25^{a,b} \pm 0.29$ (<LOQ–0.58)	0.46 ± 0.52 (<LOQ–1.04)	1–2	$0.45^{b,c} \pm 0.52$ (<LOQ–1.04)	$0.75^{b,c} \pm 0.85$ (<LOQ–1.72)
Refined prepared in pharmacy ($n=3$)	$0.48^{a} \pm 0.24$ (0.20–0.64)	0.24 ± 0.12 (0.10–0.32)	2	$0.48^{b,c} \pm 0.24$ (0.20–0.63)	$1.32^{a,b,c} \pm 0.68$ (0.55–1.81)
Bonemeal ($n=3$)	<LOQ[b]	<LOQ	6	<LOQ[f]	<LOQ[c]
Dolomite ($n=4$)	$0.57^{a} \pm 0.18$ (0.38–0.81)	0.34[3]	2.6 g[4]	$1.55^{a} \pm 0.78$[4] (0.99–2.10)	$2.33^{a} \pm 1.21$ (1.70–4.15)

Results are presented as mean ± S.D. (minimum–maximum). LOQ = limit of quantification (0.02 μg g^{-1} unit weight). [a,b,c]Means within the same column that do not show the same letter are significantly different ($p < 0.05$). Values <LOQ were considered as zero in the statistical analysis. [1]As prescribed in the product label. [2]Lead content (determined by AAS) was normalized to the calcium content of supplements (determined by AAS). [3]Results of one sample analysed in triplicate, since the other 3 samples were powder formulations. [4]Value of dolomite supplement samples (two powder formulations) that exhibited the prescribed dose in label.

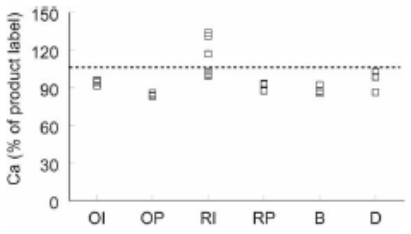

Figure 1. Content of calcium found in dietary calcium supplements according to the source of calcium and the manufacture process. OI = oyster industrialized ($n = 4$), OP = oyster prepared in pharmacy ($n = 3$), B = bonemeal ($n = 3$), D = dolomite ($n = 3$). Results are presented as a percent of the calcium levels specified in the product label. Each square represents the average content of one sample analysed in triplicate. Dashed line indicates 100% content.

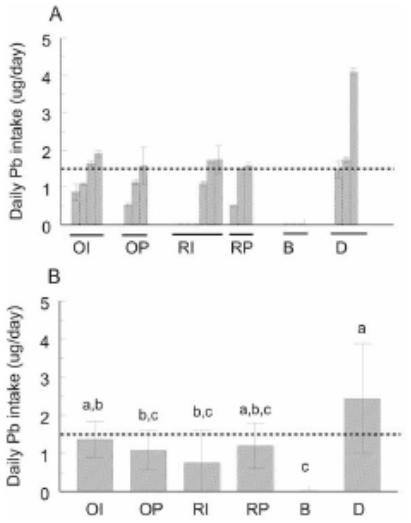

Figure 2. Daily lead intake from dietary calcium supplements. OI = oyster industrialized ($n = 4$), OP = oyster prepared in pharmacy ($n = 3$), RI = refined industrialized ($n = 6$), RP = refined prepared in pharmacy ($n = 3$), B = bonemeal ($n = 3$), D = dolomite ($n = 3$). Results are mean ± S.D of each sample analysed in triplicate (A) or the mean ± S.D of all samples from each product type (B). Considering a recommended daily calcium intake of 1000 mg and the content of calcium specified in the product label, we calculated the number of units that should be ingested per day to attain this calcium dosage. Then the lead content of supplements (determined by AAS) was normalized to this number of units (μg/day). Dashed line indicates the California (USA) limit for lead (1.5 μg/day). ^{a,b,c}In panel B bars that do not show the same letter are significantly different ($p < 0.05$). Values <LOQ were considered as zero in the statistical analysis.

liquid, we calculated the lead content (μg) per unit of supplement (Table III). Although there was no statistically significant difference in the lead content/unit among the supplement categories measured in this study, bone-based supplements had the lowest average lead concentration (<LOQ), while industrialized oyster-based products had the highest level.

Based on the suggested daily dosage listed on the product labels we calculated daily lead ingestion values (i.e. micrograms of lead ingested per prescribed daily dose, Table III). Bone-based products exhibited the lowest average lead concentration (<LOQ), while dolomite-based products had the highest levels, and the other products showed intermediate levels.

Considering that supplements exhibit a great range both in the weight of tablets and in the suggested daily dosage of calcium (0.40–1.79 g and 250–1700 mg, in the present study), the relative lead content of these products is compared more effectively when calcium content is taken into account. This presumes that consumers are seeking for a fixed amount of calcium, irrespective of form. Table III shows the lead content (μg) of supplements per g of calcium (determined by AAS).

Also, lead content of supplements per g of calcium (determined by AAS) seems to be more suitable to compare lead contamination in the different sources of calcium used in the products. Dolomite-based products had the highest lead levels per g calcium, followed by oyster-based products, supplements of refined calcium prepared in pharmacy, and refined industrialized products, while bone-based supplements had the lowest lead levels (Table III). Our results revealed no significant differences in lead levels between industrialized products and products prepared in pharmacy (Table III).

Of the 11 products evaluated six had three different lot samples analysed to determine the variability of lead levels within brands (i.e. "interlot" variability). Two out of these six products exhibited lead levels <LOQ in all three lots, and therefore were not used in the interlot variability evaluation. ANOVA (4 brands × 3 lots) with lot considered as repeated measure indicated that interlot variability of lead levels was negligible.

AAS assays revealed that some samples had calcium content slightly lower than label value (Figure 1). Since consumer intake will rely on the calcium content listed in the product label, we consider that lead content normalized to the content of calcium specified in the product label is more appropriate to estimate the daily lead intake from dietary supplements. Daily lead intake (μg per day) was calculated considering a daily calcium intake of 1000 mg (Figure 2). Dolomite-based products yielded the highest daily lead intake, bone-based products yielded the lowest intake, and the other products exhibited intermediate values (Figure 2).

Discussion

The average lead levels found in the present study were similar to or lower than those levels previously reported for various types of calcium supplements available in the USA, Canada, and Korea (Bourgoin et al. 1993, Ross et al. 2000, Scelfo and Flegal 2000, Kim et al. 2003).

Brazilian Pharmacopea establishes 0.002% as the maximum level of heavy metals in calcium carbonate used in pharmaceutical inputs and medicines. Considering the unlikely hypothesis that lead is the only heavy metal in such products, calcium supplements available in Brazil could exhibit up to 50 μg lead per g of calcium. This value is remarkably higher than limits established in other countries. Considering the lack of a specific limit for lead in calcium supplements in Brazil, results obtained will be compared to the limits established in the USA. The US federal limit for lead in calcium supplements is 7.5 $\mu g\,g^{-1}$ calcium, but some regions in the USA have a more stringent limit. In the State of California the maximum acceptable daily intake for lead from calcium supplements is <1.5 $\mu g\,g^{-1}$ calcium (2.25 μg/recommended daily dose, considering a recommended daily dose of 1500 mg calcium) (California Attorney General's Office 1997).

When lead content of calcium supplements was normalized to the calcium content specified in the product label, eight out of 22 supplements evaluated exceeded the lead limit allowed in California, USA (1.5 $\mu g\,g^{-1}$ calcium), while when lead content was normalized to the calcium content determined by AAS, 11 out of 23 supplements evaluated exceeded the limit. However, regardless of the calcium value used in the normalization, no sample exceeded the federal limit of USA (7.5 $\mu g\,g^{-1}$ calcium).

The rank order found in the present study for lead levels in calcium supplements is similar to that found by Scelfo and Flegal (2000) in the USA (dolomite > oyster shell > refined > bone meal supplements). It has been suggested that lead levels found in calcium supplements can be related to the environmental contamination. Accordingly, an evaluation of temporal variations of lead in calcium supplements revealed apparent temporal reductions in lead concentrations of most calcium supplements (Scelfo and Flegal 2000). Nevertheless, dolomite did not show temporal reductions in lead levels (Scelfo and Flegal 2000). This finding was attributed to the relative homogeneity of lead concentration in dolomite deposits (Scelfo and Flegal 2000) and unveils the importance of dolomite as a source of lead. While fresh oyster shells do not incorporate extraneous materials, the fossilized shells have the potential for heavy metal contamination during sedimentation (Bourgoin 1992). In the present study, oyster shell supplements showed intermediate lead levels, but labels of these products did not state whether they contained fossil or fresh shell. Despite the common perception that refined supplements would have fewer metal contaminants, our results show that they can still contain considerable amounts of lead, as previously observed by Ross et al. (2000). Although bone meal-based supplements have been early recognized as a worrying source of lead (Roberts 1983, Bourgoin et al. 1993) our results indicated that it is the safest source of calcium. This finding also contrasts with recent results of Kim et al. (2003) in Korea that found the highest lead levels in bone supplements. On the other hand, Scelfo and Flegal (2000) observed an apparent temporal decrease in lead concentrations in bone meal in USA, which have been attributed to the substitution of outer bone for red bone marrow that contains less lead and to the use of bones from younger animals.

Concerns about low lead level exposure have increased since some studies failed to establish a discernible threshold for some measures of sublethal lead toxicity in humans (Flegal and Smith 1995). Hence, there is some uncertainty and controversy as to the acceptable maximum oral daily intake of lead, since no risk-free blood level has been established (ATSDR 1999, US EPA 1999). Nevertheless, a provisional total tolerable weekly intake of 25 μg lead per kg of body weight has been established by FAO/WHO (1993).

Results of the present study indicate that, based solely on the ingestion of calcium supplements available in Brazil, the provisional total tolerable lead intake would not be exceeded. However, it should be stressed that this value represents the maximum amount of lead to be ingested from all dietary sources. In addition, it was deemed provisional because safe levels of lead exposure had not been identified and would likely be adjusted downward to allow for the other anticipated exposures to lead (Carrington and Bolger 1992). No estimate of the average daily lead intake by general population is available in Brazil, but in other countries daily lead intake from all oral sources is estimated to range from <5 μg in USA (Gulson et al. 2001) to >100 $\mu g\,kg^{-1}$ in Italy, UK, and Belgium (WHO 1995). Therefore, depending on the total daily intake of lead by general population, even at lead levels ≤1.5 μg/daily dose, calcium supplements could potentially contribute to a significant amount of the daily lead intake (Gulson et al. 2001). However, due to the decrease in gastrointestinal lead absorption induced by supplemental concentrations of calcium (Bruening et al. 1999), exposure risk from lead in calcium supplements is thought to be relatively small, even though their contribution to the

average total daily dietary intake may be relatively large (Bolger et al. 1991).

Conclusions

Based either on the lead content (μg) per g of calcium (determined by AAS or specified in the product label) or on the lead content (μg) per prescribed daily dose, dolomite-based products had the highest lead levels, followed by oyster and refined products, while bone-based supplements had lead levels <LOQ. Although the average lead levels found were lower than the limits established for calcium supplements in USA, it might be advisable to establish a specific lead limit for calcium supplements in Brazil, as well as a federal program to monitor such levels.

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3.2 Artigo 2

EFFECTS OF BONE DISEASE AND CALCIUM SUPPLEMENTATION ON ANTIOXIDANT ENZYMES IN POSTMENOPAUSAL WOMEN

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Effects of bone disease and calcium supplementation on antioxidant enzymes in postmenopausal women

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Abstract

Objectives: The study was aimed at investigating the effects of osteopenia and calcium supplementation on antioxidant enzyme activities (superoxide dismutase, SOD; catalase, CAT; and glutathione peroxidase, GPx) in postmenopausal women.

Design and methods: Postmenopausal women (n=75) were divided into two groups, control (no bone disease) and osteopenia, according to their bone mineral density. Each group was still divided into calcium-supplemented and nonsupplemented sub-groups. Antioxidant enzyme activities were determined in whole blood using spectrophotometric methods.

Results: CAT and SOD activities were not different among the studied groups. However, GPx activity was significantly higher in osteopenia groups as compared to control groups. Calcium supplementation had no effect on the parameters evaluated. Bone mineral density was negatively correlated with GPx activity (p<0.05).

Conclusions: Increased GPx activity could be interpreted as a defense response to counteract the overproduction of reactive oxygen species in women with osteopenia, and this effect was not prevented by calcium supplementation.

Keywords: Osteopenia; Calcium supplementation; Catalase; Glutathione peroxidase; Superoxide dismutase

Introduction

Menopause is defined as the cessation of menstruation in women and is associated with the failure of ovulation because of depletion of oocytes. Menopause is a physiological event that occurs simultaneously with the establishment of several diseases like coronary heart disease, stroke, cancer, changes in neuropsychological status and immune function, and bone diseases like osteopenia and osteoporosis [1].

Osteoporosis is characterized by low bone mass, enhanced bone fragility, and fracture risk, while osteopenia is the initial bone loss that is not associated to fracture risk [2]. The diagnosis is made by bone densitometry, which allows the use of preventive therapy before the morbidity of a fracture ensues [3]. Postmenopausal bone loss appears to be associated with the estrogen deficiency that leads to excessive osteoclastic and depressed osteoblastic activity, and possibly also impairs intestinal absorption of calcium [4,5]. In addition, in elderly females other agents seem to contribute to the net negative bone balance, such as dietary changes and reduced physical activity [6].

Calcium supplements are used for aiding in osteoporosis treatment and fracture prevention. Calcium absorption efficiency is a key factor in the maintenance of calcium balance and is reduced in postmenopausal women with vertebral fractures [7]. Although the mechanisms that decrease calcium absorption efficiency are controversial and poorly defined, bone loss has been linked to numerous cytokines, hormones, and growth factors [7,8].

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In recent years, various evidences were provided linking bone loss to reactive oxygen species [9–11]. The bone resorbing osteoclasts generate a high level of superoxide anion ($O_2^{\bullet-}$) [12,13]. Besides, agents that modulate osteoclast activity also modulate $O_2^{\bullet-}$ production: activation of osteoclasts generates $O_2^{\bullet-}$, while its inhibition results in the cessation of $O_2^{\bullet-}$ formation [12,13]. In addition, there is evidence that NO modulates bone remodeling and bone loss *in vitro* and *in vivo* [14]. NO decreases the receptor activator of nuclear factor-kB ligand (RANKL)/osteoprotegerin (OPG) equilibrium leading to a reduced osteoclastic potential and positive bone formation [15]. On the other hand, NO can induce osteoblast apoptosis through a mitochondria-dependent pathway, which involves the release of intracellular reactive oxygen species [16]. Thus, the effects of NO seem to depend on its local concentrations: at low concentrations NO promotes bone formation, whereas high concentrations may enhance bone resorption [17].

Also, other studies revealed a link between antioxidants and bone health [10,18]. Isoflavone that is a weak bone-sparing agent possesses significant antioxidant properties *in vitro* and *in vivo* [19,20]. Low dietary intake of vitamins C and E may substantially increase the risk of hip fracture in smokers [18,21]. Besides, antioxidant vitamins (A, C, and E) and the endogenous antioxidant uric acid were significantly lower in osteoporotic than in control subjects [10].

than in control subjects [10].

Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are the major enzymatic antioxidants involved in the protection against $O_2^{\bullet-}$ and H_2O_2. Despite evidence linking bone loss to oxidative stress [9], studies evaluating the activity of antioxidant enzymes in human cases of bone diseases are limited [10,22,23]. Only SOD and GPx activities were evaluated and one of these studies concerns to male osteoporosis. Moreover, data on calcium supplementation and antioxidant enzyme activities in osteoporotic women were not found. In the present study, we investigated the effects of osteopenia and calcium supplementation on the activity of antioxidant enzymes in postmenopausal women.

Methods

Subjects

This study was approved by the Ethics Committee of the Federal University of Santa Maria (CEP/CCS/UFSM no. 130/02). The subjects were selected among patients of the Rheumatology Ambulatory from Santa Maria University Hospital (Santa Maria, RS, Brazil), and all subjects gave their written consent prior to the inclusion in the study. Information about age, menopause state, hormone replacement therapy, number of children, duration of lactation, calcium supplementation, smoking habit, and alcohol use were collected through a questionnaire that was applied by a trained interviewer. Women with case history of smoking or alcoholism were excluded. All the women included were postmenopausal (>12 months of amenorrhea). Height and weight of subjects were measured and used to calculate body mass index (BMI).

The study was performed in 75 subjects that were divided into two groups according to their bone mineral density (BMD): control (no bone disease) and osteopenia. Each group was still divided into calcium-supplemented and nonsupplemented sub-groups. Calcium supplementation status was self-reported, while bone disease status was determined by dual energy X-ray absorptiometry following the diagnostic criteria proposed by the World Health Organization (WHO).

Bone mineral density

Measurements of BMD were taken at the lumbar spine (L1 to L4 a.p.) and at the femoral neck. According to WHO guidelines, osteopenia is defined as a *T* score between −1 and −2.5, where *T* score is the number of standard deviations below the mean peak bone mass of young sex-matched healthy adults.

Table 1
Characteristics of the study groups

	Control		Osteopenia	
	No Ca (n=18)	Ca (n=13)	No Ca (n=29)	Ca (n=15)
Age (years)	55.8±1.0 (49–62)	58.5±1.2 (51–65)	58.2±1.1 (49–69)	58.9±1.7 (49–68)
Years since menopause (years)	9.5±1.6 (1–21)	9.2±1.6 (1–20)	13.1±1.2 (1–27)	11.7±2.0 (2–27)
Duration of hormone therapy (months)	34.7±14.0 (0–180)	38.8±17.3 (0–180)	36.5±12.5 (0–240)	35.7±16.1 (0–228)
Number of children	2.2±0.4 (0–6)	2.6±0.4 (0–5)	2.8±0.5 (0–12)	2.5±0.5 (0–7)
Overall duration of lactation (months)	19.2±6.4 (0–84)	14.8±4.5 (0–60)	18.0±4.1 (0–96)	24.9±9.1 (0–120)
Duration of Ca supplementation (months)	0.0±0.0[b] (0–0)	8.8±2.4[a] (1–36)	0.0±0.0[b] (0–0)	14.6±5.5[a] (0.5–72)
Body mass index (kg/m^2)	28.6±1.2[a] (21.3–37.1)	29.3±1.1[a] (23.6–37.2)	27.3±0.8[a,b] (20.8–37.6)	25.9±1.0[b] (17.8–31.8)
Bone mineral density femur (g/cm^2)	0.831±0.019[a] (0.689–0.972)	0.849±0.032[a] (0.697–1.084)	0.735±0.016[b] (0.621–0.959)	0.701±0.013[b] (0.631–0.809)
Bone mineral density L1–L4 (g/cm^2)	1.042±0.033[a] (0.810–1.405)	1.082±0.035[a] (0.824–1.234)	0.910±0.015[b] (0.772–1.072)	0.868±0.025[b] (0.786–1.147)

Data are expressed as means±SE (minimum–maximum).
[a,b]Values within the same line that do not share a common superscript letter are significantly different (p<0.05).

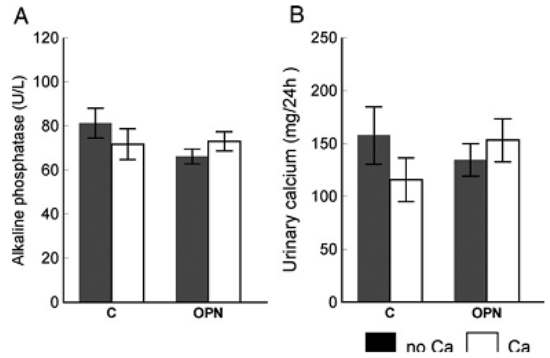

Fig. 1. Effect of osteopenia and calcium supplementation on alkaline phosphatase (A) and urinary calcium (B) in postmenopausal women. Results are mean±SE (n=13–29, as shown in Table 1). C=control; OPN=osteopenia.

Sample collection and analysis

Twenty-four-hour urine samples were collected and immediately used for calcium determination by routine kit (Roche Diagnostics, Mannheim, Germany). Heparinized blood was taken from the cubical vein after overnight fasting. Plasma was separated and immediately used for alkaline phosphatase (ALP) determination by a routine kit (Roche Diagnostics, Mannheim, Germany). Whole blood samples were immediately used for hemoglobin (Hb) determination using routine kit (Roche Diagnostics, Mannheim, Germany) and then stored at −20 °C until analysis of antioxidant enzyme activities. GPx activity was determined in a medium containing 25 mM potassium phosphate buffer, pH 7.0, 2.5 mM ethylenediaminetetraacetic acid, 0.24 U/mL glutathione reductase, 1 mM reduced glutathione, 1 mM sodium azide, 0.15 mM NADPH, and 0.4 mM hydrogen peroxide. The method is based on the oxidation of NADPH, which is indicated by the decrease in absorbance at 340 nm [24]. SOD activity was determined at 480 nm using 50 mM glycine buffer, pH 10.2, and 1 mM epinephrine at 30 °C [25]. SOD activity was expressed as the amount of enzyme that inhibits the auto-oxidation of epinephrine to adrenochrome by 50% which is equal to 1 unit. CAT activity was measured at 240 nm using 50 mM phosphate buffer, pH 7.0, and 17 mM hydrogen peroxide as substrate [26]. The pseudo-first order reaction constant (k) of the decrease in H_2O_2 absorption at 25 °C was determined and specific activity was expressed as k/g Hb.

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) (2 bone mineral density status×2 calcium supplementation status), followed by Duncan's test when appropriate. Data that did not exhibit a normal distribution were transformed (log or square root transformation) in order to meet ANOVA assumptions before analysis. The associations between variables were evaluated by Pearson's correlation for variables that had a normal distribution and by Spearman's rank order correlation for variables that did not exhibit a normal distribution. Data were analyzed using the Statistica® 6.0 software system (Statsoft Inc., 2001). Results were considered significant when $p<0.05$.

Results

Postmenopausal women were divided into four groups according to bone mineral density and the use of calcium supplementation. Characteristics of the study groups are shown in Table 1. Women enrolled in this study were on average 58 years old, were 11 years since menopause, and had 36 months of hormone therapy, 2.6 children, and 19 months of overall duration of lactation. There were no significant differences in age, years since menopause, duration of hormone therapy, number of children, or overall duration of lactation among the studied groups. However, women with osteopenia had a tendency of greater years since menopause when compared to the control group ($p<0.1$). Calcium-supplemented groups had on average 12 months of supplementation and no significant

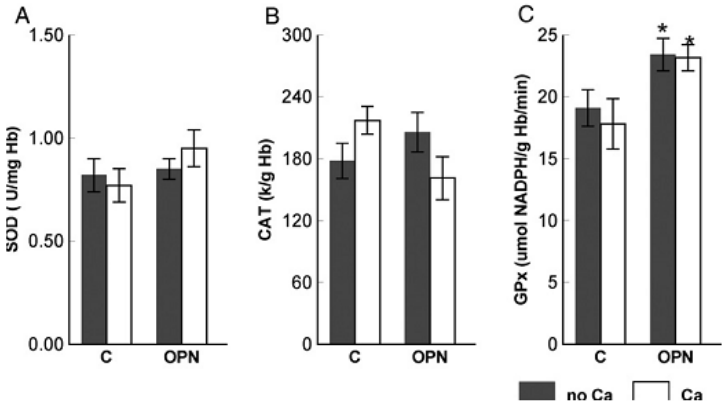

Fig. 2. Effect of osteopenia and calcium supplementation on superoxide dismutase (SOD, A), catalase (CAT, B), and glutathione peroxidase (GPx, C) activity in postmenopausal women. Results are mean±SE (n=13–29, as shown in Table 1). *Significantly different from control groups ($p<0.05$). C=control; OPN=osteopenia.

difference was observed in the duration of calcium supplementation among the two supplemented groups. ANOVA revealed a significant main effect of bone disease on BMI and BMD of postmenopausal women. The calcium-supplemented osteopenia group had lower BMI (25.9 kg/m^2) as compared to control groups (28.6 and 29.3 kg/m^2, $p<0.05$). The average BMD femur and BMD L1–L4 values of the control group were 0.838 and 1.059 g/cm^2, while osteopenia groups had average values of 0.723 and 0.895 g/cm^2, respectively.

Women studied had average values of 72.1 U/L for ALP activity and 139.5 mg/24 h for urinary calcium levels and these parameters were not different among groups (Figs. 1A and B). Also, blood SOD and CAT activities (average values of 0.85 U/mg Hb and 192.0 k/g Hb, respectively) were not significantly different among the studied groups (Figs. 2A and B). However, ANOVA revealed a significant main effect of bone disease on blood GPx activity (Fig. 2C). GPx activity was higher in both osteopenia groups (23.32 μmol NADPH/g Hb/min) as compared to controls (18.56 μmol NADPH/g Hb/min, $p<0.05$, Fig. 2C). Calcium supplementation had no effect on any evaluated parameter (Figs. 1 and 2).

Femoral BMD was negatively correlated with GPx activity ($r=-0.251$; $p<0.05$), but not with SOD or CAT activities. SOD activity was negatively correlated with age ($r=-0.32$, $p<0.05$). No significant correlations were observed between the other characteristics of the study groups or urinary calcium and antioxidant enzyme activities. In addition, femoral BMD was positively correlated with BMI ($r=0.338$; $p<0.05$), but not with the other characteristics of the study groups.

Discussion

In the present study we evaluated if osteopenia and calcium supplementation could affect the antioxidant enzymes in postmenopausal women. We found no previous study on the effect of calcium supplementation on CAT or SOD activities. SOD and CAT activities were not affected by osteopenia or calcium supplementation. In contrast, erythrocyte CAT activity was recently demonstrated to be lower in postmenopausal osteoporotic women when compared to healthy nonporotic women [27]. The absence of change in SOD activity due to bone disease is in agreement with data from Yalin et al. [28] and Ozgocmen et al. [29], but contrasts with two other studies that found decreased SOD activity in postmenopausal women [10,22]. However, in one of these studies [22], osteoporotic subjects were compared to healthy younger controls, therefore precluding the possibility of discriminating the effects of aging from those of bone disease. In fact, a reduction of SOD activity with aging has been already reported [30] and was also observed in the present study (the negative correlation between SOD and age for the whole population studied). On the other hand, the other study [10] evaluated an older population (mean age around 70 years) and an osteoporotic group with a *T* score of −3.5 or less, which may have accounted for the discrepancy between our results.

The main finding of the present study is the increased blood GPx activity in osteopenia group. It is possible that GPx, which is responsible for the degradation of H_2O_2, could be increased as an effort to counteract the overproduction of reactive oxygen species, especially H_2O_2. Bone resorbing osteoclasts generate a high level of superoxide anion [12,13], which by chemical or enzymatic dismutation yields H_2O_2. H_2O_2 not only stimulates osteoclastic differentiation and function, but also was recently demonstrated to be essential for estrogen-deficiency bone loss and osteoclast formation [11,31]. GPx is the antioxidant enzyme predominantly expressed by osteoclasts and its overexpression abrogates osteoclast formation [11]. In addition, GPx is also expressed by osteoblasts and may be relevant for protection against H_2O_2 produced by osteoclasts during bone remodeling [32]. Therefore, the increase of blood GPx activity in osteopenia might reflect reactive oxygen species-induced activation of signaling cascades that lead to increased expression of antioxidant defense proteins, which could represent a negative feedback inhibition of osteoclastic differentiation [11].

In humans, H_2O_2 is detoxified by CAT and GPx. Although, both remove the same substrate, only GPx can effectively remove organic hydroperoxides, being the major source of protection against low levels of oxidative stress [33]. In addition, a significant increase in GPx activity, but no change in CAT activity was observed in cells transfected with a copper–zinc superoxide dismutase expression vector [34], in mice with age-related oxidative stress [35], and in other models of oxidative stress [36]. These findings suggest that GPx, but not CAT, would be modulated by increased reactive species formation in various models of oxidative stress, and could help to explain our results.

The increase of GPx activity in osteopenia patients contrasts with some previous studies that found decreased GPx activity in osteoporotic patients [10,22,29]. H_2O_2 stimulates osteoclastic differentiation and is essential for estrogen-deficiency bone loss [11,31]. Hence, decreased GPx activity observed in osteoporotic patients [10,22] has been interpreted as a factor that could contribute to bone loss along with the decrease of various nonenzymatic antioxidants observed in these patients [10]. Although, in this context an increase of GPx in osteopenia patients could seem unlikely, there is *in vitro* evidence that GPx expression in osteoclasts is induced both by estrogen, which inhibits, and RANKL, which stimulates osteoclastic differentiation [11]. Besides, as discussed above for SOD, GPx activity was also demonstrated to be reduced with aging [37]. Hence, the decreased GPx activity observed by Sontakke and Tare [22] could be related to the higher mean age of the bone disease group when compared to the control group. On the other hand, discrepancies between our results and those of Maggio et al. [10] and Ozgocmen et al. [29] could be related to the higher mean age (around 70 years) and/or *T* score (<−3.5 or <−2.5) of the osteoporotic group in these studies. Oxidative stress can increase the rate of GPx mRNA transcription leading to increased GPx activity [38]. Hence, we propose that increased GPx activity in osteopenia patients may be an early protective response against the oxidative stress associated to this bone disease. With the progression of the disease to osteoporosis, we would expect an increased oxidative stress that could lead to an inhibition of GPx. In fact, a biphasic response of GPx activity was previously reported after oxidative stress induced by hyperbaric oxygen [39] and due to inflammation during periodontal wound healing [40].

Datta et al. [12] demonstrated that superoxide anion production by osteoclasts was almost completely abolished by calcitonin and partially abolished by elevated extracellular calcium. However, calcium supplementation did not prevent the increase of GPx activity, which suggests that this therapy alone could not reduce reactive oxygen species production or the imbalance between osteoclastic and osteoblastic activities.

Urinary calcium represents the fraction of the plasma water calcium that is not reabsorbed in the renal tubules and usually amounts to 1–2% of the filtered calcium [7]. Although there is some evidence that urinary calcium is inversely related to bone density and positively related to the rate of bone loss [41,42], we observed no change in urinary calcium between control and osteopenia subjects. ALP activity in blood serves as an index of bone formation [43]. In the present study, we observed no change in ALP activity due to osteopenia or calcium supplementation. This finding is in agreement with results of Kung et al. [44]. In fact, estrogen deficiency causes a significant increase in bone ALP activity, which has been demonstrated when blood ALP levels in postmenopausal women were compared to levels in premenopausal women [22,44]. Besides, calcium supplementation caused a small reduction of bone ALP [43], which may be masked in total ALP assay, since in adults bone and liver isozymes equally contribute to the total ALP.

In conclusion, higher GPx activity was observed in postmenopausal women with osteopenia, which could be interpreted as an effort to counteract the overproduction of reactive oxygen species, and this change was not prevented by calcium supplementation.

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4 MANUSCRITOS

4.1 Manuscrito 1

EFFECT OF BONE DISEASE ON BLOOD LEAD LEVELS, δ-AMINOLEVULINIC ACID DEHYDRATASE (δ-ALAD) ACTIVITY AND δ-ALAD-REACTIVATION INDEX IN POSTMENOPAUSAL WOMEN NON-OCCUPATIONALLY EXPOSED TO LEAD

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Effect of bone disease on blood lead levels, δ-aminolevulinic acid dehydratase (δ-ALAD) activity and reactivation index in postmenopausal women non-occupationally exposed to lead

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Abstract

Background: Around 90% of lead body burden is found within bone. This study was aimed at investigating the effect of bone disease on blood lead levels, δ-aminolevulinic acid dehydratase (δ-ALAD) activity and δ-ALAD reactivation index in postmenopausal women non-occupationally exposed to lead.

Methods: The study was performed in 70 postmenopausal women that were divided into three groups: control (no bone disease), osteopenia and osteoporosis, according to their bone mineral density. Blood lead was assessed by inductively coupled plasma mass spectrometry, δ-ALAD activity was determined using spectrophotometric method and δ-ALAD reactivation index was determined by measuring enzyme activity in the presence of $ZnCl_2$ and DL-dithiothreitol.

Results: No differences were observed in hematocrit, δ-ALAD activity, δ-ALAD reactivation or blood lead levels among the three study groups. δ-ALAD activity was negatively correlated with blood calcium in postmenopausal women ($r= -0.31$; $p<0.05$). Also blood lead levels and δ-ALAD reactivation index in postmenopausal women were positively correlated to the overall duration of lactation ($r= 0.36$ and 0.28, respectively; $p<0.05$).

Conclusions: Our study indicates that the bone resorption associated to osteopenia/osteoporosis does not pose a risk of lead toxicity in postmenopausal women exposed to background lead levels.

Keywords: Bone mineral density; Calcium; Lead; Osteopenia; Osteoporosis.

1. Introduction

Lead is a ubiquitous environmental toxin that induces a broad range of physiological, biochemical, and behavioral dysfunctions [1]. It interferes with several steps of the heme synthetic pathway, leading to anemia [2]. One of these steps is the condensation of two molecules of δ-aminolevulinic acid to form porphobilinogen, which is catalyzed by δ-aminolevulinic acid dehydratase (δ-ALAD) [3]. Erythrocyte δ-ALAD activity has been typically found to be inversely related to blood lead levels in exposed populations (blood lead levels > 10 μg/dL) [2]. However, δ-ALAD activity is not considered an adequate indicator for low lead-exposed populations [4,5]. The δ-ALAD reactivation index that is measured after incubation of blood samples with zinc and dithiothreitol *in vitro* has been suggested as a good alternative to assess low lead exposure [6]. This measurement is based on the replacement by zinc of lead bound to the enzyme, and allows evaluating the degree of inhibition by lead.

Increases in blood lead in elderly are particularly worrying since it was observed that blood lead levels previously thought to be safe are associated to the development of several of chronic disorders in adults, including increased blood pressure [7], impairment of renal [8], cardiovascular [9] and cognitive function [10]. Besides, it was observed that high lead body burden resulting from occupational exposures may exacerbate bone loss in postmenopausal women [11]. Lead is known to inhibit the activation of vitamin D, the uptake of dietary calcium, and several regulatory aspects of bone cell function [12-15]. It also disrupts osteoblast function [14] and alters circulating levels of hormones regulating calcium homeostasis [11]. These effects may aggravate the course of osteoporosis.

After lead enters the body, it circulates in the blood reaching the soft tissues and bone. Around 90% of the lead body burden is found within bone, where it has a half-life of decades [16]. Thus, exposure to lead over the course of a lifetime results in accumulation of lead in the skeletal compartment such that bone lead levels are generally higher among elderly [17]. Besides, older persons were exposed to higher levels of environmental lead prior to its removal from gasoline.

Bone lead stores represent a potential source for exposure of soft-tissue, even with declining environmental exposures. This occurs because physiological rises in bone turnover, such as in pregnancy, lactation [18] and in menopausal women [19-21] may cause bone lead to be release into blood, even in populations non-occupationally exposed to lead [22-27]. Indeed, significantly higher blood lead levels have been found in women after menopause [19-21,27].

Menopause is associated to hormonal and age-related changes in bone mineral metabolism that increase the risk of bone diseases like osteopenia and osteoporosis [28, 29]. These disorders, which are characterized by increased bone loss, could pose an additional risk of lead toxicity due to bone lead release. In the present study, we investigated the effect of bone disease on blood lead levels, δ-ALAD activity and δ-ALAD-reactivation index in postmenopausal women non-occupationally exposed to lead.

2. Materials and methods

2.1. Human subjects

This study was approved by the Ethics Committee of the Federal University of Santa Maria (CEP/CCS/UFSM nº 130/02). The subjects for this study were selected among patients of the Rheumatology Ambulatory from Santa Maria University Hospital and all subjects gave their written consent prior to the inclusion in the study. Study variables were collected through a questionnaire that was applied by a trained interviewer and included information about age, menopause state, hormone replacement therapy, duration of lactation, calcium supplementation, smoking habit and alcohol use. Women with case history of smoking or alcoholism were excluded. All the women included were postmenopausal (> 12 months of amenorrhea). Height and weight of subjects were measured and used to calculate body mass index (BMI). Bone disease status was determined by dual energy X-ray absorptiometry following the diagnostic criteria proposed by the World Health Organization (WHO).

The study was performed in 70 subjects that were divided into three groups according to their bone mineral density (BMD): control (no bone disease), osteopenia and osteoporosis.

2.2. Bone mineral density

Measurements of BMD were taken at the lumbar spine (L1 to L4 a.p.) and at the femoral neck. According to WHO guidelines osteopenia is defined as a T score between -1 and -2.5 and osteoporosis as a T score less than -2.5, where T score is the number of standard deviations below the mean peak bone mass of young sex-matched healthy adults.

2.3. Sample collection and analysis

Samples of heparinized blood were taken from the cubical vein after overnight fasting. Blood calcium, δ-ALAD activity, δ-ALAD-reactivation index and hematocrit were determined in fresh blood samples, and part of the samples was stored frozen (-20^{o}C) for posterior lead measurement.

Blood calcium was determined using a routine kit (Roche Diagnostic, Mannheim, Germany). Hematocrit was determined by capillary centrifugation. δ-ALAD activity was determined in whole-blood by the method of Berlin & Schaller [30] by measuring the rate of product porphobilinogen (PBG) formation, using 115 mM potassium phosphate buffer, pH 6.8, and 4.6 mM δ-aminolevulinic acid (ALA). The reaction product was determined spectrophotometrically at 555 nm using modified Ehrlich`s reagent, with a molar absorption coefficient of 6.1 x 10^4 Mol^{-1} cm^{-1} for the Ehrlich-porphobilinogen salt. The reaction was started 10 min after the addition of the enzyme preparation by adding the substrate. Incubations were carried out for 60 min at 37˚C. δ-ALAD reactivation index was determined by measuring enzyme activity in the presence of 3 mM $ZnCl_2$ and 10 mM DL-dithiothreitol (DTT). This measurement is

based on the replacement by zinc and DTT of lead bound to the enzyme, restoring activity to a maximum value. The reactivation index was calculated as follows:

$$[(\text{Zn-DTT-}\delta\text{-ALAD activity} - \delta\text{-ALAD activity}) / \delta\text{-ALAD activity}] \times 100$$

For blood lead analysis blood samples were defrosted at room temperature (25°C) and homogenized using an ultrasound bath. Subsequently 250 μL of homogenized samples were transferred to quartz vessels of a high pressure microwave digestion system (Model Multiwave 3000, Anton Paar, Austria), concentrated nitric acid (6 mL) was added, vessels were closed and the following program was carried out: 20 min at 1400 W and 20 min at 0 W for cooling. After, the samples were diluted to 25 mL with water. Blood lead was determined using inductively coupled plasma mass spectrometry (ICP-MS, Model ELAN DRC II, Perkin Elmer, USA) equipped with a cyclonic spray chamber, with nebulizer gas flow set at 1.11 L min^{-1}, radiofrequency power of 1300 W and mass charge ratio (m/z) of 207. Calibration was performed from standard analytical curve using a multi-elementar reference solution (from 50 to 1000 ng L^{-1}). Spiked samples (containing 200 ng L^{-1}) were used for accuracy check. The detection limit was 0.15 μg/dL blood and quantification limit was 0.5 μg/dL blood.

2.4. Statistical analysis

Data that did not exhibit a normal distribution were transformed (log or square root transformation) in order to meet parametrical statistics assumptions before analysis. Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's test when appropriate. The associations between variables were evaluated by Pearson´s correlation for variables that had a normal distribution and by Spearman´s rank order correlation for variables that did not exhibit a normal distribution even after transformation. A multivariate linear regression analysis was also employed to estimate the independent contribution of each individual variable to blood lead levels and δ-ALAD activity. The selection of predictors was based on (1) whether the variable was statistically significant at the $p<0.05$ level and (2) whether the inclusion of that variable increased the percentage of variance explained. Data were analyzed using the Statistica® 6.0 software system (Statsoft Inc., 2001). Only variables that had a normal distribution (before or after transformation) were included in the models.

3. Results

Table 1 shows the characteristics of the postmenopausal women included in the present study that were divided into three groups according to bone density. Age, duration of hormone therapy, overall duration of lactation, duration of calcium supplementation, and blood calcium were not significantly different among the studied groups. However, women with osteopenia and osteoporosis had a tendency of greater years since menopause when compared to the control group ($p<0.1$). ANOVA revealed a significant main effect of bone disease on BMI and BMD. Osteopenia and

osteoporosis groups had lower BMI, femoral and L1-L4 BMD as compared to control groups ($p<0.05$). Besides, osteoporosis groups had significantly lower femoral and L1-L4 BMD than osteopenia groups. Evaluation of scores T and Z revealed the same behavior of BMD (data not shown).

Markers of blood lead exposure are shown in Table 2. No differences were observed in hematocrit, δ-ALAD activity, δ-ALAD reactivation index or blood lead levels among the three studied groups. Lead in blood is mostly bound to erythrocytes [31]. However, no significant differences were observed among the studied groups even when blood lead levels were adjusted for hematocrit values, in order to control for the influence of physiological anemia (data not shown). δ-ALAD activity, δ-ALAD reactivation index and blood lead had no significant correlation with age, years since menopause, duration of calcium supplementation, BMI, femoral and L1-L4 BMD, or hematocrit. However, duration of hormone therapy had a tendency of negative correlation with blood lead levels ($r= -0.21$; $p<0.1$).

δ-ALAD activity was negatively correlated with δ-ALAD reactivation index ($r= -0.28$; $p<0.05$; Fig. 1A) and blood calcium ($r= -0.31$; $p<0.05$; Fig. 1B). In addition, blood lead and δ-ALAD reactivation index were positively correlated with the overall duration of lactation ($r= 0.36$; $p<0.05$; Fig. 2A and $r= 0.28$; $p<0.05$; Fig. 2B, respectively).

Table 3 shows the independent contribution of individual variables to δ-ALAD activity using multivariate linear regression analyses fitted to data. Blood calcium was a significant predictor for δ-ALAD activity, explaining 32% of variance. Besides, femoral BMD was also included in the model and explained 26% of variance ($p=0.06$). No significant model was generated for blood lead levels or δ-ALAD reactivation index.

4. Discussion

The present study was aimed at investigating if bone disease could affect blood lead levels, δ-ALAD activity and δ-ALAD reactivation index in postmenopausal women non-occupationally exposed to lead. The women studied had low blood lead levels, probably because they were only exposed to background lead levels. As recently reviewed by Paoliello & De Capitani [32] previous studies on reference levels of blood lead in non-occupationally exposed populations in Brazil are scarce and no study was conducted in the extreme south of Brazil (Rio Grande do Sul state). However, results found in the present study are similar to those reported in adults from the state of Paraná [32]. Besides, blood lead levels found in the present study are in agreement with the low levels found in USA in non-occupationally exposed women aged 40-74 years [27,33].

Lead content in bone or blood is directly connected with the degree of environmental pollution [34]. Lower lead concentration in children's blood was correlated to the decreasing of environmental pollution due to the introduction of lead free gasoline [35,36]. Hence, results of the present study indicate that lead environmental pollution is low in the studied area (central region of Rio Grande do Sul State, Brazil). In fact, Santa Maria is a poorly industrialized city, which may have contributed to our results. This contrasts with the worrying blood lead levels found in children from the states of São Paulo [36], Bahia [37] and Paraná [38], which are important lead contaminated areas.

Impairment of heme biosynthesis, specially the inhibition of blood δ-ALAD activity, is a well known hematological effect of lead toxicity [2]. As a consequence lead

exposure has been associated to anemia that may be indicated by decreased hematocrit values [2]. In addition, hematocrit was found to be an important determinant of blood lead levels in non-occupationally exposed women [19,20,40], since lead in blood is mostly bound to erythrocytes [31].

We found similar levels of hematocrit, δ-ALAD activity and δ-ALAD reactivation index for postmenopausal women regardless of the occurrence of bone disease. This is consistent with the absence of significant differences in blood lead levels among the postmenopausal groups studied, which is in agreement with some previous studies [21,40]. Most of the previous studies that suggested bone lead mobilization in elderly women have evaluated premenopausal women as compared to postmenopausal women, and found higher blood lead levels in the latter group [21,27,33]. Bone remodeling pattern during the first years of postmenopause mainly depends on higher turnover of trabecular bone, which increases in the perimenopausal period, leading to an accelerated loss in the first years of postmenopause and then becomes constant [41]. Our study compared only postmenopausal women with different bone densities. Hence, it is possible that lead released due to bone loss occurring in the perimenopausal period and in the early postmenopausal period could not have been detected. In fact, measurement of BMD in cross-sectional studies provides a snapshot of the balance between bone deposition and bone resorption over the preceding years, whereas blood lead levels would be expected to depend more specifically on absolute rates of ongoing bone resorption.

We found that postmenopausal blood lead levels were positively associated to the overall duration of lactation in the postmenopausal population studied. Accordingly, Latorre et al. [21] observed higher bone lead levels in postmenopausal women who breastfed in the Mexico City. They suggested that women who breastfed during the

years of high lead concentrations in the air could have incorporated additional lead during the bone gain phase that is known to follow pregnancy and lactation [21,42]. Similarly, the women enrolled in the present study also breastfed in years of higher lead environmental contamination in Brazil due to the use of leaded gasoline. In Brazil efforts to reduce lead in gasoline started in 1979, but it was completely banned only in 1993 [32].

We also found a positive correlation between δ-ALAD reactivation index in postmenopausal women and the overall duration of lactation. The δ-ALAD reactivation index has been proved to be a sensitive indicator of low lead-level exposure [43]. This is consistent with the positive association between blood lead levels and the overall duration of lactation.

Bone turnover in the adult is one of the major mechanisms for maintaining calcium homeostasis, with vitamin D and PTH as the two major regulators of calcium metabolism [44]. Due to similarities in the atomic and ionic structures, bone lead is mobilized into the blood stream concurrently with calcium [45]. We found that δ-ALAD activity was negatively associated with blood calcium levels in postmenopausal women. However, it is unlikely that this finding is secondary to changes in blood lead levels, since no association was found between blood lead and blood calcium levels or between blood calcium levels and δ-ALAD reactivation index, which is a more sensitive indicator of low lead levels than ALAD activity.

In conclusion, our study indicated that the bone resorption associated to osteopenia/osteoporosis does not pose a risk of lead toxicity in postmenopausal women exposed to background lead levels.

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Figure legends

Fig. 1. Correlation between δ-ALAD activity and δ-ALAD reactivation index (A) and between δ-ALAD activity and blood calcium (B) for all postmenopausal women studied (n=70).

Fig. 2. Correlation between log blood lead and overall duration of lactation (A) and between δ-ALAD reactivation index and overall duration of lactation (B) for all postmenopausal women studied (n=70).

Table 1. Characteristics of postmenopausal women studied

	Control (n=24)	Osteopenia (n=26)	Osteoporosis (n=20)
Age (years)	58.3 ± 3.9 (51-65)	59.0 ± 6.5 (49-69)	60.6 ± 6.4 (47-74)
Years since menopause (years)	8.6±5.8 (1-20)	12.1±7.5 (1-27)	13.3±7.5 (1-29)
Duration of hormone therapy (months)	34.0±59.6 (0-180)	34.4±67.8 (0-240)	29.4±58.6 (0-180)
Overall duration of lactation (months)	17.3 ± 21.2 (0-78)	28.0 ± 31.7 (0-120)	28.6 ± 56.7 (0-252)
Duration of calcium supplementation (months)	2.7 ± 3.0 (0-6)	8.4 ± 17.6 (0-72)	5.7 ± 11.5 (0-48)
Body mass index (kg/m^2)	30.0 ± 3.9[a] (22.9-37.1)	26.3 ± 3.7[b] (17.8-32.0)	24.7 ± 4.8[b] (16.9-36.5)
Bone mineral density femoral (g/cm^2)	0.877 ± 0.102[a] (0.737-1.084)	0.722 ± 0.090[b] (0.621-0.959)	0.657 ± 0.101[c] (0.441-0.793)
Bone mineral density L1-L4 (g/cm^2)	1.088 ± 0.102[a] (0.945-1.234)	0.886 ± 0.090[b] (0.786-1.147)	0.710 ± 0.056[c] (0.603-0.828)
[*]Blood calcium (mg/dL)	9.7 ± 0.5 (9.2 – 11.1)	9.3 ± 0.7 (7.9 – 10.4)	9.6 ± 0.7 (7.9 – 10.4)

Data are expressed as means ± SE (minimum - maximum). Values within the same line that do not share a common superscript are significantly different ($p<0.05$). [*] In blood calcium assays n= 21 for control, n=13 for osteopenia and n=14 for osteoporosis group.

Table 2. Markers of blood lead exposure in postmenopausal women

	Control (n=24)	Osteopenia (n=26)	Osteoporosis (n=20)
Hematocrit (%)	39.0±3.3 (30-44)	39.1±1.7 (35-43)	39.0±2.2 (35-43)
δ-ALAD activity (μmol PBG/min/L of erythrocytes)	13.4±5.0 (3.8-22.5)	14.4±4.7 (5.7-22.3)	12.1±4.2 (6.1-19.2)
δ-ALAD reactivation index (%)	24.7±14.8 (1.0-61.7)	23.4±13.1 (1.1-60.2)	23.3±12.7 (5.2-48.3)
Blood lead (μg/dL)	3.9±1.3 (1.9-6.2)	3.7±1.9 (2.0-8.8)	3.4±1.5 (1.0-7.3)

Data are expressed as means ± SE (minimum - maximum).

Table 3. The independent contribution of each individual variable to δ-ALAD activity by multivariate linear regression analyses

	ß	Student's t-test	*p*-value
δ-ALAD activity (μmol PBG/min/L of erythrocytes)			
Blood calcium (mg/dL)	-0.32	-2.34	0.02
BMD femur (g/cm^2)	0.26	1.89	0.06

Figures

Fig. 1

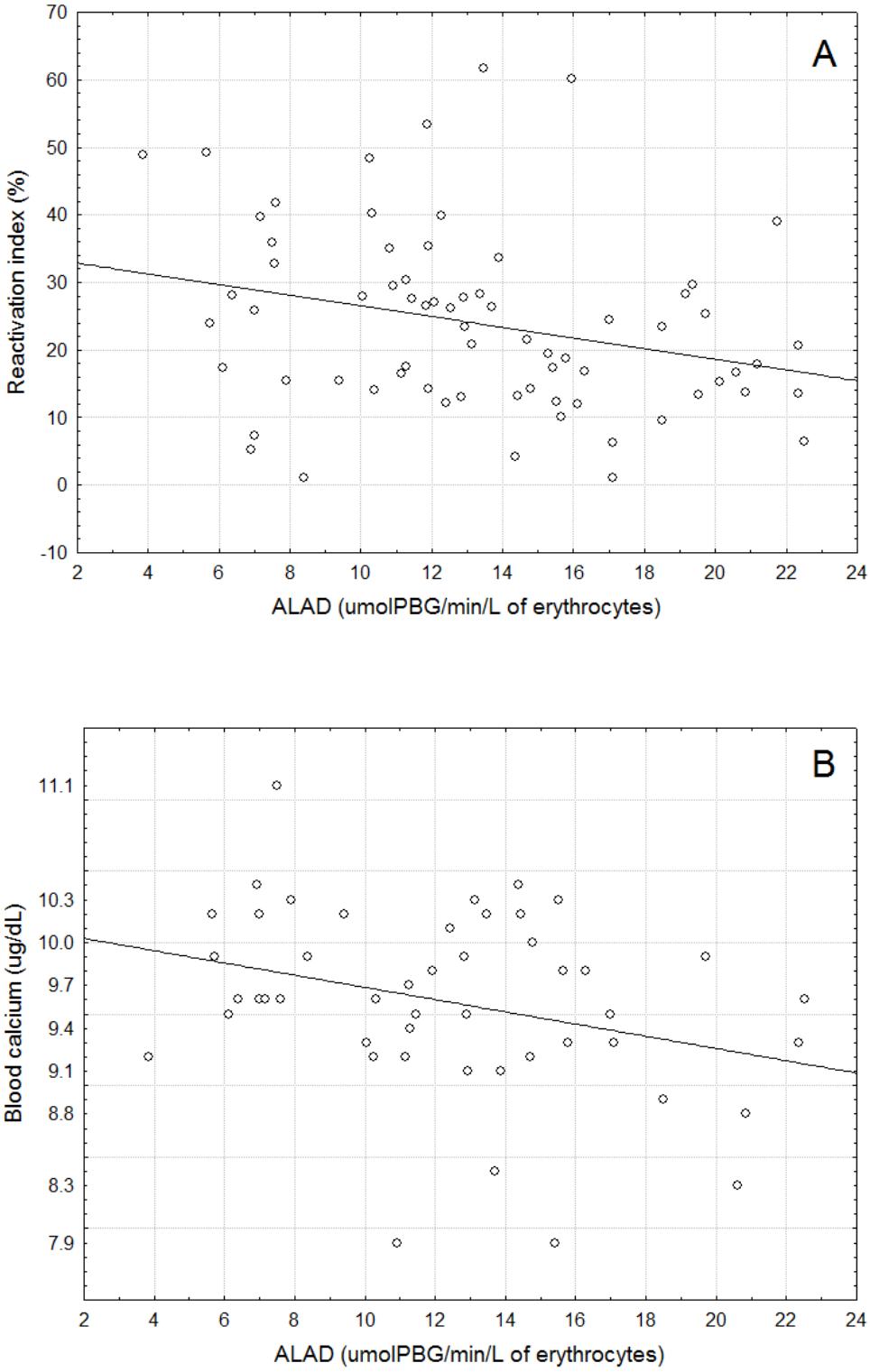
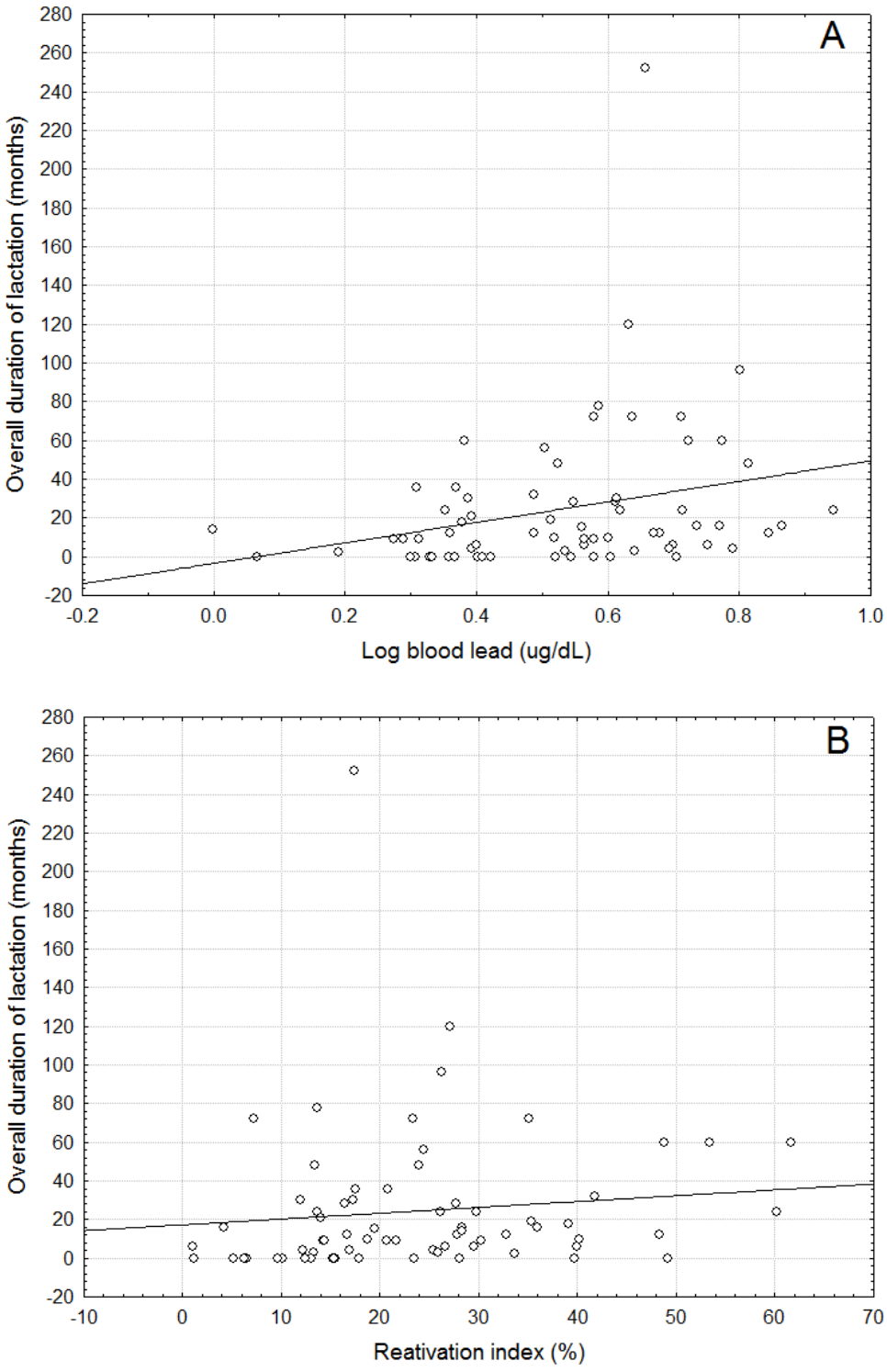


Fig. 2


A
Overall duration of lactation (months)
Log blood lead (ug/dL)
B
Overall duration of lactation (months)
Reativation index (%)

4.2 Manuscrito 2

EFFECT OF CALCIUM SUPPLEMENTATION ON BLOOD LEAD LEVELS, δ-AMINOLEVULINIC ACID DEHYDRATASE (δ-ALAD) ACTIVITY AND ANTIOXIDANT ENZYMES IN POSTMENOPAUSAL WOMEN WITH BONE DISEASE: A PROSPECTIVE STUDY

Artigo em fase final de revisão pelos autores para ser submetido à Revista *Clinical Biochemistry*

Effect of calcium supplementation on blood lead levels, δ-aminolevulinic acid dehydratase (δ-ALAD) activity and antioxidant enzymes in postmenopausal women with bone disease: a prospective study

Category: Clinical Investigation, full paper

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Abstract

Background: The study was aimed at investigating the effect of calcium supplementation and bone disease on blood lead levels, δ-aminolevulinic acid dehydratase (δ-ALAD) activity and antioxidant enzymes activities in postmenopausal women non-occupationally exposed to lead.

Methods: Thirty postmenopausal women were divided into three groups: control (no bone disease), osteopenia and osteoporosis, according to their bone mineral density. All groups received 1000 mg of a calcium carbonate supplement per day during 3 months and were evaluated for changes in δ-ALAD activity, δ-ALAD reactivation index, blood lead levels, alkaline phosphatase (ALP) activity, blood calcium, blood superoxide dismutase, catalase and glutathione peroxidase activities before starting the supplementation (0 month) and at 3 months after starting calcium supplementation. Lead was assessed by inductively coupled plasma mass spectrometry and the other parameters were assessed using spectrophotometric methods.

Results: No differences were observed in δ-ALAD activity, δ-ALAD reactivation index, blood calcium levels or blood antioxidant enzymes activities among the three studied groups along 3 months of calcium supplementation. Blood lead levels ranged from 1.2 to 7.8 µg/dL, and were not different among the three studied groups, but were enhanced in bone disease groups after 3 months of calcium supplementation ($p<0.05$). Besides, ALP activity decreased after 3 months of calcium supplementation in the three studied groups ($p<0.05$).

Conclusions: Our study suggests that 3 months of calcium supplementation contributed to a small, but not toxicologically concerning, increase of blood lead levels in postmenopausal women with bone diseases.

Keywords: Osteoporosis; Osteopenia; Bone mineral density; Catalase; Glutathione peroxidase; Superoxide dismutase.

Introduction

Menopause is defined as the permanent loss of menstruation after a period of amenorrhea lasting over one year and is linked to the loss of estrogen production [1]. Menopause is associated to hormonal and age-related changes in bone mineral metabolism. These changes increase the risk of bone diseases characterized by increased bone resorption like osteopenia and osteoporosis [1]. In recent years various evidence were provided linking these bone diseases to reactive oxygen species [2-4] and to changes in the activity of antioxidant enzymes [3,5,6].

Lead is an important environmental contaminant that accumulates mainly within the bones (around 90% of the lead body burden), where it is covalently bound to the mineral matrix, apparently in close chemical association with calcium and phosphate [7]. Bone lead levels are generally higher among elderly [8], because it has a half-life of decades [9]. Conditions of bone resorption, such as in pregnancy, lactation [10] and in menopausal women [11-13] may cause bone lead to reenter into the bloodstream where it can then re-expose the soft tissues, and, potentially, exert deleterious effects [14].

Increases in blood lead in elderly are particularly worrying since it was observed that blood lead levels previously thought to be safe are associated to the development of a number of chronic disorders in adults, including increased blood pressure [15], impairment of renal [16], cardiovascular [17] and cognitive function [18]. Besides, it was observed that high lead body burden resulting from occupational exposures may exacerbate bone loss in postmenopausal women [19]. Lead is known to inhibit the activation of vitamin D, the uptake of dietary calcium, and several regulatory aspects of

bone cell function [20-23]. It also disrupts osteoblast function [22] and alters circulating levels of hormones regulating calcium homeostasis [19]. These effects may aggravate the course of osteoporosis.

Epidemiological, experimental and clinical studies have indicated that lead impairs heme biosynthesis due to the inhibition of δ-aminolevulinic acid dehydratase (δ-ALAD) [24,25]. Although erythrocyte δ-ALAD activity is considered a sensitive and specific biochemical index of acute and chronic lead poisoning [26], δ-ALAD reactivation index is considered more appropriate to evaluate exposure to low lead levels [27]. This index is based on the replacement by zinc of lead bound to the enzyme, and allows evaluating the degree of inhibition by lead. δ-ALAD inhibition leads to accumulation of δ-aminolevulinic acid, which undergoes a process of autoxidation generating reactive oxygen species [28]. In line with this, oxidative stress and changes in the activity of antioxidant enzymes have also been implicated in the toxic effects of lead [29,30].

Calcium absorption efficiency is a key factor in the maintenance of calcium balance and is reduced in postmenopausal women with vertebral fractures [31]. Thus, adequate calcium supplementation is required to offset the obligatory losses of calcium from the skeletal reservoir that occur in osteopenia and osteoporosis [32]. Although there have been some concern about lead contamination of dietary calcium supplements in USA and Canadá [33,34], the lead content of calcium supplements available in Brazil is relatively low [35]. Besides, the consumption of calcium supplements is associated with lower blood lead levels, suggesting that the raise in calcium levels reduces mobilization of lead from the skeleton during bone demineralization in pregnancy and lactation [36,37].

In this prospective study conducted in postmenopausal women non-occupationally exposed to lead, we investigated the effect of bone disease on blood lead levels, δ-ALAD activity and antioxidant enzymes during 3 months of calcium supplementation.

Methods

Subjects

This study was approved by the Ethics Committee of the Federal University of Santa Maria (CEP/CCS/UFSM nº 130/02). The subjects for this study were selected among patients of the Rheumatology Ambulatory from Santa Maria University Hospital and all subjects gave their written consent prior to the inclusion in the study. Study variables were collected through a questionnaire that was applied by a trained interviewer and included information about age, menopause state, hormone replacement therapy, duration of lactation, number of children, dairy products intake, calcium supplementation, smoking habit and alcohol use. Women with case history of smoking or alcoholism or that were already taking calcium supplements were excluded. All the women included were postmenopausal (> 12 months of amenorrhea). Height and weight of subjects were measured and used to calculate body mass index (BMI).

Sixty subjects were recruited and divided into three groups according to their bone mineral density (BMD): control (no bone disease, n=20), osteopenia (n=20) and osteoporosis (n=20). The bone disease status was determined by dual energy X-ray absorptiometry following the diagnostic criteria proposed by the World Health Organization (WHO).

Calcium supplementation

All groups received 1000 mg of a calcium carbonate supplement per day during 3 months and were evaluated for changes in δ-ALAD activity, δ-ALAD reactivation index, blood lead levels, alkaline phosphatase activity (ALP), blood calcium, hemoglobin and antioxidant enzymes before starting the supplementation (0 month) and at 3 months after starting calcium supplementation. The calcium carbonate supplements were prepared and donated by two pharmacies from Santa Maria (RS, Brazil). The lead content of these supplements was analyzed and results were previously reported [35].

Thirty subjects stopped calcium treatment prematurely and/or did not return for blood collection after the 3 months of calcium supplementation. Thus, our study group was composed of thirty subjects that completed the treatment, being 8 for the control group, 15 for the osteopenia group and 7 for the osteoporosis group.

The duration of calcium supplementation (3 months) has been chosen in accordance with the Brazilian Decree n° 470 from the General Office of Health Assistance, from July 24th, 2002, that approves the clinic protocol and therapeutic guidelines for the treatment of osteoporosis. This decree does not establish a minimum period for the treatment with calcium carbonate, but warns that side effects, such as gastrointestinal disorders may occur after extensive use.

Bone mineral density

Measurements of BMD were taken at the lumbar spine (L1 to L4 a.p.) and at the femoral neck. According to WHO guidelines ostopenia is defined as a T score between -1 and -2.5 and osteoporosis as a T score less than -2.5, where T score is the number

of standard deviations below the mean peak bone mass of young sex-matched healthy adults.

Sample collection and analysis

Samples of heparinized blood were taken from the cubical vein after overnight fasting. Plasma was separated and immediately used for alkaline phosphatase (ALP) determination by a routine kit (Roche Diagnostics, Mannheim, Germany). Whole blood samples were immediately used for hemoglobin (Hb), hematocrit, blood calcium, δ-ALAD activity, and δ-ALAD reactivation index measurements, and another part of the samples was stored at -20^{o}C for posterior measurement of lead content and antioxidant enzymes activities.

Blood was employed for determination of Hb using routine kit (Roche Diagnostics, Mannheim, Germany) and then stored at -20^{o}C until analysis of antioxidant enzymes activity. Hematocrit was determined by capillary centrifugation. Blood calcium was determined using a routine kit (Roche Diagnostic, Mannheim, Germany). δ-ALAD activity was determined in whole blood by the method of Berlin & Schaller [38] by measuring the rate of product porphobilinogen (PBG) formation, using 115 mM potassium phosphate buffer, pH 6.8, and 4.6 mM δ-aminolevulinic acid (ALA). The reaction product was determined spectrophotometrically at 555 nm using modified Ehrlich`s reagent, with a molar absorption coefficient of 6.1 x 10^{4} Mol^{-1} cm^{-1} for the Ehrlich-porphobilinogen salt. The reaction was started 10 min after the addition of the enzyme preparation by adding the substrate. Incubations were carried out for 60 min at 37˚C. δ-ALAD reactivation index was determined by measuring enzyme activity in the presence of 3 mM $ZnCl_2$ and 10 mM DL-dithiothreitol (DTT). This measurement is

based on the replacement by zinc and DTT of lead bound to the enzyme, restoring activity to a maximum value. The reactivation index was calculated as follows:

[(Zn-DTT-δ-ALAD activity − δ-ALAD activity) / δ-ALAD activity] x 100

For blood lead analysis blood samples were defrosted at room temperature (25°C) and homogenized using an ultrasound bath. Subsequently 250 μl of homogenized samples were transferred to quartz vessels of a high pressure microwave digestion system (Model Multiwave 3000, Anton Paar, Austria), concentrated nitric acid (6 ml) was added, vessels were closed and the following program was carried out: 20 min at 1400 W and 20 min at 0 W for cooling. After, the samples were diluted to 25 ml with water. Blood lead was determined using inductively coupled plasma mass spectrometry (ICP-MS, Model ELAN DRC II, Perkin Elmer, USA) equipped with a cyclonic spray chamber, with nebulizer gas flow set at 1.11 l min^{-1}, radiofrequency power of 1300 W and mass charge ratio (m/z) of 207. Calibration was performed from standard analytical curve using a multi-elementar reference solution (from 50 to 1000 $ng.dL^{-1}$). Spiked samples (containing 200 $ng.dL^{-1}$) were used for accuracy check. The detection limit was 0.15 μg/dL blood and quantification limit was 0.5 μg/dL blood.

Antioxidant enzyme activities (glutathione peroxidase, GPx; superoxide dismutase, SOD and catalase, CAT) were determined in whole blood samples. GPx activity was determined in a medium containing 25 mM potassium phosphate buffer, pH 7.0, 2.5 mM ethylenediaminetetraacetic acid, 0.24 U/mL glutathione reductase, 1 mM reduced glutathione, 1 mM sodium azide, 0.15 mM NADPH, and 0.4 mM hydrogen peroxide. The method is based on the oxidation of NADPH, which is indicated by the decrease in absorbance at 340 nm [39]. SOD activity was determined at 480 nm using

50 mM glycine buffer, pH 10.2, and 1 mM epinephrine at 30°C [40]. SOD activity was expressed as the amount of enzyme that inhibits the auto-oxidation of epinephrine to adrenochrome by 50% which is equal to 1 unit. CAT activity was measured at 240 nm using 50 mM phosphate buffer, pH 7.0 and 17 mM hydrogen peroxide as substrate [41]. The pseudo-first order reaction constant (*k*) of the decrease in H_2O_2 absorption at 25^{o}C was determined and specific activity was expressed as *k*/g protein.

Statistical analysis

Data that did not exhibit a normal distribution were transformed (log or square root transformation) in order to meet parametrical statistics assumptions before analysis. Data were analyzed by analysis of variance (ANOVA) (3 bone disease states x 2 calcium supplementation times) with the time variable considered as a repeated measure. Post hoc comparisons were made by Duncan's test when appropriate. The associations between variables were evaluated by Pearson´s correlation for variables that had a normal distribution and by Spearman´s rank order correlation for variables that did not exhibit a normal distribution even after transformation. A multivariate linear regression analysis was also employed to estimate the independent contribution of each individual variable to δ-ALAD activity, blood lead levels, antioxidant enzyme activities and bone mineral density. The selection of predictors was based on (1) whether the variable was statistically significant at the $p<0.05$ level and (2) whether the inclusion of that variable increased the percentage of variance explained. Data were analyzed using the Statistica® 6.0 software system (Statsoft Inc., 2001). Only variables that had a normal distribution (before or after transformation) were included in the multivariate linear regression models. Results were considered significant when $p<0.05$.

Results

Postmenopausal women were divided into three groups according to bone mineral density and were evaluated before starting a calcium supplementation and at 3 months after starting supplementation. Characteristics of the postmenopausal women studied are shown in Table 1. No significant difference was observed in age, duration of hormone therapy, overall duration of lactation, number of children, dairy products intake or body mass index among the studied groups. However, control group had significantly lower years since menopause than osteopenia and osteoporosis groups. As expected, osteopenia and osteoporosis groups had lower L1-L4 and femoral BMD ($p<0.05$) as compared to control groups. Besides, osteoporosis group had significantly lower L1-L4 BMD when compared to osteopenia group.

Femoral BMD was positively correlated with the duration of hormone therapy and IMC ($p<0.05$, Table 2). L1-L4 BMD was negatively correlated with age and years since menopause ($p<0.05$), while femoral BMD showed a tendency to be negatively correlated with years since menopause ($p=0.06$; Table 2)

No differences were observed in δ-ALAD activity, δ-ALAD reactivation index or blood calcium levels among the three studied groups along 3 months of calcium supplementation (Table 3). Also, calcium supplementation did not change these parameters. The δ-ALAD reactivation index showed a tendency to be negatively correlated with δ-ALAD activity ($p=0.09$; Table 2). Blood lead levels were not different among the three studied groups. However, calcium supplementation had a significant effect on blood lead levels. Blood lead levels were significantly enhanced in bone disease groups after 3 months of calcium supplementation (Table 3). ALP activity, which is a biochemical marker of bone turnover, did not differ among the studied groups, but decreased after 3 months of calcium supplementation in the three studied

groups (Table 3). Blood GPx, CAT and SOD activities were not significantly different among the three studied groups and did not change during calcium supplementation (Table 4).

Table 5 shows the independent contribution of individual variables to bone mineral density using multivariate linear regression analysis fitted to data. Significant models were generated for femoral and L1-L4 BMD. Years since menopause was a significant predictor for femoral and L1-L4 BMD, explaining 38 and 50% of variance, respectively. Dairy products intake had a tendency to be a significant predictor for L1-L4 BMD, explaining 30% of variance.

Discussion

In agreement with previous studies regression analyses revealed that BMD decreased with increasing age and duration of menopause [1], but increased with increasing duration of hormone therapy in the studied population [42].

The present study investigated the influence of bone disease and calcium supplementation on δ-ALAD activity, blood lead levels and antioxidant enzymes activities in postmenopausal women. As observed in some previous studies [43,44] we found no significant differences in blood lead levels among control, osteopenia and osteoporosis postmenopausal women. Most of the previous studies that suggested bone lead mobilization in elderly women have evaluated premenopausal women as compared to postmenopausal women, and found higher blood lead levels in the latter group [43,45,46]. Bone remodeling pattern during the first years of postmenopause mainly depends on higher turnover of trabecular bone, which increases in the perimenopausal period, leading to an accelerated loss in the first years of postmenopause and then becomes constant [47]. Our study compared only

postmenopausal women with different bone densities. Hence, it is possible that lead released due to bone loss occurring in the perimenopausal period and in the early postmenopausal period could not have been detected.

Calcium supplementation was shown to reduce mobilization of lead from the skeleton during bone demineralization in pregnancy and lactation [36,37]. However, in the present study we found an increase of blood lead levels after 3 months of calcium supplementation in postmenopausal women with osteopenia and a tendency of increase in women with osteoporosis. These results suggest that calcium supplements could have contributed to increase blood lead levels in women with bone diseases. In fact, various previous studies revealed the concern about lead contamination of dietary calcium supplements available in other countries [33,34]. However, the lead content of the calcium carbonate supplements used in the present study was previously found to be in the range 0.51-1.58 µg per g calcium [35]. Brazil has no specific limit for lead in calcium supplements, but the lead content found is lower than the USA federal limit (7.5 µg per g calcium) and is next to the limit established in the State of California (1.5 µg per g calcium) [35]. Although the lead content of the supplements used was considered low and in accordance with international guidelines, our results indicate that their lead content contributed to increase blood lead levels in postmenopausal women with bone disease. Since this increase was observed only in women with bone diseases, it is possible that lead released due to bone resorption may have also contributed to enhance blood lead levels in these groups. Nevertheless, this contribution must be small, since no significant independent effect of bone disease was observed on blood lead levels in this study. Our results reinforce the need for a more stringent limit for lead in calcium supplements, like that established in California.

Blood lead levels found in the present study are in agreement with the low levels found in USA in non-occupationally exposed women aged 40-74 years [45,46]. This result is consistent with exposure to background lead levels and suggests a low environmental pollution in the studied area (central region of Rio Grande do Sul State, Brazil). Although calcium treatment increased blood lead levels, values attained after a 3-months supplementation were not of toxicological concern. Accordingly, neither δ-ALAD activity or δ-ALAD reactivation index, which are biochemical indicators of lead exposure [26,27] were changed due to bone disease or calcium supplementation. Also, antioxidant enzymes activities that may be affected after lead exposure [29,30] were not changed in the present study.

We have recently reported the effect of calcium supplementation on the activity of antioxidant enzymes in control and osteopenia postmenopausal women in a cross-sectional study and observed no changes in SOD or CAT activities [48]. Accordingly, in the present prospective study SOD and CAT activities were not affected by bone disease or calcium supplementation. In contrast, erythrocyte CAT activity was recently demonstrated to be lower in postmenopausal osteoporotic women when compared to healthy non-porotic women [49]. The absence of change in SOD activity due to bone disease is in agreement with data from Yalin et al. [50] and Ozgocmen et al. [51].

As observed for SOD and CAT activities, GPx was also not affected by bone disease or calcium supplementation in the present study. This finding contrasts with our previous study where an increased GPx activity was observed in osteopenia women when compared to the control group [48]. This discrepancy probably occurred due to the lower number of subjects enrolled in this prospective study, since before

starting calcium supplementation GPx activity of the osteopenia group was higher than that of control group, but it did not reach statistical significance.

Blood ALP activity, which represents the sum of liver and bone ALP isoenzymes, has been used as an index of bone formation [52,53]. We observed no differences in ALP activity due to bone disease, but a significant decrease after 3 months of calcium supplementation in all studied groups. This decrease was expected because a reduction in bone resorption is followed by a parallel reduction in bone formation when a new steady state is reached. This finding is in agreement with previous studies that observed a reduction of bone ALP activity after calcium supplementation in postmenopausal women reviewed by [52].

Blood calcium or extracellular calcium represents the pool into which calcium enters from the gut, by absorption, and from bone, by resorption, and from which it leaves via the gastrointestinal tract, the kidneys, the skin, and into bone by formation [31]. We observed no differences in blood calcium levels between control and bone disease groups. Also, no changes were observed during the 3 months of calcium supplementation. This finding was expected, since the organism is clearly concerned to maintain blood calcium levels. Actually, the increased calcium loss in postmenopausal women leads to an increased bone resorption to maintain blood calcium levels, or must be counteracted by an increased calcium intake [31].

In conclusion, our study suggested that 3 months of calcium supplementation contributed to a small, but not toxicologically concerning increase of blood lead levels in postmenopausal women with bone diseases.

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Table 1. Characteristics of the postmenopausal women studied

	Control (n=8)	Osteopenia (n=15)	Osteoporosis (n=7)
Age (years)	58.5 ± 1.4	61.1 ± 1.1	63.7 ± 1.6
Years since menopause (years)	8.6 ± 1.8[a]	15.8 ± 1.7[b]	17.7 ± 2.6[b]
Duration of hormone therapy (months)	31.5 ± 23.0	14.7 ± 7.4	17.1 ± 17.1
Overall duration of lactation (months)	9.1 ± 1.8	15.8 ± 4.1	20.0 ± 10.2
Number of children	3.0 ± 0.4	4.0 ± 0.7	4.0 ± 0.8
Dairy products intake (units/week)	21.9 ± 3.3	26.1 ± 4.7	17.0 ± 2.6
Body mass index (kg/m^2)	30.3 ± 1.6	27.9 ± 1.3	26.4 ± 2.1
Bone mineral density L1-L4 (g/cm^2)	1.078 ± 0.040[a]	0.877 ± 0.017[b]	0.716 ± 0.020[c]
Bone mineral density femur (g/cm^2)	0.882 ± 0.044[a]	0.714 ± 0.019[b]	0.661 ± 0.049[b]

Data are expressed as means ± SE (minimum - maximum).

[a,b,c] Values within the same line that do not share a common superscript letter are significantly different ($p \leq 0.05$).

Table 2. Simple correlation between the study variables

	n	r	*p*-value
Square root of bone mineral density femur (g/cm^2)			
IMC (kg/m^2)	30	0.42	0.02
Years since menopause (years)	30	-0.35	0.06
Duration of hormone therapy (months)	30	0.38	0.04
Bone mineral density L1-L4 (g/cm^2)			
Age (years)	30	-0.38	0.04
Years since menopause (years)	30	-0.44	0.01
Log of δ-ALAD reactivation index (%)			
δ-ALAD (μmol PBG/min/L of erythrocytes)	60	-0.23	0.09

Table 3. δ-ALAD activity, δ-ALAD reactivation index, blood lead levels, blood calcium and ALP activity of the three study groups during calcium supplementation

	Control (n=8)		Osteopenia (n=15)		Osteoporosis (n=7)	
Duration of Ca supplementation	0 months	3 months	0 months	3 months	0 months	3 months
δ-ALAD (μmolPBG/min/L of erythrocytes)	13.4 ± 0.5	9.1 ± 1.8	12.7 ± 1.0	8.2 ± 1.2	13.2 ± 2.0	11.3 ± 1.7
δ-ALAD reactivation index (%)	18.0 ± 2.6	29.0 ± 4.2	26.1 ± 5.2	27.4 ± 4.2	16.6 ± 3.0	20.7 ± 5.9
Blood lead (μg/dL)	3.8 ± 0.4[a,b]	3.7 ± 0.4[a,b]	3.3 ± 0.3[b]	4.6 ± 0.4[a]	3.2 ± 0.5[b]	3.9 ± 0.6[a,b]
Blood calcium (mg/dL)	9.6 ± 0.1	10.0 ± 0.3	9.6 ± 0.1	9.6 ± 0.1	9.7 ± 0.2	9.6 ± 0.1
Alkaline phosphatase (U/L)	71.9 ± 8.1[a]	66.2 ± 8.7[b]	71.1 ± 6.2[a]	67.1 ± 6.5[b]	86.1 ± 7.0[a]	83.9 ± 5.3[b]

Data are expressed as means ± SE (minimum – maximum).

[a,b] Values within the same line that do not share a common superscript letter are significantly different ($p<0.05$).

Table 4. Antioxidant enzymes in three study groups during calcium supplementation

Duration of Ca supplementation	Control (n=8)		Osteopenia (n=15)		Osteoporosis (n=7)	
	0 months	3 months	0 months	3 months	0 months	3 months
GPx (μmol NADPH/g Hb/min)	21.9 ± 2.2	23.1 ± 3.6	24.4 ± 1.8	21.6 ± 1.9	22.8 ± 1.2	19.4± 2.2
CAT (k/g Hb)	212.1 ± 13.9	235.8± 16.7	238.7 ± 21.2	216.3± 16.5	187.4 ± 31.2	241.8±38.4
SOD (U/mg Hb)	1.1±0.1	1.2±0.2	1.1±0.1	1.1±0.1	0.9±0.1	1.1±0.2

Data are expressed as means ± SE (minimum – maximum).

Table 5. The independent contribution of each individual variable to enzymes antioxidants and bone mineral density by multivariate linear regression analyses

	ß	Student`s *t*-test	*p*-value
Square root of bone mineral density femur (g/cm^2)			
Years since menopause (years)	-0.38	-2.14	0.04
Dairy products intake(units/week)	0.20	1.14	0.26
Bone mineral density L1-L4 (g/cm^2)			
Years since menopause (years)	-0.50	-3.00	0.006
Dairy products intake (units/week)	0.30	1.82	0.08

5 DISCUSSÃO

O chumbo é quimicamente semelhante ao cálcio, podendo mimetizar a ação deste em muitos processos fisiológicos (GODWIN *et al*., 2001). Pode substituí-lo nos ossos, onde apresenta uma meia vida de aproximadamente 27 anos (WHO, 1995) e pode retornar a circulação em casos de desmineralização óssea, tal como a menopausa (HERNANDEZ-AVILA *et al*., 2000). O uso de suplementos de cálcio parece reduzir a absorção gastrintestinal de chumbo e limitar os níveis sanguíneos desse metal na pós-menopausa (WEYERMANN & BRENNER, 1998). No entanto, trabalhos realizados nos Estados Unidos revelaram a presença de níveis preocupantes de chumbo em suplementos de cálcio (ROSS *et al*., 2000; SCELFO & FLEGAL, 2000) e, além disso, não foram encontrados trabalhos avaliando os níveis de chumbo em suplementos comercializados no Brasil.

No presente estudo determinou-se a quantidade de chumbo presente nos suplementos de cálcio comercializados no Brasil e constatou-se que a quantidade de chumbo nestes variou bastante em função do tipo de matéria prima utilizada. Por exemplo, os suplementos de cálcio de ostra industrializados apresentaram valores de chumbo de 0,91 a 2,0 µg por grama de cálcio, enquanto os suplementos de cálcio de ostra fabricados em farmácias de manipulação apresentaram 0,64 a 1,90 µg de chumbo por grama de cálcio. O carbonato de cálcio industrializado teve o limite inferior abaixo do limite de detecção do método (0,02 µg.g-1) e o limite superior foi de 1,72 µg de chumbo por grama de cálcio; já o carbonato de cálcio manipulado apresentou uma variação de 0,55 a 1,81 µg de chumbo por grama de cálcio. Os suplementos a base de cálcio de ossos apresentaram quantidade de chumbo inferior ao limite de detecção do método, enquanto aqueles a base de dolomita apresentaram uma faixa de 1,70 a 4,15 µg de chumbo por grama de cálcio. Além disso, 8 das 23 amostras avaliadas excederam os limites de chumbo permitidos no estado da Califórnia (EUA; 1,5 µg. g^{-1} de cálcio), quando o cálculo foi baseado na quantidade de cálcio especificado na bula do produto, e quando esse cálculo foi

ajustado para quantidade de cálcio determinada por espectrometria de absorção atômica, 11 das 23 amostras avaliadas excederam os limites de chumbo permitidos no estado da Califórnia. No entanto, nenhuma das amostras avaliadas excedeu os limites da Federação dos Estados Unidos (7,5 µg. g^{-1} de cálcio).

No Brasil não existe legislação específica que regulamente os níveis de chumbo em suplementos de cálcio. A Farmacopéia Brasileira estabelece que o teor máximo de metais pesados em carbonato de cálcio utilizado em insumos farmacêuticos e medicamentos pode ser de 0,002%. Considerando a hipótese de que o chumbo seja o único metal pesado presente nesses suplementos de cálcio comercializados no Brasil, nós poderíamos ingerir uma média de 50 µg de chumbo por grama de cálcio, ao consumirmos tais suplementos.

Em um estudo transversal avaliamos o efeito da suplementação de cálcio e da doença óssea sobre a atividade de enzimas antioxidantes em mulheres na pós-menopausa. A atividade das enzimas SOD, CAT e GPx não foi afetada pela suplementação de cálcio. Nós também não encontramos nenhum estudo prévio que tenha avaliado o efeito da suplementação de cálcio sobre a atividade da SOD, CAT e GPx. Mas, um estudo recente mostrou diminuição na atividade da CAT em mulheres pós-menopáusicas com osteoporose quando comparadas ao grupo controle (OZGOCMEN *et al*., 2007). A doença óssea não afetou a atividade da CAT ou da SOD. Nossos resultados em relação a atividade da SOD estão de acordo com alguns estudos prévios (YALIN *et al*., 2006; OZGOCMEN *et al*., 2007), mas contrastam com dois outros estudos, que encontraram diminuição na atividade da SOD e em mulheres na pós-menopausa com osteoporose (SONTAKKE & TARE, 2002; MAGGIO *et al*., 2003). Encontramos um significativo aumento na atividade da GPx no grupo com osteopenia quando comparado ao grupo sem doença óssea. Esse aumento na atividade da GPx em mulheres pós-menopáusicas com osteopenia é contrário a outros estudos prévios, que encontraram diminuição na atividade da GPx em mulheres pós-menopáusicas com osteoporose (SONTAKKE & TARE, 2002; MAGGIO *et al*., 2003; OZGOCMEN *et al*., 2007). Porém, esses estudos possuem algumas diferenças relevantes em relação ao nosso, a primeira diferença foi em relação ao estágio da doença óssea; e a segunda diferença, foi em relação à média de idade das participantes, que foi de aproximadamente 70 anos (MAGGIO *et al*., 2003), sendo que a média de idade das mulheres do nosso estudo foi de aproximadamente 59 anos.

A atividade enzimática da GPx é um dos mais eficientes meios de controle dos níveis de H_2O_2 (YU, 1994). Esta enzima é expressa pelos osteoclastos, ao quais produzem quantidades relativamente altas de H_2O_2 durante a reabsorção óssea (DREHER *et al*., 1998). Em humanos, a remoção do H_2O_2 é realizada pela CAT e pela GPx, mas recentemente foi comprovado que somente a GPx pode remover efetivamente os hidroperóxidos, por ser mais eficiente em proteger as células contra baixos níveis de estresse oxidativo (MATÉS *et al*., 1999). Nosso achado contrasta com vários estudos prévios, que encontraram diminuição na atividade da enzima GPx em pacientes com osteoporose (MAGGIO *et al*., 2003; SONTAKKE & TARE, 2002; OZGOCMEN *et al*., 2007).

Em outro estudo transversal nós também avaliamos se a doença óssea pode contribuir para o aumento dos níveis de chumbo e com isso alterar a atividade da δ-ALAD em mulheres na pós-menopausa não expostas ocupacionalmente ao chumbo. Verificamos que a atividade da δ-ALAD e o índice de reativação da δ-ALAD não foram diferentes entre os grupos com e sem doença óssea. Sabe-se que a δ-ALAD é a principal proteína responsável pela ligação de chumbo nos eritrócitos (BERGDAHL *et al*., 1997) e que, em humanos, ela sofre inibição a partir de concentrações sanguíneas de chumbo superiores a 5 $\mu g.dL^{-1}$ (CAMPAGNA et al., 1999). A média dos níveis de chumbo sanguíneo das mulheres do nosso estudo foi de 3,7 $\mu g.dL^{-1}$, o que explica a ausência de inibição na atividade da enzima δ-ALAD. Esses resultados estão de acordo com estudos anteriores (MULDOON *et al*., 1994; DONANGELO & DÓREA, 1998) e também com outros estudos realizados em mulheres entre 40 e 74 anos, que não foram expostas ao chumbo (KORRICK *et al*., 2002; NASH *et al*.,2004); os quais são plausíveis de comparação com o nosso estudo, que foi composto por mulheres com média de idade de 59 anos, que também não foram expostas ao chumbo.

A quantidade de chumbo do osso e do sangue é diretamente ligada ao grau de contaminação ambiental (JURKIEWICS *et al*., 2005). Portanto, nossos resultados indicam que a região estudada, parte central do estado do Rio Grande do Sul, apresenta baixa contaminação ambiental de chumbo. Nós encontramos uma correlação positiva entre os níveis sanguíneos de chumbo e o tempo de lactação. Esses dados estão de acordo com LATORRE *et al* (2003), que observou níveis de chumbo sanguíneo aumentados em mulheres pós-menopáusicas que amamentaram. Além disso, os anos em que as mulheres do nosso estudo

amamentaram foram os anos de maior contaminação ambiental de chumbo no Brasil, devido ao uso de chumbo tetraetila na gasolina, o qual só foi totalmente removido da gasolina na década de noventa.

As enzimas antioxidantes constituem o principal mecanismo de defesa antioxidante intracelular, pois protegem as células aeróbicas de injúrias oxidativas causadas por espécie reativas de oxigênio (EROs) (FRIDOVICH, 1978), eliminando $O_2^{\cdot -}$, H_2O_2 e hidroperóxidos (YU, 1994). Por outro lado, as EROs constituem o sistema pró-oxidante, que é formado por um grande número de moléculas quimicamente reativas e derivadas do oxigênio, como por exemplo o ânion radical superóxido ($O_2^{\cdot -}$), peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($^{\cdot}OH$) entre outros (NORDBERG & ARNÉR, 2001).

Através de um estudo prospectivo nós investigamos se a suplementação de cálcio e a doença óssea podem afetar os níveis sanguíneos de chumbo, a atividade da δ-ALAD, a reativação da δ-ALAD e a atividade de enzimas antioxidantes em mulheres na pós-menopausa não expostas ocupacionalmente ao chumbo, que foram submetidas à terapia com cálcio por três meses. Não foi observada nenhuma diferença significativa na atividade da δ-ALAD ou na reativação dessa enzima ao longo do estudo. Além disso, não houve diferença significativa nos níveis de chumbo sanguíneo entre as mulheres com ou sem doença óssea. Mas, observamos um pequeno, porém significativo aumento dos níveis de chumbo sanguíneo nas mulheres com osteopenia e osteoporose, após três meses de tratamento, sugerindo que a suplementação de cálcio pode ter contribuído para o aumento dos níveis de chumbo na corrente sanguínea das mulheres com doença óssea.

Muitos estudos têm sugerido que os níveis de chumbo sanguíneo estão mais elevados em mulheres na pós-menopausa do que em mulheres na pré-menopausa (KORRICK *et al*., 2002; LATORRE *et al*., 2003; NASH *et al*.,2004), sugerindo que o aumento na reabsorção óssea, característico e mais pronunciado no período pós-menopáusico, mas que se inicia no período pré-menopáusico, contribui com a retirada do chumbo depositado nos ossos e conseqüentemente com a elevação dos níveis de chumbo na corrente sanguínea dessas mulheres. As mulheres que participaram do nosso estudo foram todas pós-menopáusicas, sem doença óssea ou em diferentes estágios da doença óssea, assim nós não podemos concluir se o período menopáusico contribuiu com o aumento nos níveis de chumbo sanguíneo juntamente com os suplementos de cálcio.

Observamos também uma significativa diminuição na atividade da enzima fosfatase alcalina nos três grupos do estudo após os três meses de tratamento com cálcio. A fosfatase alcalina é um dos principais marcadores de formação óssea, que refletem a atividade osteoblástica e apresenta-se diminuída com a redução na atividade dos osteoblastos (STEIN & LIAN, 1993). Nosso resultado está de acordo com outros estudos, que observaram uma diminuição na atividade da fosfatase alcalina em mulheres na pós-menopausa, após o uso de suplementação de cálcio (WEISMAN & MATKOVIC, 2005). Os níveis de cálcio sanguíneo não apresentaram mudanças ao longo do tratamento com cálcio. Os níveis do cálcio sanguíneo são mantidos tanto pela absorção no intestino delgado quanto pela reabsorção dos ossos, sendo mantido sempre sob um rígido controle homeostático (COHEN & ROE, 2000). Assim, o cálcio sanguíneo não serve como um indicador da homeostase esquelética, mas sim como um bom indicador do estado de nutrição de cálcio. A terapia de cálcio contribui com o cálcio sanguíneo e ainda diminui a atividade osteoclástica, a qual está aumentada em mulheres na pós-menopausa (NORDIN,1997). Existem evidências da presença de receptores de estrogênio ligados ao transporte do cálcio no intestino humano (ARJMANDI *et al.*, 1993), o que pode explicar a diminuição da absorção do cálcio em mulheres na pós-menopausa, e o aumento da retirada deste mineral do osso para manter a homeostase do cálcio sanguíneo (GALLAGHER *et al*., 1979; 1980).

No estudo prospectivo as atividades das enzimas antioxidantes não foram diferentes entre os grupos com e sem doença óssea e também não apresentaram mudanças ao longo do tratamento com cálcio. Nós não encontramos estudos que tenham avaliado o efeito da suplementação de cálcio sobre a atividade das enzimas antioxidantes. Estudos recentes, que avaliaram o efeito da doença óssea sobre a atividade da SOD também não encontraram diferenças significativas (YALIN *et al*., 2006; OZGOCMEN *et al*., 2007), mas outros dois estudos, demonstraram diminuição na atividade da SOD e da GPx em mulheres na pós-menopausa (SONTAKKE & TARE, 2002; MAGGIO *et al*., 2003).

Em ambos os estudos, transversal e prospectivo os grupos osteopenia e osteoporose tiveram menor DMO L1-L4 e femoral quando comparados ao grupo controle ($p<0,05$). Além disso, o grupo osteoporose teve significativamente menor DMO L1-L4 quando comparado ao grupo osteopenia. Está estabelecido que a DMO sofre progressiva diminuição com o avanço da idade (SILBERGELD & FLAWS,

1999). Os resultados do estudo prospectivo confirmaram essa afirmativa através de uma correlação negativa entre a DMO e a idade e também mostraram uma correlação positiva entre a DMO e a terapia de reposição hormonal. A acelerada perda de massa óssea tem como causa principal a reabsorção óssea aumentada (atividade osteoclástica) que ultrapassa os limites e fica em desequilíbrio com a osteogênese (atividade osteoblástica) (TURNER *et al.*, 1994). Estudos demonstram que a ação do estrogênio seria mediada por receptores de estrogênio presente nos osteoblastos, implicando na sua ação sobre o metabolismo ósseo (MANO *et al.*, 1996; MITRA *et al.*, 2006).

Os resultados do estudo transversal indicaram que a reabsorção óssea, característica em mulheres na pós-menopausa associada à osteopenia/osteoporose não representa um risco à saúde para essas mulheres no que diz respeito à toxicidade do chumbo. Mas, por outro lado, a suplementação de cálcio, que normalmente está presente, tanto na prevenção como no tratamento de doenças ósseas, pode representar um risco à saúde de mulheres, não só na pós-menopausa, como para mulheres em diferentes etapas da vida, onde o uso de suplementos de cálcio venha a ser recomendado, pois através dos nossos resultados podemos constatar que os suplementos de cálcio atuam como uma fonte de baixos níveis de chumbo, mas que ao longo da vida e com o prolongamento do seu uso pode vir a exercer efeitos deletérios, principalmente em mulheres na pós-menopausa. Sabe-se que esse metal pesado inibe mecanismos importantes para a prevenção da doença óssea, como a ativação da vitamina D, a diferenciação osteoblástica e a ação de hormônios responsáveis pela homeostase do cálcio.

É importante ressaltar que a absorção desse metal sofre influência de fatores como concentração e tempo de exposição (SCHIFER *et al*., 2005), dieta e estado nutricional do organismo (MIDIO & MARTINS, 2000), pois a baixa ingestão de cálcio, fósforo e ferro podem provocar aumento na absorção de chumbo (PAOLIELLO & CHASIN, 2001), o que ressalta a importância do uso de suplementos de cálcio. Por esse motivo, e considerando ainda a importância dos suplementos de cálcio na prevenção e tratamento de doenças ósseas, faz-se necessário a intervenção dos órgãos responsáveis pela saúde pública, no que diz respeito à regulamentação dos níveis máximos permitidos de chumbo em suplementos de cálcio, através de uma legislação específica.

6 CONCLUSÕES

Os resultados do presente trabalho indicam que:

⇛ Embora a média dos níveis de chumbo encontrados nas amostras analisadas tenha sido inferior aos limites estabelecidos para suplementos de cálcio nos Estados Unidos, pode ser aconselhável o estabelecimento de limites específicos de chumbo para suplementos de cálcio no Brasil, assim como um programa de monitoramento de tais níveis.

⇛ O aumento observado na atividade da enzima GPx em mulheres pós-menopáusicas com osteopenia, pode ser interpretado como um esforço contra a super produção de EROs, e essa mudança não foi prevenida pela suplementação de cálcio.

⇛ A osteopenia e a osteoporose não contribuíram para o aumento dos níveis de chumbo ou alteração de indicadores bioquímicos de exposição ao chumbo em mulheres pós-menopáusicas expostas a baixos níveis desse metal e os nossos resultados indicam que a região estudada, parte central do estado do Rio Grande do Sul, apresenta baixa contaminação ambiental de chumbo.

⇛ A terapia de três meses de suplementação de cálcio contribuiu para o aumento dos níveis de chumbo na corrente sanguínea de mulheres na pós-menopausa expostas a baixos níveis desse metal, atuando como uma possível fonte de chumbo, e o que reforça a necessidade da regulamentação de uma legislação específica para os níveis permitidos de chumbo em suplementos de cálcio no Brasil.

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8 ANEXOS

8.1 ANEXO 1

Questionário aplicado às mulheres participantes deste estudo

Este questionário foi aplicado por um pesquisador treinado que explicava às pacientes cada uma das questões.

FICHA DA PACIENTE

CÓDIGO DE IDENTIFICAÇÃO:_____

Nome: _____

Endereço: _____

Cidade: _____

Estado: _____ **CEP:** _____

Telefone:_____

1. Data de nascimento: _____

2. Raça: ( ) negra ( ) branca ( ) amarela

3. Menstruação: ( ) normal ( ) irregular ( ) menopausa – Há quanto tempo?_____

4. Histórico obstétrico (filhos): _____

5. Tempo de aleitamento: _____

6. Pressão arterial: _____

7. Fumante: ( ) Sim ( ) Não nº de cigarros por dia? _____

8. Consumo de bebida alcoólica: ( ) Sim ( ) Não nº de doses por semana?____

9. Prática de exercício físico: ( ) Não Faz ( ) Leve ( ) Moderado ( ) Intenso

10. Doenças diagnosticadas: ( ) já tratadas ( ) em tratamento

( ) diabetes. Qual? _____

( ) doenças endócrinas (Tireóide). Qual (s)?_____

( ) doenças renais. Qual (s)? _____

( ) doenças da medula óssea. Qual (s)? _____

( ) doenças cardíacas /ou vasculares (varizes). Qual (s)? _____

( ) doenças pulmonares. Qual (s)? _____

( ) outras doenças. Qual (s)? _____

11. Medicações em uso :

12. Terapia de Reposição com Hormônios: ( ) Sim ( ) Não

Há quanto tempo? _____

13. Usa Cálcio (suplemento): ( ) Sim ( ) Não Quanto tempo? _____

Tipos de Cálcio:

( ) industrializado

( ) industrializado com vitamina D

( )manipulado

( ) manipulado com vitamina D

14. Usa ou usou Corticóides: ( ) Sim ( ) Não Quanto tempo? _____

15. Após os 45 anos já sofreu algum tipo de fratura em algum dos locais abaixo citados:

( ) quadril ( ) coluna vertebral ( ) fêmur ( ) punho

16. Consome regularmente algum (s) desses alimentos abaixo citados? Com que freqüência?

( ) Leite (n° copos por dia): _____

( ) Leite em pó (nº de colheres por dia): _____

( ) Iogurte (unidades por dia): _____

( ) Queijo (nº fatias por dia): _____

( ) Peixes (sardinha, linguado...) (porções por semana): _____

( ) Vegetais verdes escuros (porções por dia): _____

( ) Amêndoas (unidades por semana): _____

( ) Soja (porções por semana): _____

( ) Melado (n° colheres por dia): _____

Avaliações antropométricas (para cálculo do índice de massa corporal – IMC):

17. Peso: _____

18. Altura: _____