UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
Instituto de Ciências Básicas da Saúde
Departamento de Bioquímica Prof. Tuiskon Dick
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Efeito redox ativo da vitamina D em coração, fígado e cérebro de ratos

Aline Longoni dos Santos

Dissertação apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica, da Universidade Federal do Rio Grande do Sul, como requisito para obtenção do título de Mestre em Ciências Biológicas: Bioquímica.

Porto Alegre, 28 de fevereiro de 2013

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Orientadora: Profª Drª Cristiane Matté

Coorientador: Dr. Adriano Martimbianco de Assis

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Banca Examinadora:

1. Prof. Dr. Carlos Severo Dutra-Filho - Membro do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, ICBS/UFRGS (relator);
2. Profª Drª Rosane Gomez – Departamento de Farmacologia, ICBS/UFRGS;
3. Profª Drª Ana Paula Horn -Instituto de Ciências Biológicas – Morfologia, Universidade Federal de Rio Grande (FURG).

Suplente:

Profª Drª Christianne Gazzana Salbego - Membro do Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, ICBS/UFRGS

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“Às vezes quando a gente ganha, a gente perde. Às vezes quando a gente perde, a gente ganha.”

Um amor além da vida

Sumário

RESUMO

A vitamina D é um pró-hormônio que, além de desempenhar um papel essencial na homeostase do cálcio e do metabolismo mineral ósseo, recentemente tem sido relacionada a uma grande variedade de funções biológicas, tais como a regulação da proliferação e da diferenciação celular, a modulação imunológica, o desenvolvimento cerebral e a redução do risco de desenvolvimento de alterações cardiovasculares. Por outro lado, existe atualmente um descontrole na utilização de suplementação vitamínica, incluindo de vitamina D, o que pode levar a intoxicações, cujos efeitos celulares ainda são pouco estudados. Nesse sentido, o objetivo deste estudo foi verificar o efeito de diferentes doses e concentrações de colecalciferol e calcitriol em estudos *in vivo* e *in vitro*, respectivamente, sobre parâmetros de estresse oxidativo em coração, fígado e cérebro de ratos. Inicialmente, verificamos se a administração sistêmica de vitamina D, por 21 dias, afeta as defesas antioxidantes e os parâmetros de dano oxidativo em ratos Wistar. Foi utilizada suplementação de vitamina D3 em uma dose limítrofe e uma dose suprafisiológica (5.000 ou 30.000 UI/Kg/dia, respectivamente). Nossos dados demonstram que a suplementação de vitamina D3 possui efeitos tóxicos quando administrada em doses suprafisiológicas, e parece ser segura quando em doses limítrofes. O músculo cardíaco apresentou apenas uma redução dos produtos de lipoperoxidação em ratos tratados com a dose de 30.000 UI/Kg/dia, enquanto a menor dose não alterou nenhum dos parâmetros avaliados. O fígado apresentou um aumento nos níveis de carbonilas em ambas às concentrações, bem como um desequilíbrio na atividade antioxidante enzimática em animais tratados com a dose mais alta. O cérebro, especialmente o córtex, foi afetado em animais tratados com a dose de 30.000 UI/Kg/dia, apresentando um desequilíbrio nas defesas antioxidantes, apesar de termos verificado uma redução na oxidação de lipídeos e proteínas em córtex e hipocampo. Nosso próximo objetivo foi detectar a contribuição do 1α,25$(OH)_2$D, o metabólito mais ativo da vitamina D, sobre as alterações causadas pela administração da vitamina D no status redox dos mesmos tecidos. Foi avaliado o efeito da incubação, à 37ºC em banho metabólico, de fatias de coração, fígado, córtex cerebral e hipocampo provenientes de ratos saudáveis, em meio contendo calcitriol (1α,25$(OH)_2$D) nas concentrações de 50-1.000 nM. Nossos resultados mostraram, que mesmo altas concentrações de 1α,25$(OH)_2$D protegem o tecido cardíaco do dano oxidativo e incrementam o status antioxidante, enquanto o fígado foi pouco afetado. O cérebro sofreu um significante desequilíbrio nos parâmetros de estado redox, evidenciadas por alterações oxidativas em lipídeos e na modulação da atividade de enzimas antioxidantes. Nossos dados permitem concluir que a 1α,25$(OH)_2$D modula o estado redox de forma diferente no cérebro, fígado e coração. Em adição, os efeitos verificados *in vivo*, por meio da administração da vitamina D3, são conflitantes quando comparados com os dados obtidos pela ação direta do calcitriol, indicando que possivelmente os efeitos sistêmicos da vitamina D não são atribuídos apenas ao 1α,25$(OH)_2$D, e podem ser o resultado de um efeito combinado dos metabólitos da vitamina D. Os achados aqui expostos trazem um importante alerta em relação à suplementação com vitamina D, que em altas doses pode causar alterações oxidativas em cérebro e fígado.

ABSTRACT

Vitamin D is a prohormone that plays an essential role in calcium homeostasis and bone mineral metabolism, however, is now recognized as part of a wide variety of biological functions, such as regulating cell differentiation and proliferation, modulating immune system and brain development, as well as reducing the risk of cardiovascular diseases. Nowadays, the use of vitamin supplementation is unmanageability, which can lead to poisoning, whose cellular effects are still poorly understood. Accordingly, the objective of this study was to investigate the effect of different doses and concentrations of cholecalciferol and calcitriol (1α,25$(OH)_2$D) in experimental models *in vivo* and *in vitro*, respectively, on parameters of oxidative stress in heart, liver, and brain of rats. Initially, we determine whether systemic administration of vitamin D, for 21 days, affects antioxidant defenses and oxidative damage parameters in treated-animal tissues. We used vitamin D3 supplementation in a borderline dose and in a supraphysiological doses (5,000 or 30,000 IU/kg/day, respectively). Our data demonstrate that supplementation with vitamin D3 have toxic effects when given in supraphysiological doses, and appears to be safe when the lowest doses were adopted. The cardiac muscle showed only a reduction in lipid peroxidation products, in rats treated with a dose of 30,000 IU/kg/day, while the administration of 5,000 IU/kg/day did not change any of the parameters evaluated. The liver showed increased levels of carbonyls at both concentrations, as well as an imbalance in the antioxidant enzymes network, in animals treated with the highest dose. The brain, especially the cortex, was affected in animals treated with the dose of 30000 IU/Kg/day, an imbalance in the antioxidant defenses was found, although we have observed a reduction in oxidation of lipids and proteins in cortex and hippocampus. Our next goal was to detect the contribution of1α,25$(OH)_2$D, the more active metabolite of vitamin D, on the metabolic changes caused by vitamin D administration on the redox status of heart, liver, and brain. We evaluated the effect of incubation (37 ° C in a metabolic water bath) of tissue slices from heart, liver, cerebral cortex, and hippocampus obtained from healthy rats in a medium containing calcitriol at a final concentrations of 50-1000 nM. Our results showed that even high concentrations of 1α,25$(OH)_2$D protected cardiac muscle from oxidative damage and increased the antioxidant status, while the liver was unaffected. The brain has suffered a significant imbalance in the redox state parameters, evidenced by oxidative changes in lipids and the modulations of antioxidant enzymes activities. In conclusion, our data shows that 1α,25$(OH)_2$D modulates the redox state differently in the brain, liver and heart. In addition, the effects observed in vivo, by administration of vitamin D3, are conflicting when compared with results obtained by the direct action of the vitamin most active metabolite, calcitriol, possibly indicating that the systemic effects of vitamin D are not allocated only to calcitriol, and may be the result of the combined effect of vitamin D metabolites. The findings shown here bring us an important warning, regarding to vitamin D supplementation, which in high doses can cause oxidative changes in brain and liver.

LISTA DE ABREVIATURAS

BHE - barreira hematoencefálica

CAT – catalase

DBP – proteína ligadora de vitamina D

DCFH – 2′,7′-diclorofluoresceína

DRI – recomendações de ingestão diária

ERO – espécies reativas de oxigênio

ERN – espécies reativas de nitrogênio

GPx – glutationa peroxidase

GSH – glutationa

NO – óxido nítrico

NOS – óxido nítrico sintase

H_2O_2 – peróxido de hidrogênio

$1,24,25(OH)_3D$ – 1,24,25-triidroxivitamina D

$1\alpha,25(OH)_2D$ – 1α,25-diidroxivitamina D

$1,25(OH)_2D$ – 1,25-diidroxivitamina D

1α-OHase – 1α-hidroxilase

24-OHase – 24-hidroxilase

25-OHase – 25-hidroxilase

$24,25(OH)_2D$ – 24,25-diidroxivitamina D

25(OH)D– 25-hidroxivitamina D

PKA – proteína cinase A

PKC – proteína cinase C

PLC – fosfolipase C

PTH – paratormônio

RXR – receptor X retinoico

SNC – sistema nervoso central

SOD – superóxido dismutase

TBARS – espécies reativas ao ácido tiobarbitúrico

UVB – radiação ultravioleta B

VDR – receptor de vitamina D

VDRE – elemento de resposta à vitamina D

VDRm – receptor de vitamina D de membrana

VDRn – receptor de vitamina D nuclear

1. INTRODUÇÃO

1.1. Vitamina D

A vitamina D é um composto lipossolúvel essencial para manter o equilíbrio nos níveis plasmáticos de cálcio e fosfato do organismo, sendo considerado um hormônio esteroide. Como podemos observar na Figura 1, a sua forma ativa pode ser produzida endogenamente. Nessa via, o 7-desidrocolesterol localizado na pele é convertido em vitamina D_3(colecalciferol), por ação da radiação ultravioleta B (UVB) e do calor. A excessiva exposição ao sol não causa hipervitaminose, em função do controle do metabolismo do colecalciferol e da degradação do 7-desidrocolesterol em excesso (Haddad et al., 1993; Lips 2001). Por volta de 80% da vitamina D é obtida através da via endógena, dependente da exposição aos raios ultravioletas, e apenas 20% das necessidades corporais diárias são supridas pela alimentação. Este fato diferencia a vitamina D das demais vitaminas, cuja principal fonte é a dieta (Huotari e Herzig 2008).

A vitamina D obtida da dieta é suprida pela ingestão de nutrientes de origem animal (vitamina D3 ou colecalciferol), tais como peixes de água salgada e gema de ovo, ou de origem vegetal (vitamina D2 ou ergocalciferol), tais como cereais. A vitamina da dieta é absorvida no intestino e incorporada aos quilomícrons, sendo transportada pelo sistema linfático até a circulação venosa, alcançando o fígado (Hollis 2005; Holick 2006).

O ergocalciferol e o colecalciferol são transportados no plasma por uma proteína ligadora de vitamina D (DBP) até o fígado, onde são hidroxilados pela enzima 25-hidroxilase (25-OHase), produzindo 25-hidroxivitamina D (25(OH)D) (Figura 2). Em seguida, a enzima 1α-hidroxilase (1α-OHase) converte o

25(OH)D em 1,25-diidroxivitamina D (1,25$(OH)_2$D ou calcitriol), que é a forma mais ativa da vitamina. Essa reação ocorre principalmente nos rins, apesar da presença dessa enzima em diversos tecidos. Além disso, os rins produzem uma forma considerada inativa da vitamina D, o 24,25-diidroxivitamina D (24,25$(OH)_2$D), por ação da enzima 24-hidroxilase (24-OHase) (Deeb et al., 2007).

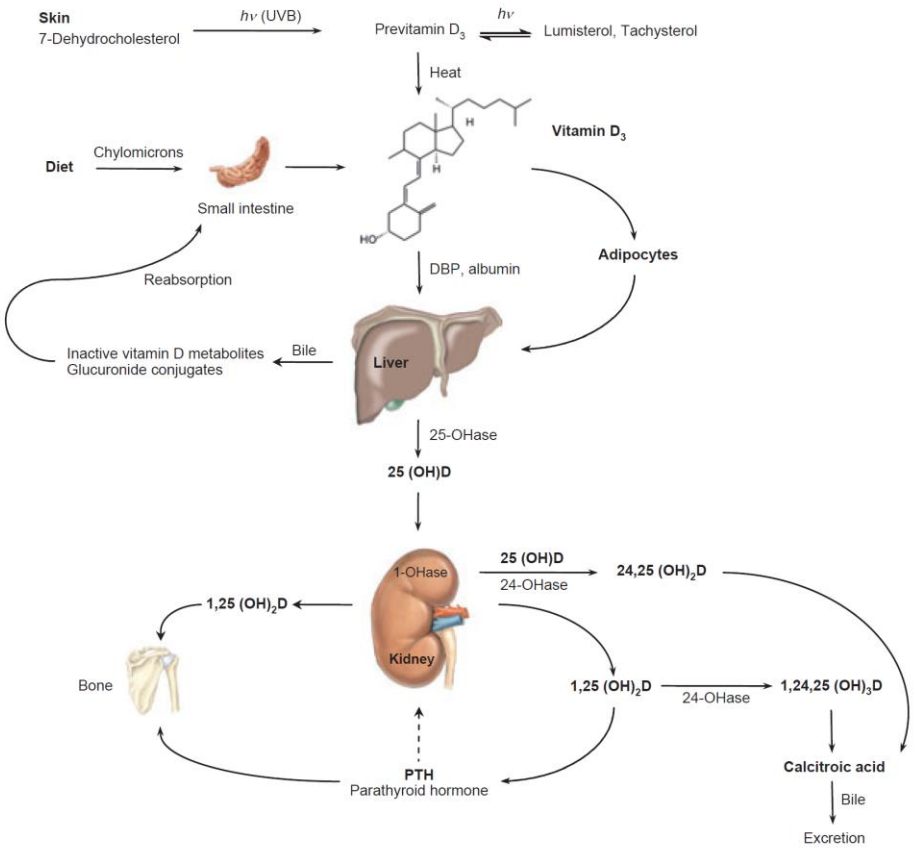

Figura 1. Metabolismo da vitamina D (Stokes et al., 2013). DBP: proteína ligadora de vitamina D; 1,24,25$(OH)_3$D: 1,24,25-triidroxivitamina D; 1,25$(OH)_2$D: 1,25-diidroxivitamina D; 24,25$(OH)_2$D: 24,25-diidroxivitamina D; 25(OH)D: 25-hidroxivitamina D; 24-OHase: 24-hidroxilase.25-OHase: 25-hidroxilase; UVB: radiação ultravioleta B

O calcitriol liberado liga-se ao receptor de vitamina D (VDR), amplamente distribuído no organismo, onde estimula a síntese de uma gama de proteínas relacionadas à regulação do metabolismo de cálcio e fósforo, modula a atividade do sistema imune, a síntese e secreção de insulina, a

proliferação e diferenciação celular, apoptose e angiogênese (Nagpal e col. 2005). Sendo capaz de diminuir a proliferação celular, tanto em células normais, como em células cancerosas e induzir a diferenciação celular terminal. Este fator seria a possível explicação para a diminuição do risco de morte em pacientes com câncer que apresentam níveis altos de 25(OH)D (Hollis 2005).

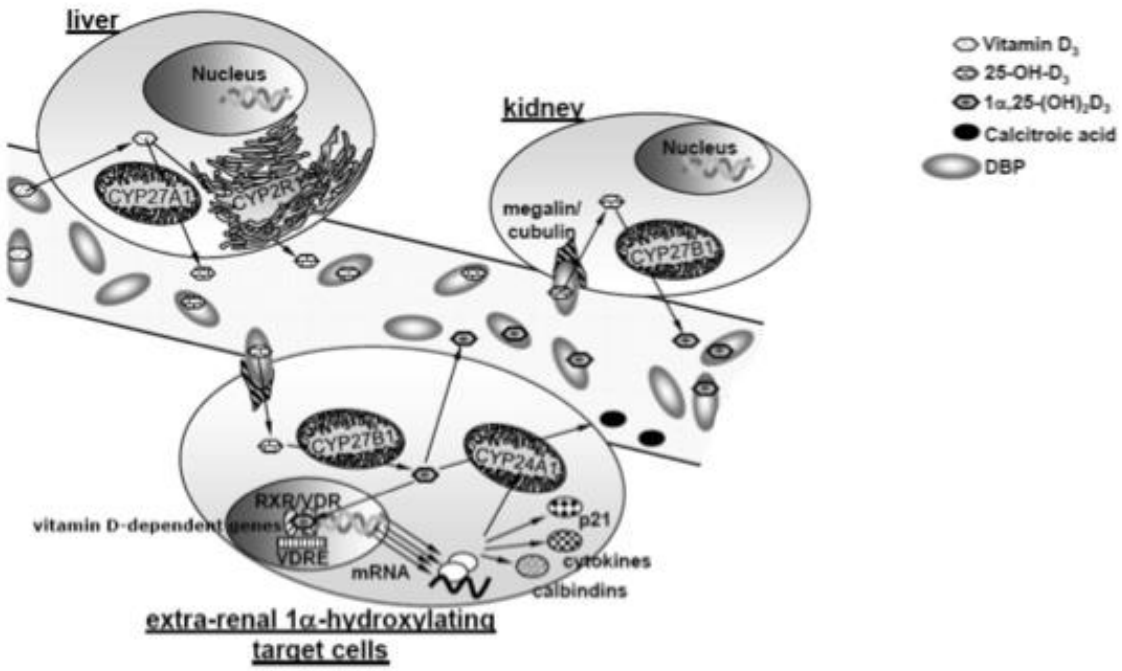

Figura 2. Mecanismo de ação da vitamina D (Jones, 2010). DBP: proteína ligadora de vitamina D; 1α,25(OH)$_2$D: 1α,25-diidroxivitamina D; 25(OH)D: 25-hidroxivitamina D; CYP27B1 (1α-OHase): 1α-hidroxilase;CYP24A1 (24-OHase): 24-hidroxilase;CYP2R1 e CYP27A1 (25-OHase): 25-hidroxilases RXR: receptor X retinoico; VDR: receptor de vitamina D; VDRE: elemento de resposta à vitamina D

A fim de manter em equilíbrio os níveis de cálcio e fósforo plasmáticos, a vitamina D, em conjunto com o PTH, age regulando a absorção intestinal e a eliminação e reabsorção renal desses minerais, bem como modulando a mineralização óssea (Fu et al., 2009).

A produção desta vitamina é regulada pelos níveis de paratormônio (PTH), cálcio e fósforo plasmáticos. Considerando que a principal ação

da1α,25$(OH)_2$D é manter os níveis de cálcio e de fósforo normais no plasma, a hipocalcemia e a hipofosfatemia atuam de forma direta aumentando a atividade da enzima 1α-OHase, bem como de forma indireta, aumentando a secreção de PTH (Holick 2007).A síntese de calcitriol pelos rins também pode ser regulada por feedback negativo, onde o calcitriol se liga ao VDR e promove uma inibição na síntese de 1α-OHase renal e na secreção de PTH pela glândula paratireoide. O acúmulo de 25(OH)D no fígado e no tecido adiposo, estimula a conversão desse em 24,25$(OH)_2$D por ação da enzima 24-OHase renal, que é excretado pelos rins (Galea e Blundell, 2011).

1.1.1. Deficiência de Vitamina D

A principal causa de raquitismo em crianças e de osteomalácia em adultos é a deficiência de vitamina D. Ainda, essa deficiência pode causar maior risco de fraturas, osteoporose, fraqueza muscular, exacerbação da osteopenia e aumento no risco de doenças cardiovasculares (Artaza et al., 2009). A tabela 1 resume os fatores associados à deficiência de vitamina D, entre eles encontramos alterações no metabolismo da vitamina, síntese inadequada de 7-desidrocolesterol, pouca exposição solar, deficiência dietética, diminuição na absorção de vitamina D lipossolúvel ou ainda resistência do órgão alvo ao 1α,25$(OH)_2$D (Holick 2006b). Os níveis dessa vitamina ainda podem ser afetados pelo uso de suplementos alimentares (aumento), medicamentos que interferem nos níveis séricos de vitamina D (redução), assim como por doenças que afetam o trato gastrointestinal, fígado, rins e pele.

Não existe um consenso em relação aos níveis ótimos de vitamina D, medidos pela concentração plasmática de 25(OH)D Especialistas utilizam o

limite de 20 ng/mL para definir o diagnóstico de hipovitaminose, enquanto níveis entre 20 ng/mL e 30 ng/mL são considerados como insuficientes, e acima de 30 ng/mL (75-80 nM), suficientes (Vieth et al., 2007; Huotari e Herzig, 2008). Por outro lado, Lips (2001) definiu como hipovitaminose D uma concentração de 25(OH)D menor que 20 ng/mL e propôs que a deficiência desta vitamina fosse considerada leve quando apresentasse valores entre 10 e 20 ng/mL, moderada entre 5 e 10 ng/mL e grave quando<5 ng/mL.

Tabela 1. Fatores de risco para hipovitaminose D (adaptado de Premaor, 2008)

Pouca exposição à luz UVB
Uso excessivo de roupas
Países de pouca insolação (alta altitude)
Pouca penetração da luz UVB durante o inverno na atmosfera
Uso de bloqueadores solares
Confinamento em locais onde não há exposição à luz UVB
Diminuição da capacidade de sintetizar vitamina D pela pele
Envelhecimento
Fototipo (?)
Raça amarela
Doenças que alteram o metabolismo da 25-hidroxivitamina D ou 1,25-diidroxivitamina D
Fibrose cística
Doenças do trato gastrointestinal
Doenças hematológicas
Doenças renais
Insuficiências cardíaca
Imobilização

A hipovitaminose D é mais frequente do que se imagina, principalmente na população idosa e de países nórdicos (Holick 2006). Recentemente, Santos et al. (2012) mostraram que uma população saudável de mulheres jovens do sul do Brasil, com idades entre 7 e 18 anos, apresentou uma prevalência de 54,3% de insuficiência nos níveis de 25(OH)D (20-29 ng/mL), enquanto 36,3% apresentam deficiência (<20 ng/mL), estando associada a três polimorfismos do gene do VDR. Em estudo transversal com médicos residentes do hospital

de clinicas de Porto Alegre, Premaor et al. (2008) descreveram que a média dos níveis séricos de 25(OH)D foi de 17.9 ng/mL, e 57,4% dos participantes apresentaram níveis abaixo de 20ng/mL.

Um dos tratamentos indicado para a hipovitaminose consiste na exposição dos braços e pernas ao sol, fonte de calor e UVB, durante cinco a trinta minutos, dependendo do horário do dia, da estação do ano e da latitude, entre dez horas da amanhã e três horas da tarde duas vezes por semana (Holick, 2006). Como existe o risco de desenvolvimento de câncer de pele associado à exposição solar nesses horários, o uso de suplementação de vitamina D via oral é mais aceito pelos especialistas.

1.1.2. Excesso de Vitamina D

Os dados da literatura apresentam informações conflitantes e pouco claras sobre a toxicidade de altas dosagens de vitamina D. A *American Academy of Pediatrics* caracteriza como hipervitaminose níveis acima de 250 nmol/L, enquanto concentrações de 25(OH)D plasmáticos que alcançam 700 nmol/L ocorrem em casos de alta ingesta dessa vitamina, cujos pacientes apresentam como principais sintomas relatados hipercalcemia, hipercalciúria, calcificações em tecidos moles e alterações metabólicas e cognitivas secundárias (Hathcock et al., 2007), bem como aumento do risco de desenvolvimento de doenças alérgicas (Back et al., 2009). A ingestão de 100 UI/dia promove um aumento em torno de 2,5 nmol/L nos níveis sanguíneos dessa vitamina (Heaney, 2008).

A ingestão de vitamina D em doses acima de 250 µg ou 10.000 UI/dia (1 µg de colecalciferol = 40 UI) podem levar à hipervitaminose. Intoxicação pode ocorrer em decorrência do uso desta substância em altas doses como

suplemento vitamínico, como tratamento da deficiência de vitamina D, e ainda em animais que se alimentam com plantas que contenham substâncias carcinogênicas análogas a vitamina D, como *Nierembergia veitchii*, *Solanum torvum*,*Cestrum diurnum* e *Trisetum flavescens* (Smith et al., 2008; Peterlik et al., 1977).Em humanos,Down et al.(1979), descreveram a intoxicação por vitamina D em uma família que ingeriu alimentos cozidos com o óleo de nozes contendo 5 milhões de UI de vitamina D3/mL. Todos apresentaram sintomas de hipercalcemia. Os níveis plasmáticos do pai e da mãe foram, respectivamente, 55 e 60 UI/mL (os valores normais oscilam de 0 a 1,6 UI/mL). Onze anos depois,os três pacientes estavam bem, mas a biopsia renal de um deles apresentou nefrocalcinose persistente. Em outro relato (Hoppe et al., 1992), quatro crianças se intoxicaram com vitamina D, por meio da ingestão de 7,5 mg de vitamina D3 por via oral, durante 4 semanas (a dose padrão para a profilaxia de raquitismo é de 400 UI/dia no primeiro ano de vida). Hipercalcemia, hipercalciuria, nefrocalcinose medular e hematúria devido à passagem de um cálculo, foram observadas. Dois lactentes (2 e 18 meses de idade), de famílias diferentes, foram intoxicados por vitamina D devido a erro na administração por seus familiares. Um recebeu 9.000.000 UI em 15 dias e o outro 4.200.000 UI em 7 dias. Os sinais clínicos foram similares, náusea, vômito, poliúria, desidratação, redução do tônus muscular, hipercalcemina, hipercalciuria e distúrbios na habilidade de concentração renal (Molina e col. 1984). Parfitt (1977), observou que uma dose de 2.100 mg (84.000 UI)/ dia durante 5 anos esta associado com a diminuição da função renal, nefrolitiase e nefrocalcinose.

1.1.3. Recomendações terapêuticas

De acordo com Cashman e Kiely (2011), é provável que as concentrações séricas de 25(OH)D entre 30 e 80 nM são capazes de impedir efeitos adversos de ambos os níveis, excessivamente altos ou baixos e irá promover a saúde ao longo da vida.

Tabela 2. Resumo das doses recomendadas de ingestão diária (DRIs;adaptado de Institute of Medicine, 1997)

200UI	Recomendado para todos até 50 anos
400UI	Recomendado para todos entre 50 e 70 anos
600UI	Recomendado para todos após 70 anos
800UI	Recomendado para prevenir osteoporose
1.000UI	Recomendado para tratamento de raquitismo
2.000UI	São doses excessivas para crianças ou otimizar os efeitos da osteoporose
3.000 – 4.000UI	Recomendado para melhorar os efeitos do raquitismo
20.000UI	Doses excessivas para adultos

Entre os órgãos oficiais não existe um consenso quanto à suplementação indicada. As recomendações de ingestão diária (DRI) do *Institute of Medicine* (1997; Tabela 2), a dose recomendada de vitamina D é de 10 mg ou 400 UI/dia para homens, mulheres e crianças. O limite superior em homens é de 20 mg ou 800 UI/dia e em mulheres é de 17,2 mg ou 686 UI/dia. A organização mundial da saúde reúne periodicamente um grupo de especialistas que reavaliam e definem as DRIs de vitaminas e minerais para humanos. De acordo com o mais recente documento desse grupo, publicado em 2001, a suplementação com vitamina D deve ser realizada com 5 mg/dia em crianças, adultos, grávidas e lactantes, enquanto idosos podem utilizar 10-15 mg. Heaney et al. (2003) demonstraram que 500 UI de vitamina D via oral

eram suficiente para manter os níveis sanguíneos de 25(OH)D em 70 nmol/L em homens durante o inverno.

1.1.4. Receptores de Vitamina D

As ações da vitamina D são mediadas pelo VDR. O VDR nuclear (VDRn) atua como um fator de transcrição, através da regulação da transcrição de diversos genes, tais como os correspondentes à calbindina e à osteopontina, por exemplo (Figura 2). O VDR é capaz de se ligar a vários metabólitos da vitamina D, mas sua maior afinidade é pelo 1,25$(OH)_2$D, o que induz uma mudança conformacional que permite ao VDR formar um heterodímero com receptor X retinoico (RXR) (Deluca e Cantorna 2001; Hibler et al., 2010). O VDRn possui um domínio do tipo dedo de zinco, que liga com uma sequência específica do DNA conhecida como elementos de resposta à vitamina D (VDRE), localizados nas regiões promotoras de genes que são ativados pela vitamina D. Logo após a ligação ao DNA, ocorre um recrutamento de outras proteínas nucleares para o complexo transcricional (Yamada et al., 2001).

O VDR de membrana (VDRm) é responsável por ações rápidas de vitamina D, e poderia ser a explicação para o aumento acelerado na absorção do cálcio pelos enterócitos, em resposta ao aumento dos níveis plasmáticos de vitamina D, segundo Premaor e Furlanetto (2006). Os efeitos rápidos da vitamina D são mediados pelas vias de sinalização mediadas pela fosfolipase C (PLC), proteína cinase A (PKA), proteína cinase C (PKC), bem como ativação de canais de cálcio (Farach-Carson e Nemere, 2003; Khanal e Nemere, 2007).

1.1.5. Ações da vitamina D no cérebro

A vitamina D está associada com a adequação da anatomia, fisiologia e função do cérebro. Estudos recentes têm demonstrado que a vitamina D possui inúmeras funções no sistema nervoso central (SNC), e não seria um equívoco nomear esta vitamina como um “neuro-esteroide”. O cérebro, incluindo o córtex cerebral e o hipocampo, expressam VDR, bem como a enzima 1α-OHase, responsável pela síntese do calcitriol (Zehnder e Hewison,1999).

Levando em conta a capacidade já demonstrada da vitamina D em elevar os níveis de dopamina no SNC, esta vem sendo estudada como um potente agente terapêutico em modelos da doença de Parkinson. Nesse sentido, Cass et al. (2006) demonstraram que o pré-tratamento com $1,25(OH)_2D$ é capaz de restaurar os níveis de dopamina em modelos experimentais onde a dopamina é encontrada depletada. Além disso, a administração de vitamina D por 8 dias restaurou a locomoção de ratos que receberam administração de 6-hidroxidopamina no feixe anterior medial cerebral (Wang et al., 2001), atuando como uma substância neuroprotetora, aumentando os níveis de dopamina, possivelmente devido ao aumento da enzima tirosina hidroxilase. Em adição, estudos in vitro mostraram que a vitamina D protege neurônios contra a toxicidade da 6-hidroxidopamina e do peróxido de hidrogênio (H_2O_2) (Wang et al., 2001). A administração sistêmica de $1,25(OH)_2D$ previne danos oxidativos no locus ceruleus causados pela administração local de ferro (Chen et al., 2003). Outro estudo recente demonstrou que, além do ferro, o zinco também pode induzir danos oxidativo na substância nigra. O zinco promoveu peroxidação lipídica e apoptose dos neurônios dopaminérgicos e reduziu a concentração de dopamina no estriado.

Contudo, estes efeitos são atenuados por um pré-tratamento com 1,25$(OH)_2$D (Lin et al., 2003).

1.1.6. Ações da vitamina D no coração e no fígado

O primeiro relato relacionando a vitamina D e as doenças cardiovasculares foi publicado em 1981 (Scragg, 1981). Neste trabalho o autor observou que um aumento à exposição aos raios ultravioletas estava associado com um menor risco para o desenvolvimento de doenças cardiovasculares. Alguns trabalhos posteriores a este, confirmaram uma ligação entre uma maior exposição à luz solar, doses adequadas de vitamina D e uma menor incidência de doenças cardiovasculares (Judd et al., 2009; Pell e Cobbe, 1999). Em adição, baixos níveis plasmáticos de 25(OH)D são um fator independente para infarto e morte súbita (Pilz et al., 2012). Recentemente, a potencia de ação da suplementação com vitamina D tem sido comparada à ação das estatinas, no que tange aos benefícios na prevenção de doenças cardiovasculares (Wilding, 2012). Acredita-se que a cardioproteção medida pela vitamina esteja relacionada à regulação de uma série de vias de sinalização, incluindo NFkB, Wnt/β-catenina e o sistema renina-angiotensina (Li, 2012).

A deficiência de vitamina D também parece estar relacionada a manifestações hepáticas, tais como osteodistrofia hepática e doenças hepáticas crônicas (cirrose e doença hepática colestática), onde os níveis plasmáticos de 25(OH)D estão inversamente correlacionados com a gravidade da patologia (Stokes et al., 2013)

1.2. Estresse Oxidativo

A produção de espécies reativas de oxigênio (ERO) e de nitrogênio (ERN) é parte integrante do metabolismo celular, desempenhando funções bioquímicas variadas, tais como quando são produzidas pelos fagócitos para eliminar um agente agressor, pelo endotélio para regular a contratilidade da musculatura lisa vascular, pelo músculo esquelético por estímulo do exercício físico e pelo SNC atuando como neurotransmissor (Halliwel e Gutteridge, 2006). O metabolismo dessas espécies reativas é controlada por sistemas antioxidantes enzimáticos e não-enzimáticos. Dentre as enzimas, destacamos a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), e dentre os não-enzimáticos, a glutationa (GSH), tocoferois, ascorbato, β-caroteno e a transferrina (Halliwel e Gutteridge, 2006).

Estresse oxidativo é um termo usado na situação onde ocorre uma formação de espécies reativas ou radicais livres, que são definidos como uma espécie química com um ou mais elétrons desemparelhados no seu último orbital; que excede a capacidade de transformação destas moléculas em outras não oxidantes por meio das defesas antioxidantes, resultando em desequilíbrio entre o sistema pró- e antioxidante (Shimizu et al., 1984; Halliwell e Gutteridge, 2006).

Em um recente estudo, foi demonstrado que a admistração de $1,25(OH)_2D$ atenua os efeitos deletérios de uma lesão aguda oxidativa no giro dentado (Goudarzvand et al., 2010). Em condições de estresse oxidativo, as concentrações fisiológicas de $1,25(OH)_2D$ podem aumentar o potencial antioxidante no cérebro, como os níveis de GSH (Chen et al., 2003). Nesse

contexto, Garcion et al. (2002) mostraram que a 1,25$(OH)_2$D pode inibir a síntese de óxido nítrico (NO) pela óxido nítrico sintase (NOS), bem como aumentar a síntese de GSH, indicando um efeito redox ativo dessa vitamina no SNC. Níveis fisiológicos de 1,25$(OH)_2$D também são capazes de bloquear a neurotoxicidade induzida por glutamato e por H_2O_2 em neurônios mesencefálicos. O mecanismo exato para este efeito ainda não foi elucidado, contudo parece que a transcrição e a síntese proteica são necessárias para esse efeito protetor, sugerindo assim uma regulação genômica mediada pela vitamina D (Ibi et al., 2001). Embora as ERO sozinhas induzam dano não especifico a membranas lipídicas, há alguns indícios que apoiam uma interação mais direta e reversível entre essas moléculas e a 1,25$(OH)_2$D. Já o NO, H_2O_2 e o peroxinitrito inibem a sinalização nuclear da vitamina D agindo, de maneira dose-dependente, diretamente na ligação entre o VDR e o seu co-receptor RXR (Kroncke et al., 2002).

2. OBJETIVOS

2.1. Objetivo Geral

A cada ano que passa novos estudos demonstram mais funções bioquímicas atribuídas à vitamina D, o que torna crucial a manutenção dos níveis séricos de vitamina D e seus metabólitos sem uma estreita janela de concentração onde possua o máximo de efeitos benéficos e o mínimo de efeitos adversos. Levando em conta esses aspectos e a ampla utilização de suplementos vitamínicos pela população de uma forma não controlada, o objetivo geral deste trabalho foi determinar o efeito de diferentes doses e concentrações de colecalciferol e de calcitriol em estudos *in vivo* e *in vitro*, respectivamente, sobre parâmetros de estresse oxidativo em coração, fígado e cérebro de ratos.

2.2. Objetivos Específicos

Inicialmente, nos estudos *in vivo*, verificamos o efeito da administração crônica, por via oral, de vitamina D3(5.000 e 30.000 UI/Kg/dia) em ratos, sobre o estado redox tecidual, por meio da avaliação de produção de espécies reativas, dano a lipídeos e proteínas, atividades das enzimas antioxidantes e níveis de GSH, em coração, fígado, hipocampo e córtex cerebral.

O objetivo dos ensaios *in vitro* foi determinar o efeito de diversas concentrações (50 a1000nM) de calcitriol, o metabólito mais ativo da vitamina D, sobre parâmetros de estresse oxidativo, tais como produção de espécies reativas, dano às biomoléculas e status antioxidante nos mesmos tecidos citados acima, a fim de definir o papel desse metabólito no efeito mediado pela administração da vitamina D sistemicamente.

3. RESULTADOS

Os resultados serão descritos em dois capítulos, correspondentes a um trabalho a ser submetido para publicação (capítulo I) e a um segundo manuscrito submetido à publicação (capítulo II).

3.1. Capítulo I: Vitamin D_3 supplementation alters redox parameters in heart, liver, and brain from adult rats

Manuscrito a ser submetido ao periódico Applied Physiology, Nutrition and Metabolism

Vitamin D_3 supplementation alters redox parameters in heart, liver, and brain from adult rats

Aline Longoni[1], Karen Yurika Kudo[2], Vinícius Stone[2], Thiago Beltram Marcelino[2], Guilhian Leipnitz[1,2], Adriano Martimbianco de Assis[1], Cristiane Matté[1,2]

[1] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90035-003, Brazil;

[2] Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90035-003, Brazil;

* Corresponding author: Cristiane Matté

Department of Biochemistry, St Ramiro Barcelos 2600 Annex,

Federal University of Rio Grande do Sul, 90035-003,

Porto Alegre, RS, Brazil.

Tel.: +55 51 3308 5548

E-mail: matte@ufrgs.br

Abstract

Vitamin D is pro-hormone which, besides playing an essential role in calcium homeostasis and bone mineral metabolism, is now recognized to sub serve a wide range of fundamental biological functions in cell proliferation and differentiation, immunological modulation, cardiovascular protection, and cerebral development. The aim of this study was to determine whether vitamin D supplementation in rats affects redox status of heart, liver and brain. Adult Wistar rats received, by gavage, during 21 days vitamin D3 in limitrophe or supraphysiological doses (5,000 or 30,000 IU/Kg/day, respectively). Twenty-four hours after the last administration, the rats were euthanized and the tissue samples dissected. Our results showed that vitamin D3 supplementation has diverse effects when administered in limitrophe or supraphysiological doses. Rats treated with 5,000 IU/Kg present beneficial effects on cerebral cortex and hippocampus, verified by a reduction on lipid peroxidation and protein oxidation; while heart was not affected, and liver presented an indicative of protein oxidation. On the other hand, daily administration of 30,000 IU/Kg promotes crucial changes in antioxidant enzymes activities, compatible with reduced reactive oxygen species elimination, in liver and cerebral cortex; although the effect on the oxidation of lipids and protein has been maintained. Our study clearly brings new data on vitamin D supplementation, and places an alert on the use of high doses of cholecalciferol as a dietary supplement.

Key words:vitamin D supplementation; redox status; antioxidant; protein oxidation; lipid peroxidation, oxidative stress

Introduction

Vitamin D, a pro-hormone liposoluble, is obtained from dietary sources or synthesized in the skin, a process ultraviolet irradiation-dependent that varies with the season of the year and geographic latitude (Dusso and Brown, 1998). The first step to metabolic activation of vitamin D is an hydroxylation of carbon 25 producing 25-hydroxyvitamin D (25(OH)D), mediated by a cytochrome P450-dependent system, which occurs mainly in the liver. The second step, which take place predominantly in kidney, is the formation of 1,25-dihydroxyvitamin D ($1\alpha,25(OH)_2D$), also known as calcitriol, by the enzyme 1α-hydroxylase (Dusso, et al. 2005; Zehnder et al. 2001).

The available evidence shows that an adequate intake of vitamin D contributes to many biological functions beyond maintenance of calcium homeostasis, preventing cardiovascular problems (Artaza et al. 2009], acting as an inflammatory response modulator, controlling insulin synthesis and release (Chagas et al. 2012), potentially playing a role in brain development, cerebral function, and neurodegeneration (Kiraly et al. 2006; Smolders et al. 2011).

Calcitriol exerts its effects via two pathways (Khanal and Nemere, 2007): the classical genomic pathway that involves interaction with nuclear vitamin D receptor (nVDR), a member of the nuclear receptor superfamily of ligand-activated transcription factors (Carlberg, 2004); and a non-genomic pathway (Haussler et al. 2011), through the binding to a membrane-associated vitamin D receptor (mVDR), which is involved with many signaling pathways (Fleet, 2004). VDR is widely distributed in many tissues, including liver, heart, and different structures of brain, as hippocampus and cerebral cortex (Pilz et al. 2012; Barchetta et al. 2012; Veenstra et al. 1998). The genomic pathway is involved

in a host of cellular processes, including the protection of cells against oxidative damage, control of cell cycle arrest, regulating cell proliferation, increasing apoptosis and promoting cell differentiation (Banakar et al. 2004). Literature is sparse in regard to the action of vitamin D on redox state. In this context, recent works have demonstrated the potential of vitamin D3 in reducing oxidative stress in different animal models and tissues (George et al. 2012; Dong et al. 2012).

Vitamin D toxicity is rare, however could be a result of supplementation with excessively high doses. The tolerable upper consumption level established in Europe and North America is 2,000 IU/day (Hathcock et al. 2007), mean while many clinical trials indicates that prolonged intake of until 10,000 IU/day of vitamin D3 probably poses no risk. Although some authors propose that a cumulative supplementation of at least 600,000 IU might be used in deficient patients (Haines and Park, 2012), doses more than 30,000 IU/day raise levels of 25(OH)D to more than 120 ng/ mL, which are associated with hypercalcemia, hyperphosphatemia, hypertension, pain, vomiting, poor mental concentration, chills, thirst, fever, anorexia, (Hathcock et al. 2007; Vieth, 2004).

Considering that literature is scarce about the redox active character of vitamin D3, the objective of this study was to evaluate the effect of vitamin D supplementation, in limitrophe and supraphysiological doses used in clinic, on redox state of heart, liver, and brain.

Material and Methods

Ethics Statement

The experiments were approved by local Ethics Commission (Comissão de Ética no Uso de Animais/Universidade Federal do Rio Grande do Sul - CEUA/UFRGS) under the number 20613, and followed the National Institute of Health “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 80-23, revised 1996). We further attest that all efforts were made to minimize the number of animals used and their suffering.

Animals

Adult Wistar rats (male, middle weight = 300g) from the Central Animal House of the Department of Biochemistry were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room temperature (22±2 °C). Rats had free access to a 20% (w/w) protein commercial chow and water. These conditions were maintained constant throughout the experiments. **Chemicals**

ADDERA D3 (cholecalciferol - vitamin D3. Farmasa, SP, Brazil) was purchased in commercial pharmacies from Porto Alegre, Brazil. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Drug preparation and treatment

Eighty-four adult male Wistar rats per experiment were treated daily during noon (i.e., when the animals are drowsy, calm, and remained so another 7 hours without an increased amount of feed intake) to ensure a maximum absorption of the vitamin. The vitamin or vehicle were administered once daily for 21 days. The vitamin D3 (ADDERA D3) was dissolved in sunflower oil in the concentrations of 5,000 IU/Kg/day and 30,000 IU/Kg/day in a final volume of

500 µL. The animals were divided into three groups as follow: group 1) control rats (n = 9) received the vehicle (sunflower oil) in a final volume of 500 uL, group 2) vitamin D 5,000 IU (n = 9) received 5000 IU/kg/day, and 3) vitamin D 30,000 IU (n = 10) received 30,000 IU/kg/day of cholecalciferol (vitamin D3) orally by gavage, using a metal stomach tube.

Sample preparation

The animals were sacrificed 24 hours after the last administration of vitamin D. Heart, liver, cerebral cortex, and hippocampus of rats were dissected on ice immediately after sacrifice and stored at -80 ˚C for subsequent analysis of biochemical parameters. The structures used to measure redox parameters were homogenized in 10 volumes (1:10, w / v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The homogenates were centrifuged at 1,000 x g for 10 min at 4°C to discard nuclei and cell debris. The pellet was discarded and the supernatant was taken for biochemical assays described herein. All results were normalized by protein content using bovine serum albumin as standard (Lowry, et al. 1951).

Hepatic Lipids

In order to measure the hepatic lipid content, frozen liver samples were thawed on ice and homogenized in de ionized water. Extraction and isolation of lipids to yield dried lipid extracts was performed using the technique described by Folch et al. (1957). The hepatic cholesterol and triglyceride content (de Assis et al. 2012) of the lipid extracts was then assayed enzymatically by commercial kits (Labtest, MG, Brazil).

Redox status parameters

Thiobarbituric acid reactive species

In order to assess the extent of lipoperoxidation, we detected thiobarbituric acid reactive species (TBARS) formation through a heated and acidic reaction. This is widely adopted as a method for the measurement of lipid redox states, as previously described (Draper and Hadley, 1990). The samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid and were then heated in a boiling water bath for 25 min. The level of TBARS was determined by measuring the absorbance of each sample at 532 nm. The concentration of TBARS in the samples was then determined from a calibration curve using 1,1,3,3-tetramethoxypropane (which had been subjected to the same treatment as the supernatants) as a standard. Results are expressed as nanomoles of TBARS per milligram of protein.

Measurement of protein carbonyl content

The oxidative damage to proteins was measured by quantifying the levels of carbonyl groups by reaction with 2,4-dinitrophenylhydrazine (DNPH). Proteins were precipitated by the addition of 20% TCA and were ressuspended in 10mM DNPH, and the absorbance of the resulting solution at 370 nm was recorded (Levine et al. 1994). Results are expressed as nmol of carbonyl per milligram of protein.

2′,7′-Dihydrodichlorofluorescein oxidation

Production of reactive oxygen/nitrogen species was measured following the method of Lebel et al. (1992), which is based on 2′,7′-dihydrodichlorofluorescein (DCFH) oxidation. 2′,7′-dihydrodichlorofluorescein-diacetate (DCFH-DA) is cleaved by cellular esterases and the DCFH formed is

eventually oxidized by reactive oxygen species (ROS) or reactive nitrogen species (RNS) present in samples. Briefly, DCFH-DA was incubated for 30 min at 37 °C with an aliquot of the supernatant obtained from tissue. Fluorescence was measured using excitation and emission wavelengths of 488 nm and 525 nm, respectively. A calibration curve was performed with standard DCF and the levels of reactive species were calculated as nmol of DCF per mg of protein.

Reduced glutathione levels

Glutathione (GSH) levels were measured according to Browne and Armstrong (1998). Tissue supernatants with approximately 0.3 mg of protein were diluted in 20 volumes (1:20, v/v) of 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation was incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol per mg of protein.

Superoxide dismutase assay

Superoxide dismutase (SOD) activity was evaluated by quantifying the inhibition superoxide-dependent autoxidation of epinephrine, verifying the absorbance of the samples at 480 nm (Misra and Fridovich 1972). In a plate with 96 wells, we added tissues homogenates (30 uL), 150 uL of 50 mM glycine buffer pH 10.2, and 10 uL of 10 uM catalase. In the auto-oxidation wells were added 180 uL of 50 mM glycine buffer pH 10.2 and 10 uL of 10 uM catalase. The reaction was initiated by adding 10 uL of 60 mM epinephrine. The absorbance was taken at 480 nm, being recorded for 10 min, at 32 °C. Units of

SOD activity was defined as the amount of enzyme required to decrease in 50% the oxidation of epinephrine by superoxide. The specific activity was expressed in units per mg of protein.

Catalase activity

Catalase (CAT) activity was measured as previously described (Aebi, 1984). The rate of decrease in absorbance at 240 nm was measured as a function of H_2O_2 degradation by catalase. Tissues homogenates (40uL) from each sample were added on 150 uL of 125 mM phosphate buffer in different wells. The reaction was initiated by adding 10 uL of H_2O_2 (0.5 mM). Blank was prepared with 190 uL of phosphate buffer and 10 uL of H_2O_2 (0.5 mM). The rate of decrease in optical density due to the degradation of H_2O_2 was measured for 3 min, at 240 nm. Units of CAT was defined as the amount of enzyme that decomposed 1 µM H_2O_2 per minute at 37 °C. The specific activity was expressed as units per mg of protein.

Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was measured according to Wendel (1981) using tert-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM ethylenediaminetetracetic acid (EDTA), pH 7.2, 2 mM GSH, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, 0.1 mM NADPH. One GPx unit is defined as 1 µmol of NADPH consumed per minute. The specific activity was calculated as unit per mg of protein.

Statistical Analyses

Data are expressed as the mean ± S.D. All analyses were performed using the Prism GraphPad 5.0 Software (San Diego, CA, USA). Results were analyzed using one-way ANOVA followed by Tukey's post hoc test, when F-test was significant. *P*-values were considered significant when $p<0.05$.

Results

Vitamin D supplementation did not affect body weight and lipid liver content

In Table 1, we can observe the effects of vitamin D_3 administration on body weight and lipid liver content. Vitamin administration during 21 days did not alter rat body weight, as well as triglyceride and cholesterol concentration in liver, when compared to control ($p>0.05$).

Effects of vitamin D supplementation on heart and liver redox status

The supplementation of vitamin D_3 (30,000 IU/kg) by 21 days induced a significant decrease ($p=0,0122$) in levels of lipid peroxidation in heart, in relation to control group (Figure 1A). Liver did not present any alteration on TBARS levels ($p>0.05$; Figure 1A), however protein carbonyl content analysis (Figure 1B) showed an increment in protein oxidation ($p=0.0010$) observed in both vitamin D dosages (5,000 and 30,000 IU/kg/day). Carbonyl levels were not altered in heart, when compared to control group ($p>0.05$; Figure 1B). Considering that reactive species could be responsible by carbonyl formation, we evaluated DCF oxidation. Our results showed no differences between groups vitamin-treated and control in this parameter in both organs ($p>0.05$; Figure 1C).

Figures 2 and 3 show the effect of vitamin D supplementation on the antioxidant status in liver and heart. The administration of limitrophe or high doses of vitamin D did not elicit any alteration on heart antioxidant network in rats treated by 21 days ($p>0.05$; Figure 2). On the other hand, liver presents a reduction of CAT ($p=0.0086$; Figure 2B) and GPx ($p=0.0018$; Figure 2C) activities, when animals were supplemented with 30,000 IU/Kg/day, while SOD

was not affected (p>0.05; Figure 2A). In addition, the administration of 5,000 IU/Kg/day, considered a secure dose, did not alter antioxidant parameters in liver (p>0.05; Figure 2). Although we have found an inhibition of CAT and GPx activities in liver of vitamin-treated rats (30,000 IU/Kg/day), we did not verify any alteration on the ratio SOD/(CAT+ GPx) (p>0.05; Figure 2D), in heart and liver of rats.

We also measured reduced GSH levels, the substrate of GPx and an important non-enzymatic antioxidant found in cells. Vitamin administration did not affect GSH levels in liver neither heart (p>0.05; Figure 3).

Effects of vitamin D supplementation on brain redox status

Administration of vitamin D3, in the doses of 5,000 and 30,000 IU/kg/day, induced a significant decrease in levels of lipid peroxidation (p=0.0002 for both structures; Figure 4A) and carbonyl levels (p=0.0018 for cerebral cortex; p=0.0053 for hippocampus; Figure 4B) in rats treated for 21 days, when compared to the control animals. We also evidenced a reduction on DCF oxidation in cerebral cortex (p=0.0005; Figure 4C), however, only in rats treated with 30,000 IU/kg/day. Hippocampal DCF oxidation in vitamin treated-rats was not different from control, in neither doses supplemented (p>0.05; Figure 4C).

In the figure 5 we could observe the results regarding antioxidant enzymes activities in cerebral cortex and hippocampus from rats treated with 5,000 or 30,000 IU of vitamin D/Kg/day. Cortical and hippocampal SOD activity was not altered by vitamin administration (p>0.05; Figure 5A), while CAT and GPx were modulated differently in each CNS structure studied. Catalase activity was reduced in cerebral cortex (p=0.0316; Figure 5B) and increased in hippocampus (p=0.0271; Figure 5B) isolated from rats supplemented with the

highest dose (30,000 IU/Kg/day). In addition, GPx was inhibited in cerebral cortex ($p=0.0005$; Figure 5C), and was not altered in hippocampus ($p>0.05$; Figure 5C). As a possible consequence of GPx modulation, we found increased levels of GSH in cerebral cortex ($p=0.0346$; Figure 6), and a concentration similar to control in hippocampus ($p>0.05$; Figure 6). Notwithstanding the supplementation with 30,000 IU of vitamin D/Kg/day has changed the activities of CAT and GPx, the ratio SOD/(CAT+ GPx) was not affected in cerebral cortex and hippocampus ($p>0.05$; Figure 5D).

Finally, it is important to emphasize that the supplementation of 5,000 IU of vitamin D/Kg/day, considered a non-toxic dose, did not result in significant changes in the antioxidant status of the CNS ($p>0.05$; Figures 5 and 6).

Discussion

In the present study we showed that vitamin D3 supplementation has diverse effects when administered in limitrophe or supraphysiological doses. Adult rats treated daily for 21 days with 5,000 IU/Kg present beneficial effects on cerebral cortex and hippocampus, verified by a reduction on lipid peroxidation and protein oxidation; while heart was not affected, and liver presented an indicative of protein oxidation. On the other hand, daily administration of 30,000 IU/Kg promotes crucial changes in antioxidant enzymes activities, compatible with reduced ROS elimination, in liver and cerebral cortex; although the effect on the oxidation of lipids and protein has been maintained. Our study clearly brings new data on vitamin D supplementation, thereby contributing to the sparse literature on the issue.

Vitamin D3 is a lipophilic molecule similar to its closely related lipid precursor cholesterol. In order to evaluate if the vitamin D3 supplementation could alter hepatic lipid content, we realize the measurement of hepatic cholesterol and triglyceride levels. Chronic administration of vitamin independent of the doses administered did not alter hepatic lipid content, as well as the increment in body weight during the treatment. These results eliminate certain biases that might interfere with the redox parameters evaluated.

Vitamin D is transported in the blood by the vitamin D binding protein (DBP) to the liver, where it is hydroxylated by a cytochrome P450-dependent system. Considering that liver possess a central role on 25(OH)D synthesis, and that hydroxylation of vitamin D could be related to superoxide and other ROS production (Hanukoglu, 2006), we evaluated the hepatic redox status of

vitamin D3 treated-rats. We have shown that 5,000 IU/Kg promotes an increase on carbonyl content, a marker of protein modification, such as glycation by sugars, binding of aldehydes formed during lipid peroxidation, or by direct oxidation by reactive species (Levine, 2002). When 30,000 IU/Kg was administered we verify a more pronounced damage effect, with protein oxidation, and inhibition of antioxidant enzymes responsible by hydrogen peroxide elimination, CAT and GPx. By Fenton reaction, the excess hydrogen peroxide might be converted into $OH^{\bullet}$, which could cause protein modification (Halliwell and Gutteridge, 2006). Interestingly, although we have observed the reduction of antioxidant enzymes and protein oxidation, we did not found any alteration on DCF oxidation, a fluorescent probe when in the presence of reactive species.

In accord to literature, one of the most widespread effects of vitamin D is the reduction of the risk of cardiovascular disease development (Salusky and Goodman, 2002; Artaza et al. 2009). Brøndum-Jacobsen et al. (2012) recently showed an inverse correlation between levels of vitamin D and cardiovascular risk. In contrast, our results demonstrated that only high doses of vitamin D3 reduce lipid peroxidation on heart of rats, while 5,000 IU/Kg did not alter any parameter related to cardiac redox status. Corroborating with our data, have been reported a reduction on products of lipid peroxidation in subjects receiving high doses of cholecalciferol (Tarcin et al. 2009; Harbuzova, 2002). These effects probably are related to intrinsic antioxidant activity of cholecalciferol and its metabolites, and the ability to interact and stabilize the cell membrane (Wiseman, 1993). Thus, we showed here that higher doses of vitamin D presents a cardio-protector effect.

Brain cells, including Purkinje cells and other neurons in the cerebral cortex and hippocampus, express VDR and the major gene product, 1α-hydroxylase; which converts 25(OH)D to 1,25$(OH)_2$D (Zehnder et al. 1999; 2001), suggesting that calcitriol could be synthesized in situ. The supplementation of 5,000 IU/Kg appears to protect lipids and proteins from oxidation in cerebral cortex and hippocampus of rats. Wiseman (1993) explains the antioxidant property of the vitamin D based on the presence of hydroxyl groups and its lipophilic chemic structure, which permits the membrane interaction and stabilization, with consequent action as a membrane antioxidant preventing lipid peroxidation elicited by iron. Corroborating with our results, Moore et al. (2005) demonstrated that supplementation of vitamin D3 promote beneficial effects in hippocampus, although this effect was based in anti-inflammatory properties. Izquierdo et al. (2012), recently showed that the VDR activator, paricalcitol, when administered for 3 months to hemodialysis patients reduced oxidative stress parameters in peripheral blood, evidenced by reduced lipid peroxidation products and carbonyl groups content. Furthermore, calcitriol reduces UV-induced DNA damage (Song et al. 2012), as well as reduced oxidative stress markers and ameliorates antioxidant defense system in children with nutritional rickets (Dogan et al. 2012).

In contrast, the administration of high doses of cholecalciferol disrupts the brain antioxidant network. Hippocampus presents an increased CAT activity, without affect SOD and GPx; while cerebral cortex presented a reduction of CAT and GPx, similar to liver. Reduced GSH was increased in cortex, probably as a result of GPx inhibition. In addition, both structures presented reduced levels of TBARS and carbonyl groups, even in rats treated

with 30,000 IU/Kg, as well as a reduction of DCF oxidation on cortex. Literature brings us conflicting results in regard to the effect of vitamin D and its metabolites on antioxidant defenses. Kidney is responsible by the final activation of vitamin D, synthesizing 1α,25$(OH)_2$D, the most active vitamin D metabolite. Moreover, 24,25$(OH)_2$D is also produced when calcitriol concentration is sufficient to maintain the calcium homeostasis. In fact, the plasma concentration of 24,25$(OH)_2$D is higher than calcitriol. These metabolites have distinct functions and even antagonist actions (Khanal and Nemere, 2007; Farach-Carson and Nemere, 2003). Interestingly, one of the binding proteins for 24,25$(OH)_2$D is the antioxidant enzyme CAT, which is inhibited by this compound (Khanal and Nemere, 2007; Larsson et al. 2006), in agreement to our findings in liver and cerebral cortex. In opposite, increased CAT activity in erythrocytes was reported by Javanbakht et al. (2010), in atopic dermatitis patients that received 1600 IU daily for 60 days.

In our experimental conditions, we did not know if the metabolic modulation verified are a result of one or more cholecalciferol metabolite, such as 1α,25$(OH)_2$D; 24,25$(OH)_2$D; and even 25$(OH)_2$D, a neglected product of vitamin metabolism, that now has been recognized as an important mediator of the vitamin D-effects (Khanal and Nemere, 2007). In summary, the current study provided the first evidence that supplementation with 5,000 IU of vitamin D3/Kg/day appears to be safe, at least for heart and brain of rats, playing an antioxidant role. In addition, liver presented minor oxidative effects in this dose, probably related to the normal metabolism of cholecalciferol by hydroxylases. However, when 30,000 IU/Kg/day were administered, we verify redox disturbance effects, mainly on antioxidant enzymes on liver and cerebral cortex.

Our data places an alert on the use of high doses of cholecalciferol as a dietary supplement.

Acknowledgments

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Legends

Figure 1. Effects of vitamin D3 supplementation on thiobarbituric acid reactive substances (A), carbonyl levels (B), and dichlorofluorescein oxidation (C) in heart and liver of adult rats. Results are presented as mean + S.D. (n=8-10 per group), and was statistically analyzed by one-way ANOVA followed by Tukey's post hoc test (*p<0.05; ***p<0.001).

Figure 2. Effects of vitamin D3 supplementation on antioxidant enzymes activities in heart and liver of adult rats: superoxide dismutase (A), catalase (B), glutathione peroxidase (C), and SOD/(CAT+ GPx) ratio (D). Results are presented as mean + S.D. (n=6-10 per group), and was statistically analyzed by one-way ANOVA followed by Tukey's post hoc test (**p<0.01).

Figure 3. Effect of vitamin D3 supplementation on reduced glutathione levels in heart and liver of adult rats. Results are presented as mean + S.D. (n=7-10 per group), and was statistically analyzed by one-way ANOVA followed by Tukey's post hoc test (p>0.05).

Figure 4. Effects of vitamin D3 supplementation on thiobarbituric acid reactive substances (A), carbonyl levels (B), and dichlorofluorescein oxidation (C) in cerebral cortex and hippocampus of adult rats. Results are presented as mean + S.D. (n=7-10 per group), and was statistically analyzed by one-way ANOVA followed by Tukey's post hoc test (**p<0.01; ***p<0.001).

Figure 5. Effects of vitamin D3 supplementation on antioxidant enzymes activities in cerebral cortex and hippocampus of adult rats: superoxide dismutase (A), catalase (B), glutathione peroxidase (C), and SOD/ (CAT+ GPx) ratio (D). Results are presented as mean + S.D. (n=7-10 per group), and was

statistically analyzed by one-way ANOVA followed by Tukey's post hoc test (*p<0.05; ***p<0.001).

Figure 6. Effect of vitamin D3 supplementation on reduced glutathione levels in cerebral cortex and hippocampus of adult rats. Results are presented as mean + S.D. (n=8-10 per group), and was statistically analyzed by one-way ANOVA followed by Tukey's post hoc test (*p<0.05).

Table 1. Effect of vitamin D3 supplementation on body weight and liver lipids content.

	Control	Vit D_3 5,000 IU	Vit D_3 30,000 IU
Body parameters			
Initial body weight (g)	345,1 ± 31,5	330,3 ± 33,7	324,5 ± 35,5
Final body weight (g)	383,2 ± 30,7	371,1 ± 26,2	371,9 ± 29,8
Body weight gain (g)	38,10 ± 13,8	40,70 ± 16,6	47,40 ± 14,2
Lipids in liver			
Triglyceride (mg%)	0,81 ± 0,19	0,79 ± 0,12	0,78 ± 0,31
Cholesterol (mg%)	0,40 ± 0,03	0,39 ± 0,02	0,41 ± 0,01

Data are expressed as mean ± S.D., for ten experiments (animals). There are no significant differences between groups, evaluated by one-way analysis of variance ($p>0.05$).

Figure 1.

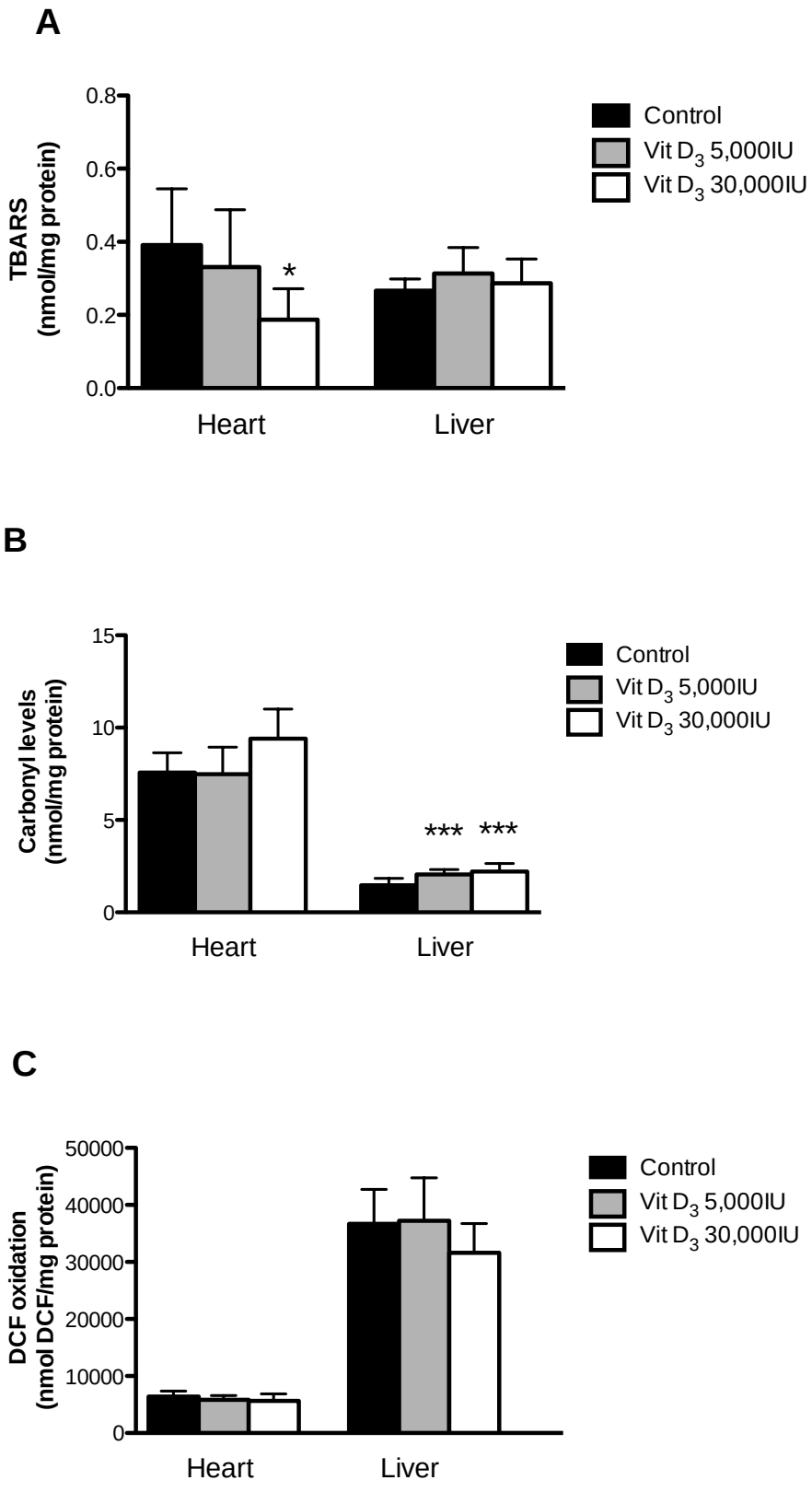

A
TBARS (nmol/mg protein)
0.8
0.6
0.4
0.2
0.0
*
Heart
Liver
Control
Vit D3 5,000IU
Vit D3 30,000IU
B
Carbonyl levels (nmol/mg protein)
15
10
5
0
*** ***
Heart
Liver
Control
Vit D3 5,000IU
Vit D3 30,000IU
C
DCF oxidation (nmol DCF/mg protein)
50000
40000
30000
20000
10000
0
Heart
Liver
Control
Vit D3 5,000IU
Vit D3 30,000IU

Figure 2.

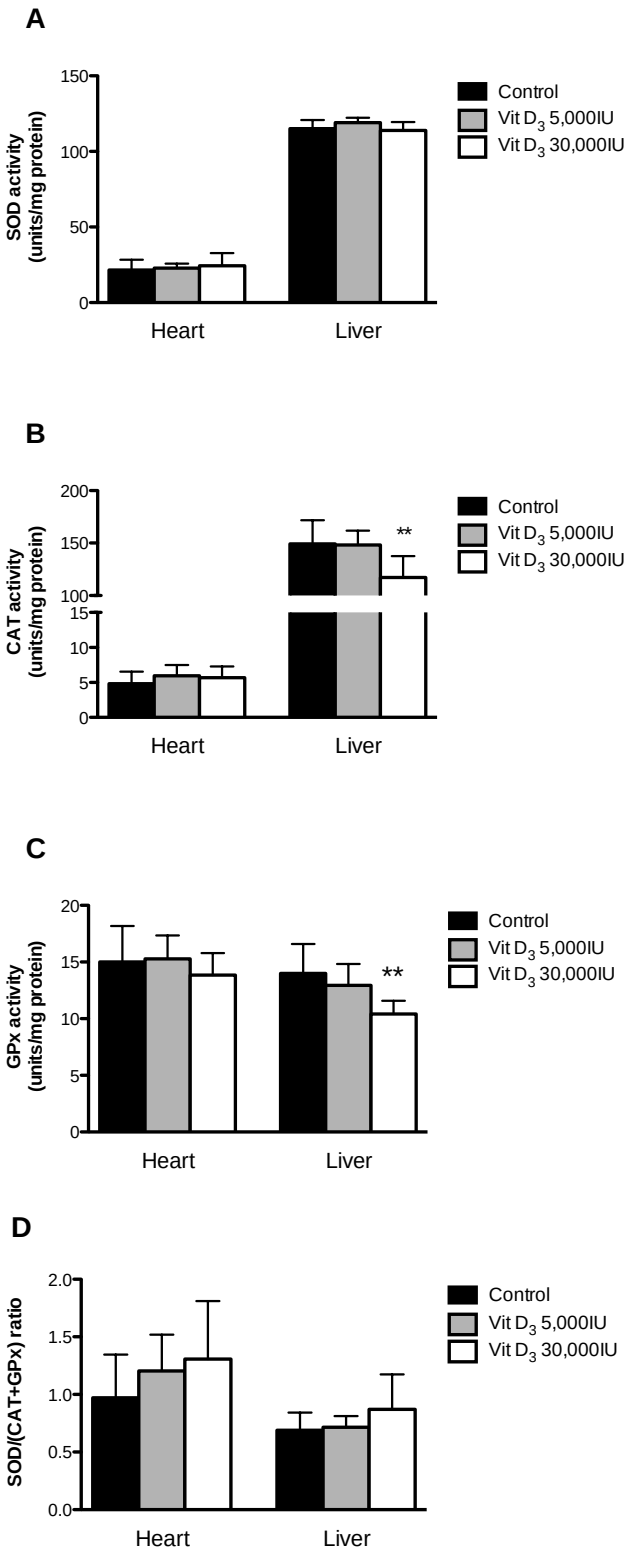

A
SOD activity (units/mg protein)
150
100
50
0
Heart
Liver
Control
Vit D3 5,000IU
Vit D3 30,000IU
B
CAT activity (units/mg protein)
200
150
100
15
10
5
0
**
Heart
Liver
Control
Vit D3 5,000IU
Vit D3 30,000IU
C
GPx activity (units/mg protein)
20
15
10
5
0
**
Heart
Liver
Control
Vit D3 5,000IU
Vit D3 30,000IU
D
SOD/(CAT+GPx) ratio
2.0
1.5
1.0
0.5
0.0
Heart
Liver
Control
Vit D3 5,000IU
Vit D3 30,000IU

Figure 3.

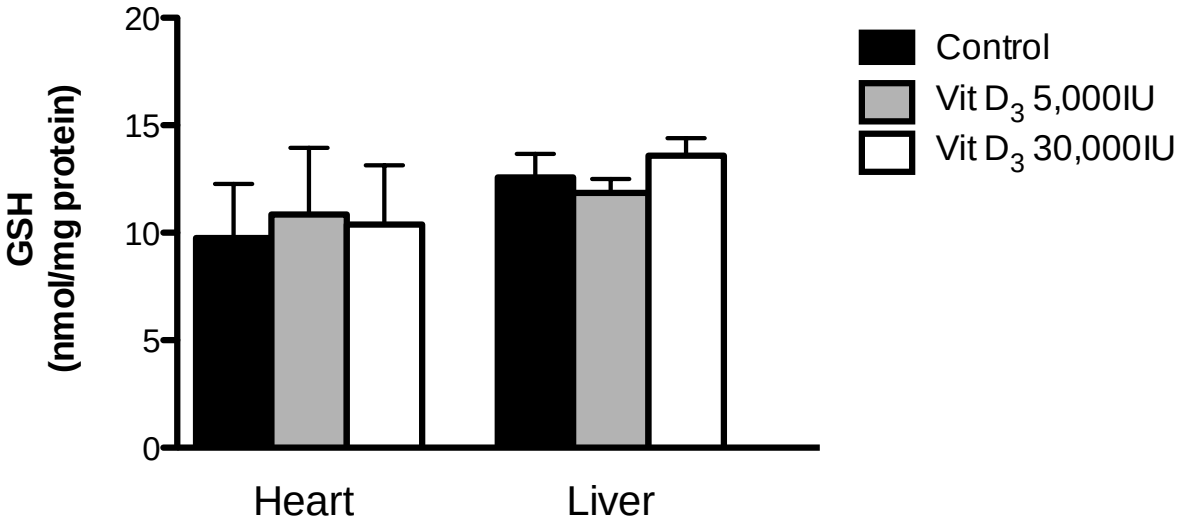

GSH
(nmol/mg protein)
20
15
10
5
0
Heart
Liver
Control
Vit D3 5,000IU
Vit D3 30,000IU

Figure 4.

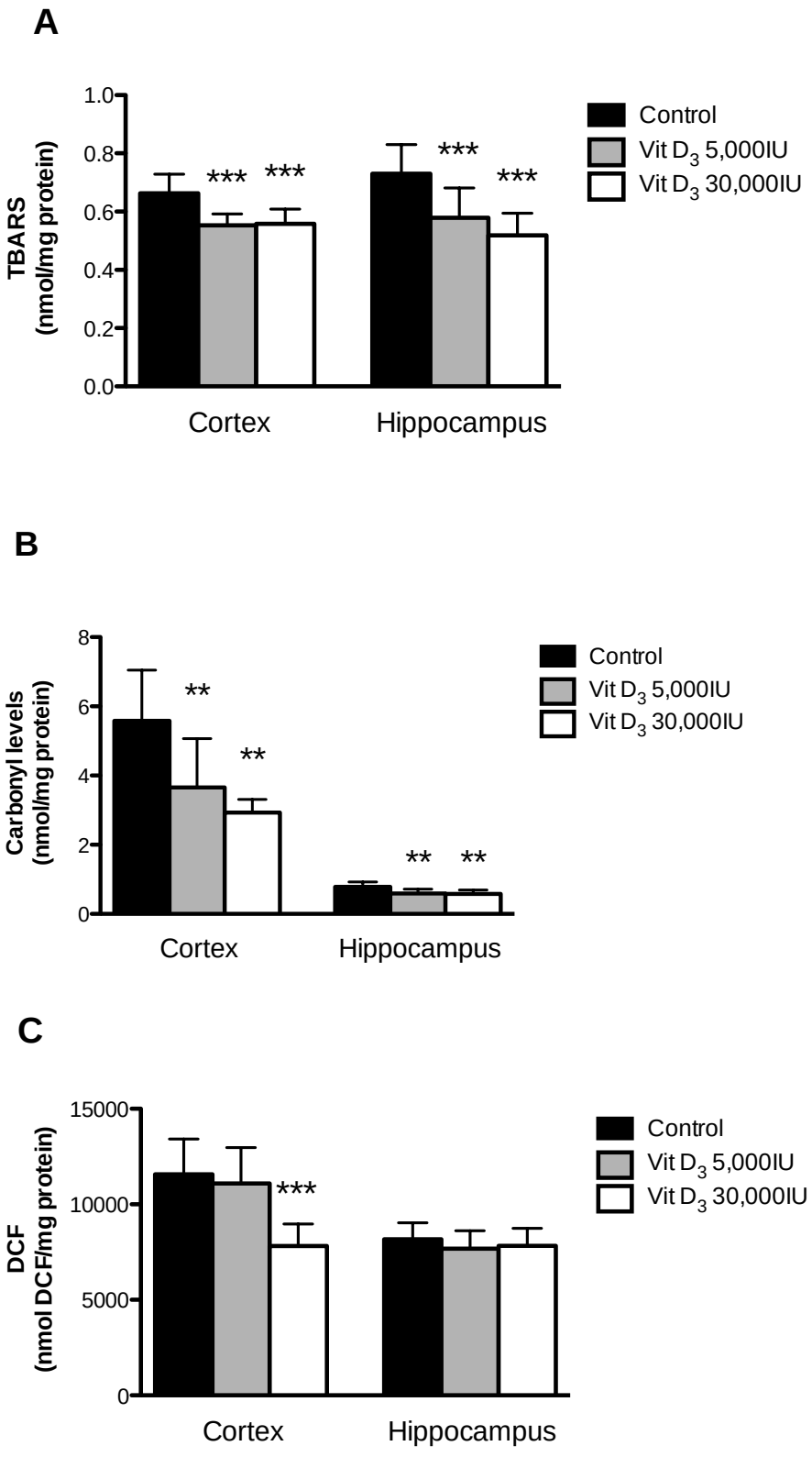

A
TBARS (nmol/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
***
Cortex
Hippocampus
Control
Vit D3 5,000IU
Vit D3 30,000IU
B
Carbonyl levels (nmol/mg protein)
8
6
4
2
0
**
**
**
**
Cortex
Hippocampus
Control
Vit D3 5,000IU
Vit D3 30,000IU
C
DCF (nmol DCF/mg protein)
15000
10000
5000
0
***
Cortex
Hippocampus
Control
Vit D3 5,000IU
Vit D3 30,000IU

Figure 5.

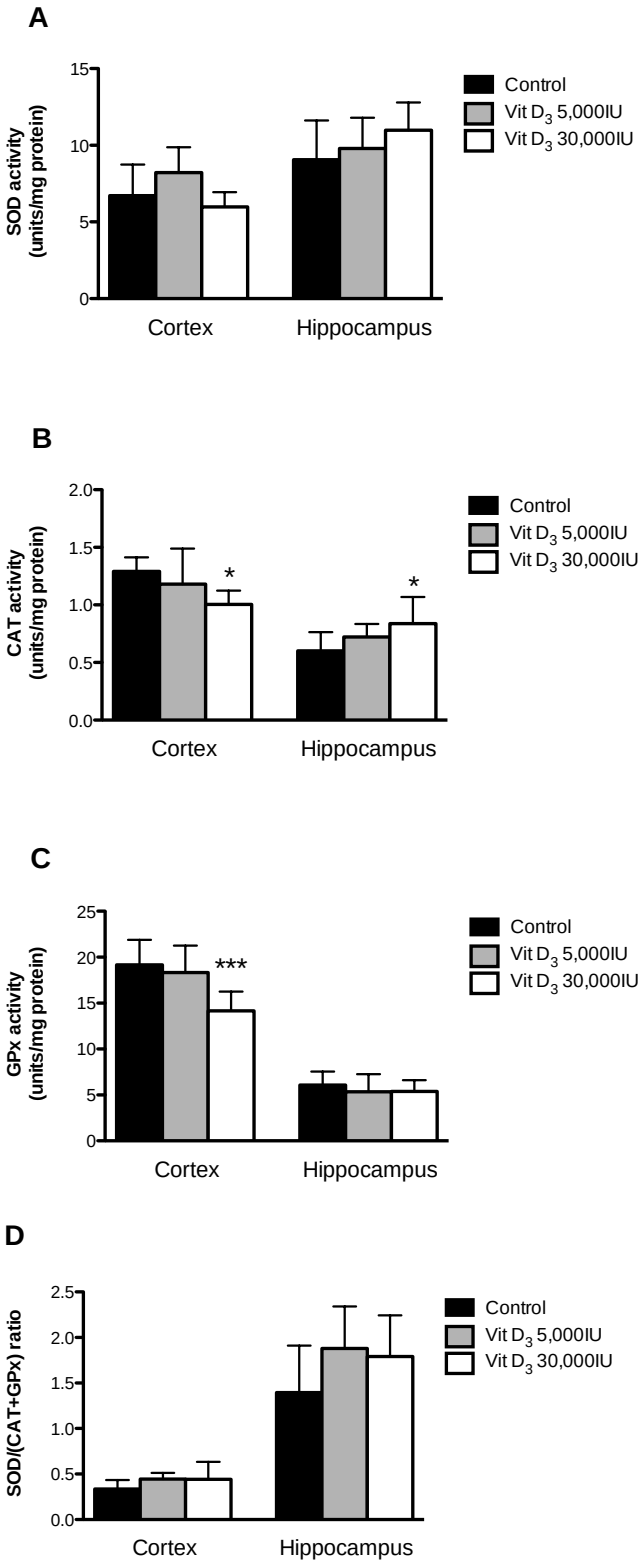
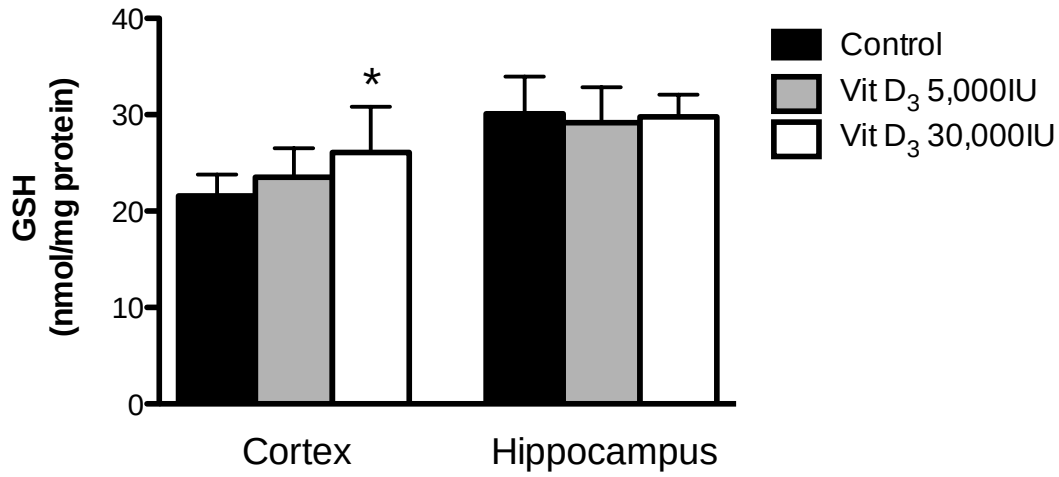

A
SOD activity (units/mg protein)
15
10
5
0
Cortex
Hippocampus
Control
Vit D3 5,000IU
Vit D3 30,000IU
B
CAT activity (units/mg protein)
2.0
1.5
1.0
0.5
0.0
*
*
Cortex
Hippocampus
Control
Vit D3 5,000IU
Vit D3 30,000IU
C
GPx activity (units/mg protein)
25
20
15
10
5
0
***
Cortex
Hippocampus
Control
Vit D3 5,000IU
Vit D3 30,000IU
D
SOD/(CAT+GPx) ratio
2.5
2.0
1.5
1.0
0.5
0.0
Cortex
Hippocampus
Control
Vit D3 5,000IU
Vit D3 30,000IU

Figure 6.


GSH
(nmol/mg protein)
40
30
20
10
0
*
Cortex
Hippocampus
Control
Vit D3 5,000IU
Vit D3 30,000IU

3.2. Capítulo II: *In vitro* effect of 1α,25-dihydroxyvitamin D(3)on redox state in central nervous system, liver, and heart of rats

Manuscrito submetido ao periódico Nutrition Research

In vitro effect of 1α,25-dihydroxyvitamin D(3)on redox state in central nervous system, liver, and heart of rats

Aline Longoni[1a],Adriano M. de Assis[1a], Anderson Rech[2], Karen Y. Kudo[2], Thiago B. Marcelino[2], Vinicius Stone[2],Guilhian Leipnitz[1,2], Cristiane Matté[1,2]

[1] Programa de Pós graduação em Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90035-003, Brasil;
[2] Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 90035-003, Brasil;

Running heads:
- Calcitriol induces oxidative stress
- Calcitriol increased lipid peroxidation, although has reduced reactive species production in heart
- Heart appears to be positively affected by 1α,25$(OH)_2$D3, that improves enzymatic and non-enzymatic antioxidant status
- Liver was minimally affected by calcitriol, presenting reduced catalase activity, which was compensated by increased glutathione-peroxidase
- Cerebral cortex and hippocampus was negatively affected by calcitriol, which misbalance antioxidant defenses and induces lipid peroxidation

World Count: 4519

[a]Both authors had contributed equally.

* Corresponding author: Cristiane Matté
Department of Biochemistry, St Ramiro Barcelos 2600 Annex,
Federal University of Rio Grande do Sul, 90035-003,
Porto Alegre, RS, Brazil.
Tel.: +55 51 3308 5548
E-mail: matte@ufrgs.br

Abbreviations:

1α,25$(OH)_2$D3 - 1α,25-dihydroxyvitamin D3;

VDR - vitamin D receptor;

CNS - central nervous system;

ROS - reactive oxygen species;

RNS - reactive nitrogen species;

DCFH-DA - 2′,7′-dihydrodichlorofluorescein;

TBARS - thiobarbituric acid reactive species;

DNPH - 2,4-dinitrophenylhydrazine;

TCA - trichloroacetic acid;

SOD - Superoxide dismutase;

CAT – Catalase;

GPx - Glutathione peroxidase;

GSH – Glutathione;

VDRm - membrane isoform of vitamin D receptor.

Abstract

Vitamin D status is positively correlated with prevention of health conditions such as cancer, immunity disorders, diabetes, and cardiovascular disease. However, questions about vitamin D safety and adequacy have become subjects of great discussion between nutrition communities and regulatory authorities. The present study was undertaken the in vivo effects of different concentrations of 1α,25-dihydroxyvitamin D3 (1α,25$(OH)_2$D3) on redox state of central nervous system, heart, and liver of rats. Adult Wistar rats are killed by decapitation and the tissues were dissected, and sliced. The samples were incubated in 1.0 mL Dulbecco buffer pH 7.4 at 37°C for 1h, in a final concentration of 50nM, 100nM, 500nM, and 1000nM of 1α,25$(OH)_2$D3. Our data showed that 1α,25$(OH)_2$D3 altered the oxidative status of cerebral cortex, increasing the reactive species production and lipid peroxidation, as well as unbalancing antioxidant defenses, verified by inhibition of catalase and glutathione- peroxidase. In contrast, the hippocampus presented a reduction on reactive species production associated to an increment of superoxide-dismutase and catalase activities, while glutathione-peroxidase was inhibited by 1α,25$(OH)_2$D3. In heart, our results have showed an increased lipid peroxidation induced by high levels of 1α,25$(OH)_2$D3, a reduction in reactive species and a compensatory effect of antioxidant status, increasing superoxide-dismutase and glutathione-peroxidase activities, as well as glutathione content. Liver was less affected, presenting reduced catalase activity, which was compensated by increased glutathione-peroxidase. Our data enable us to conclude that 1α,25$(OH)_2$D3 modulates the redox status differently on brain, heart, and liver, affecting the production of reactive species and the tissue antioxidant status.

Keywords: 1α,25-dihydroxyvitamin D(3), oxidative stress, brain, heart, liver.

1. Introduction

Vitamin D3 is essential for numerous physiological functions including the maintenance calcium/phosphate homeostasis, the regulation of cell growth and differentiation [1]. Although the exact physiologic function of 1α,25$(OH)_2$D3 in the brain, heart, pancreas, mononuclear cells, activated lymphocytes, and skin remains unknown, its major biologic function has been identified as a potent antiproliferative and prodifferentiation hormone [2,3,4].Therefore, vitamin D receptor (VDR) plays a central role in the biological actions of vitamin D. The 1α,25$(OH)_2$D3 acts by binding to VDR, a member of the steroid hormone receptor family [5], taking the transcriptional regulation of target genes [6]. The activation of VDR is directly related with many genes, promoting an up regulation (e.g., CYP24A1, CaBP-D28k, osteocalcin, and Rankl) or a down regulation (e.g., PTH and CYP27B1) of several genes involved on vitamin D metabolism [7].

Changes in circulating levels of 1α,25$(OH)_2$D3, the most active metabolite, are generally small and unreliable, therefore the levels of 25(OH)D are considered the indicator of vitamin D status. Normal serum levels of vitamin D are above 75 to 80 nmol/L, while 10,000IU appear to be well tolerated, for humans, increasing serum levels to 220 nmol/L [8,9]. On the other hand, literature data show conflicting information concerning upper reference levels of vitamin D [8]. Reports indicated that 700 nmol/L is a limit to toxicity [8,10]. The adverse effects of D hypervitaminosis are probably largely mediated by hypercalcemia, although some evidences suggest the relation of high concentrations of vitamin D and hypertension, and the development of renal stones [8]. In addition, the prolonged ingestion of excessive amounts of vitamin

D and the accompanying hypercalcemia can cause metastatic calcification of soft tissues, including the kidney, blood vessels, heart, and lungs [11.12]. The central nervous system (CNS) may also be involved: a severe depressive illness has been noted in D hypervitaminosis [13].

Oxidative stress may result from an overload of oxidants, particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS), with respect to the antioxidant defense system developed by cells to counteract oxidation. Oxidative insult may disrupt cell structures and functions, insofar as they are dependent on critical redox balance [14,15]. Low vitamin D status have been correlated to increased circulating oxidative stress markers [16,17], while vitamin D supplementation maintain DNA integrity [18,19].

Many studies have suggested that hypervitaminosis is related with premature aging [20] and oxidative stress [21], however, the mechanism that leads to vitamin to alter redox state and accelerate the aging is unknown. Although there are many studies in the literature on vitamin D, most of them were dedicated to study the deficiency of this vitamin, leaving in the background the study of hypervitaminosis. Recent studies have demonstrated the importance of the effects of hypervitaminosis D and even more the importance of its prevention, although studies focus on the relationship between vitamin status and hypercalcemia. Thus, the present study was undertaken the *in vitro* effects of different concentrations of 1α,25-dihydroxyvitamin D(3)on redox state in cerebral cortex, hippocampus, heart, and liver of rats.

2. Material and Methods

2.1 Animals and chemicals

Adult Wistar rats (total number = 42) male, from the Central Animal House of the Department of Biochemistry were maintained under a standard dark–light cycle (lights on between 7:00 a.m. and 7:00 p.m.) at room temperature (22±2 °C). These conditions were maintained constant throughout the experiments. The experiments were approved by local Ethics Commission (Comissão de Ética no Uso de Animais/Universidade Federal do Rio Grande do Sul - CEUA/UFRGS) under the number 20613, and followed the National Institute of Health Guide for the Care and Use of Laboratory Animals “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 80-23, revised 1996).

1α,25$(OH)_2$D3 and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Tissue preparation and in vitro incubation with 1α,25-dihydroxyvitamin D3

The rats were killed by decapitation and the tissues were dissected, weigh immediately and cut into slices (0.3mm) using a tissue chopper. Heart, liver, hippocampus and cerebral cortex slices (100-120 mg) were incubated in 1.0 mL Dulbecco buffer pH 7.4 containing 5.0mM D-glucose. The slices were incubated at 37°C for 1h in a Dubnoff metabolic shaker (60breaths/min) according to the method of Dunlop et al. [22], the reaction medium was aerated with 95% O_2:5% CO_2. The tissue slices were incubated in five different groups**:** control group (C) containing Dulbecco buffer, ethanol control group (CE), which contained 10% ethanol (the vehicle of 1α,25$(OH)_2$D3), and 1α,25$(OH)_2$D3 in the

final concentrations of 50nM, 100nM, 500nM, and 1000nM. These concentrations are based in previous works [45]. After the incubation the samples were washed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 1,000 x g for 10 min at 4ºC, to discard nuclei and cell debris. The pellet was discarded and the supernatant was taken to biochemical assays. The homogenates used were from individual animals, and they were never pooled.

2.3 Oxidative stress parameters

2.3.1 2',7'-Dihydrodichlorofluorescein oxidation

Production of reactive oxygen/nitrogen species was measured following the method of LeBel et al. [23], which is based on DCFH oxidation. 2′,7′-dihydrodichlorofluorescein diacetate (DCFH-DA) is cleaved by cellular esterases and the DCFH formed is eventually oxidized by ROS or RNS present in samples. Briefly, DCFH-DA was incubated for 30 min at 37 °C with an aliquot of the supernatant obtained from the in vitro assay. Fluorescence was measured using excitation and emission wavelengths of 488 nm and 525 nm, respectively. A calibration curve was performed with standard DCF and the levels of reactive species were calculated as nmol of DCF/mg protein.

2.3.2 Thiobarbituric acid reactive species (TBARS)

In order to assess the extent of lipoperoxidation, we detected thiobarbituric acid reactive species (TBARS) formation through a heated and acidic reaction. This is widely adopted as a method for the measurement of lipid redox states, as previously described [24]. The samples were mixed with 0.6 mL of 10% TCA and 0.5 mL of 0.67% thiobarbituric acid and were then heated in a boiling water bath for 25 min. The level of TBARS was determined by

measuring the absorbance of each sample at 532 nm. The concentration of TBARS in the samples was then determined from a calibration curve using 1,1,3,3-tetramethoxypropane (which had been subjected to the same treatment as the supernatants) as a standard. Results are expressed as nmol of TBARS/mg protein.

2.3.3 Measurement of protein carbonyl

The oxidative damage to proteins was measured by quantifying the number of carbonyl groups by reaction with 2,4-dinitrophenylhydrazine (DNPH). Proteins were precipitated by the addition of 20% trichloroacetic acid (TCA) and were ressuspended in 10mM DNPH, and the absorbance of the resulting solution at 370 nm was recorded [25]. Results are expressed as nmol carbonyl/mg protein.

2.3.4 Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline autooxidation by recording the absorbance of the samples at 480 nm [26]. SOD activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit. The data were expressed as Units/mg protein.

2.3.5 Catalase activity assay

Catalase (CAT) activity was assayed according to Aebi [27] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100 and 10 mM potassium phosphate buffer, pH 7.0. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity is reported as units/mg protein. The data were expressed as Units/mg protein.

2.3.6 Glutathione peroxidase activity assay

Glutathione peroxidase (GPx) activity was measured according to the method described by Wendel [28] using *tert*-butyl hydroperoxide as substrate. NADPH disappearance was monitored at spectrophotometrically at 340 nm in a medium containing 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as 1 µmol of NADPH consumed per minute and the specific activity is represented as units/mg protein.

2.3.7 Reduced glutathione levels

Glutathione (GSH) levels were measured according to Browne and Armstrong [29]. Tissue supernatants with approximately 0.3 mg of protein were diluted in 20 volumes (1:20, v/v) of 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation was incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was performed with standard GSH (0.001–0.1 mM), and GSH concentrations were calculated as nmol of GSH/mg protein.

2.4. Statistical analyses

Biochemical data are expressed as the mean ± S.D. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 16.0, Chicago, IL, USA) software. Likewise, the differences between all groups were analyzed using one-way ANOVA, followed by Tukey's post hoc test, when F-test was significant. *P*-values were considered significant when $p<0.05$.

3. Results

3.1 Effect of 1α,25-dihydroxyvitamin D(3) on oxidative stress parameters in liver and heart

In Table 1, we verify the effect of 1α,25$(OH)_2$D3 on oxidative damage in liver and heart slices. In heart, we observed a significant increase ($p<0.05$) in lipid peroxidation in the highest evaluated concentration, 1α,25$(OH)_2$D3 1000nM. The same concentration elicited a significant reduction ($p<0.05$) in reactive species production and protein damage, evaluated by DCFH and carbonyl techniques, respectively. Further, 50nM of 1α,25$(OH)_2$D3 also reduced carbonyl levels in heart.

Antioxidant defenses of heart were affected by the incubation with 1α,25$(OH)_2$D3. Fifty - 1000 nM increased SOD activity ($p<0.05$), while CAT activity was increased in 50 nM and decreased in 500 nM of 1α,25$(OH)_2$D3. GPx activity were increased by 1000 nM of 1α,25$(OH)_2$D3, as well GSH levels ($p<0.05$). In order to better understand the redox balance, we calculated a SOD/(CAT+ GPx) ratio. Figure 1A shows the effect of 1α,25$(OH)_2$D3 on balance SOD/(CAT+ GPx) in heart, where, although we have observed alterations in individual enzymes activities, the ratio was not altered by vitamin D metabolite.

Liver was minimally affected by the incubation with 1α,25$(OH)_2$D3, where DCF oxidation, carbonyl levels, and lipid peroxidation were not altered ($p<0.05$, Table 1). SOD activity was not altered, while CAT was reduced and GPx was increased by the incubation with 1000 nM of 1α,25$(OH)_2$D3 (Table 2). Nevertheless, the ratio SOD/(CAT+ GPx) was not altered (Figure 1B).

3.2 Effect of 1α,25-dihydroxyvitamin D(3) on oxidative stress parameters in central nervous system

Table 3 shows the effect of 1α,25$(OH)_2$D3on oxidative damage parameters in CNS. In cerebral cortex slices we could observe a significant increase ($p<0.05$) in lipid peroxidation by 50nM 1α,25$(OH)_2$D3, as well in reactive species production, measured by DCF oxidation, when the slices were incubated with 100 nM of 1α,25$(OH)_2$D3 ($p<0.05$).

Antioxidant enzymes was inhibited by 1α,25$(OH)_2$D3 in cerebral cortex (Table 4). CAT activity was reduced by 500 and 1000nM, while GPx was inhibited in all tested concentrations ($p<0.05$). In addition, we showed a significant increase in GSH levels by 100 and 500nM, probably related to reduced GPx activity. We also calculated the ratio of SOD/(CAT+ GPx) in cerebral cortex. Figure 2A shows an increase in the ratio by 100 and 500nM of 1α,25$(OH)_2$D3 ($p<0.001$), indicating an imbalance in antioxidant enzymes.

We also evaluated the same oxidative parameters in hippocampus, a brain structure largely related to memory processing. Hippocampus presented a reduction on DCF oxidation in all concentration tested ($p<0.05$; Table 3). TBARS was not altered by 1α,25$(OH)_2$D3 incubation ($p>0.05$). Regarding the antioxidant enzymes, hippocampus presented increased SOD (100nM of 1α,25$(OH)_2$D3) and CAT (50 nM of 1α,25$(OH)_2$D3) activities, while GPx was inhibited (100, 500 and 1000 nM of 1α,25$(OH)_2$D3). Furthermore, we observed that incubation with 1α,25$(OH)_2$D3 promoted a significant increase ($p<0.05$) in SOD/(CAT+ GPx) ratio in the highest concentration tested, suggesting an imbalance in ROS elimination.

4. Discussion

Adequate dietary intake of vitamin D is essential to building and maintaining many physiological processes. Recent evidences have indicated an interrelationship between vitamin D and health besides the action on bone, including effects on cell proliferation and on immune system [30,31]. Calcitriol (1α,25$(OH)_2$D3), the biologically active metabolite of vitamin D, which has been approved for the prevention and treatment of osteoporosis, mediates its actions via VDR [32]. In a recent study, researchers showed that the signaling pathway of calcitriol might be mediated by a membrane isoform of VDR (VDRm) in cerebral cortex of young rats, and could be a pharmacological target for the treatment of neuro pathological conditions [33]. VDR was found including in the CNS, in rat hippocampus [34] and cortex [35], having the capacity to binding specifically to DNA. These studies suggest that the VDR might mitigate processes related to cellular homeostasis, perhaps through a calcium buffering mechanism. Li et al. [36], showed that 1α,25$(OH)_2$D3 could protect rat primary cortical cells from neurotoxins, like cyanide. Calcitriol blocks ROS formation by mitochondrial respiratory chain, reducing uncoupling proteins expression via NF-κB pathway inhibition [36].

There are many studies evaluating the deficiency of vitamin D in the current literature, however, there is a lack of studies to demonstrate the response of different organs to its overdoses. According to the American Academy of Pediatrics, serum vitamin D levels above 250 nmol/L (100 ng/mL) are considered as hypervitaminosis D, whereas serum levels above 375 nmol/L (150 ng/mL) are associated with vitamin D intoxication [37]. In the present study, we investigated for the first time the effects of high concentrations of

1α,25(OH)$_2$D3 on redox state in slices of two brain structures (cerebral cortex and hippocampus) and two organs of peripheral system (heart and liver).

Beyond its well-known skeletal functions, vitamin D is necessary for the normal functioning of other human body systems including those related to immunity, and the cardiovascular system [38]. A couple of studies suggest that hypervitaminosis D may be a key factor in the accelerated aging phenotype of Fgf-23 and Kl-deficient mice [39,40] related to ROS production. In other study, researchers used vitamin D plus insulin in order to prevent oxidative stress induced by diabetes in liver, kidney, and heart of rats. Inversely, they found an increased lipid peroxidation, and did not find any alteration on antioxidant enzymes in heart and liver [41]. In accord, we showed that heart was affected by incubation with 1000 nM of calcitriol, increasing TBARS, an index of lipid peroxidation. On the other hand, the remaining parameters evaluated suggest that 1α,25(OH)$_2$D3 possess antioxidant properties in this tissue. We have shown an improvement on antioxidant status, verified by SOD, CAT, and GPx activities, as well as by GSH levels, even in high calcitriol concentrations. Corroborating with these data, we also verified a reduction in reactive species production and carbonyl levels, an index of protein oxidation.

In liver we demonstrated a misbalance in antioxidant system where reduction in CAT activity, was compensated by GPx activation. The incubation was performed with the active metabolite of vitamin D, excluding the possible ROS generation by hydroxylation naturally occurring when vitamin D is used to produces 1α,25(OH)$_2$D3 [42]. Hepatocarcinogenesis promoted by diethylnitrosamine induced DNA damage, that was prevented by 1α,25(OH)$_2$D3

supplementations. However, rats treated only with calcitriol also induced enzymatic antioxidant system and increased lipid peroxidation in liver [43].

The brain possesses enzymatic antioxidant defenses, which act against ROS, in a lesser amount than liver or heart (Halliwel and Gutteridge, 2006) SOD plays a key role in detoxifying superoxide anions, while CAT and GPx play a key role in detoxifying hydrogen peroxide in water. Our results showed that 1α,25$(OH)_2$D3 altered the oxidative status of the CNS. Cerebral cortex presented an increment in reactive species production and lipid peroxidation by calcitriol. Antioxidant enzymes, CAT and GPx, were inhibited by 1α,25$(OH)_2$D3, suggesting an impair removal of hydrogen peroxide. Fenton reaction could convert hydrogen peroxide in the most harmful free radical, hydroxyl, which could be responsible by the oxidative effects verified in slices of cortex. Reduced GSH was increased in cerebral cortex, and might be related to the inhibition of GPx. In a contrasting way, hippocampus presented a reduction in DCF oxidation, indicating decreased reactive species synthesis. Contributing with our results obtained in hippocampus, Anya et al. [44] showed that vitamin D3 have antioxidant effect *in vitro* model in brain homogenates incubated with zinc-induced oxidative stress. SOD and CAT was increased by calcitriol, while GPx was inhibited. These data suggest that calcitriol affect negatively the redox status of cortex and hippocampus. Thereby, the brain appears to be more affected by calcitriol than peripheral tissues evaluated here.

In summary, the current study provided the evidence that 1α,25$(OH)_2$D3acts differently according to the tissue and concentration studied and may act as an antioxidant or as a pro-oxidant. Further studies, however,

are necessary to better understand the effects of vitamin D on the oxidant and antioxidant systems in CNS and peripheral tissues.

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Legends.

Figure 1. Antioxidant enzymes ratio in Heart (A) and Liver (B).

The SOD/(CAT+ GPx) ratio is calculated based in % of controls of each enzyme and expressed as mean ± S.D. (n = 8 per group). There are no statistical difference from control ($p>0.05$), evaluated by one way ANOVA, followed Tukey's post-hoc test.

Figure 2. Antioxidant enzymes ratio in Cerebral Cortex (A) and Hippocampus (B).

The SOD/(CAT+ GPx) ratio is calculated based in % of controls of each enzyme and expressed as mean ± S.D. (n = 8 per group). Different from control (*$p<0.05$; ***$p<0.001$), evaluated by one way ANOVA, followed Tukey's post-hoc test.

Table 1. Oxidative Damage in Liver and Heart

	HEART			LIVER		
	DCFH	TBARS	CARBONYL	DCFH	TBARS	CARBONYL
Control	100	100	100	100	100	100
1α,25-$(OH)_2$ D3 50nM	97,5 ± 18,4	94,8 ± 20,6	78,9 ± 8,5*	106 ± 11,1	97,7 ± 16,8	76,1 ± 8,1
1α,25-$(OH)_2$ D3 100nM	86,7 ± 7,7	97,2 ± 23,4	106 ± 16,4	82,9 ± 17,7	106 ± 22,9	79,4 ± 18,3
1α,25-$(OH)_2$ D3 500nM	78,9 ± 24,2	126,13 ± 22,6	82,7 ± 10	107 ± 25,1	121 ± 24,2	126 ± 44,5
1α,25-$(OH)_2$ D3 1000nM	74,9 ± 16,8*	155,69 ± 30,3*	66,7 ± 21,6*	103 ± 26,2	94,6 ± 15,9	121 ± 84,1

Values are expressed in % of control and are represented as the mean ± S.D. (n = 8 per group). Different from control, *p<0.05, evaluated by one way ANOVA, followed by Tukey's post-hoc test.

Table 2. Oxidative Damage in Central Nervous System

	CEREBRAL CORTEX		HIPPOCAMPUS	
	DCFH	TBARS	DCFH	TBARS
Control	100	100	100	100
1α,25-$(OH)_2$ D3 50nM	107 ± 31,7	120 ± 16,1*	65,5 ± 17,3*	93,9 ± 20,9
1α,25-$(OH)_2$ D3 100nM	161 ± 61,1*	104 ± 9,0	43,5 ± 13,2*	92,2 ± 13,7
1α,25-$(OH)_2$ D3 500nM	135,4 ±51,5	89,4 ± 16,5	40,6 ± 5,6*	85,5 ± 8,1
1α,25-$(OH)_2$ D3 1000nM	147 ± 31,6	93,8 ± 5,2	39,6 ± 22,1*	83,1 ± 17,9

Values are expressed in % of control and are represented as the mean ± S.D. (n = 8 per group). Different from control, *$p<0.05$, evaluated by one way ANOVA, followed by Tukey's post-hoc test.

Table 3. Antioxidant Defense in Liver and Heart

	HEART				LIVER			
	SOD	CAT	GPx	GSH	SOD	CAT	GPx	GSH
Control	100	100	100	100	100	100	100	100
1α,25-$(OH)_2$ D3 50nM	129 ± 9,8*	124,5 ± 22,7*	113 ± 25,6	89,7 ± 26,7	90,3 ± 23,4	106 ± 12,5	88,5 ± 48,2	82,5 ± 14,2
1α,25-$(OH)_2$ D3 100nM	150 ± 9,1*	118 ± 23,8	112 ± 19,6	122 ± 13,5	90,7 ± 17,3	103,3 ± 18,6	73,6 ± 29,5	75,1 ± 19,5
1α,25-$(OH)_2$ D3 500nM	89,7 ± 26,8	86,2 ± 26,9*	70,7 ± 21,7	103 ± 15,1	94,8 ± 9,8	89 ± 12,6	111,3 ± 8,6	87,3 ± 19,5
1α,25-$(OH)_2$ D3 1000nM	112 ± 6,7*	119 ± 26,1	146,9 ± 33,4*	148 ± 56,8*	106 ± 18,7	82,7 ± 19,1*	179 ± 51*	87,9 ± 16,5

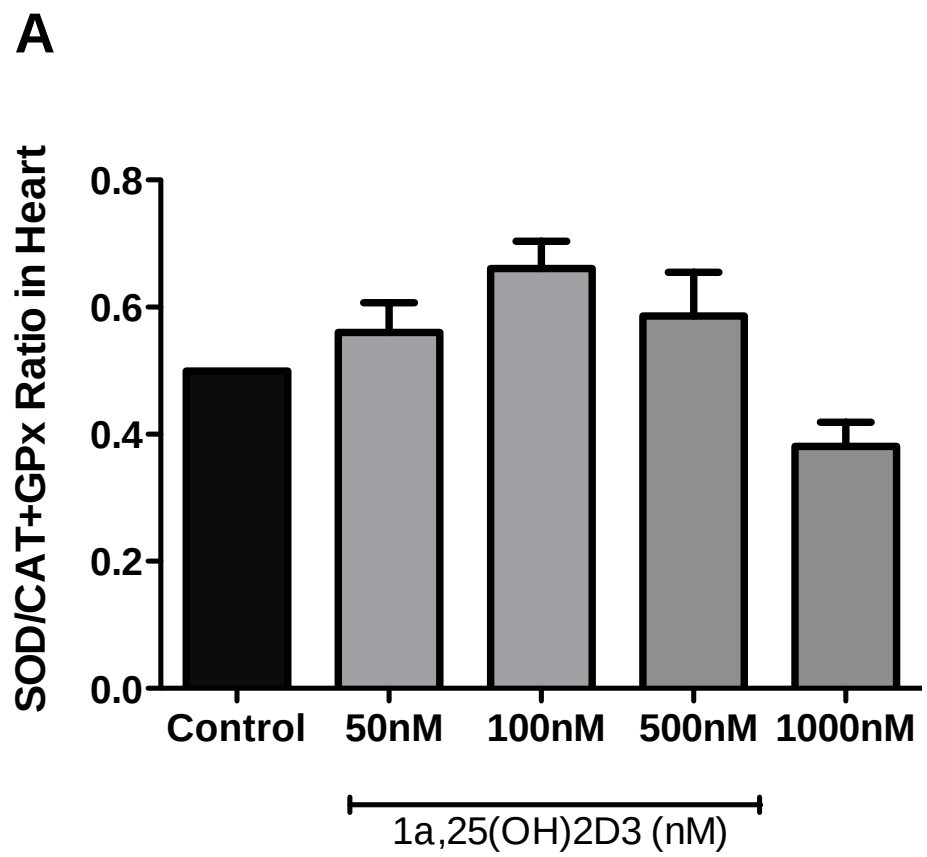
Values are expressed in % of control and are represented as the mean ± S.D. (n = 8 per group). Different from control, *$p<0.05$, evaluated by one way ANOVA, followed by Tukey's post-hoc test.

Table 4. Antioxidant Defense in Central Nervous System

	CEREBRAL CORTEX				HIPPOCAMPUS			
	SOD	CAT	GPx	GSH	SOD	CAT	GPx	GSH
Control	100	100	100	100	100	100	100	100
1α,25-$(OH)_2$ D3 50nM	101 ± 13,7	104 ± 23,8	71,8 ± 10,9*	148 ± 23,1	110 ± 6,8	172 ± 84,2*	82,4 ± 18,6	118 ± 37,4
1α,25-$(OH)_2$ D3 100nM	121 ± 12,2	88,5 ± 23,5	57,4 ± 11,5*	160 ± 41,3*	122 ± 19,5*	136 ± 45,1	76,3 ± 6,3*	120 ± 34
1α,25-$(OH)_2$ D3 500nM	84,2 ± 9,1	44,7 ± 19,9*	44,1 ± 12,2*	164 ± 47,2*	88,4 ± 13,8	94,2 ± 13,3	41 ± 5,1*	97 ± 18,6
1α,25-$(OH)_2$ D3 1000nM	98,1 ± 14,1	62,4 ± 21,2*	78,5 ± 21,2*	145 ± 35,9	101 ± 14,1	69,5 ± 34,5	75,6 ± 25,6*	99 ± 27,6

Values are expressed in % of control and are represented as the mean ± S.D. (n = 8 per group). Different from control, *$p<0.05$, evaluated by one way ANOVA, followed by Tukey's post-hoc test.

Figure 1.


A
SOD/CAT+GPx Ratio in Heart
0.8
0.6
0.4
0.2
0.0
Control
50nM
100nM
500nM
1000nM
1a,25(OH)2D3 (nM)

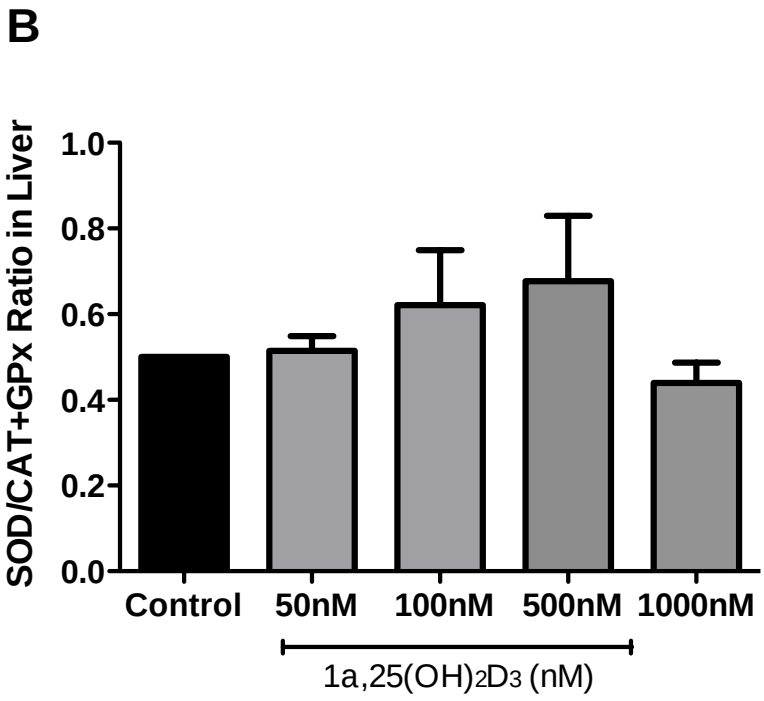
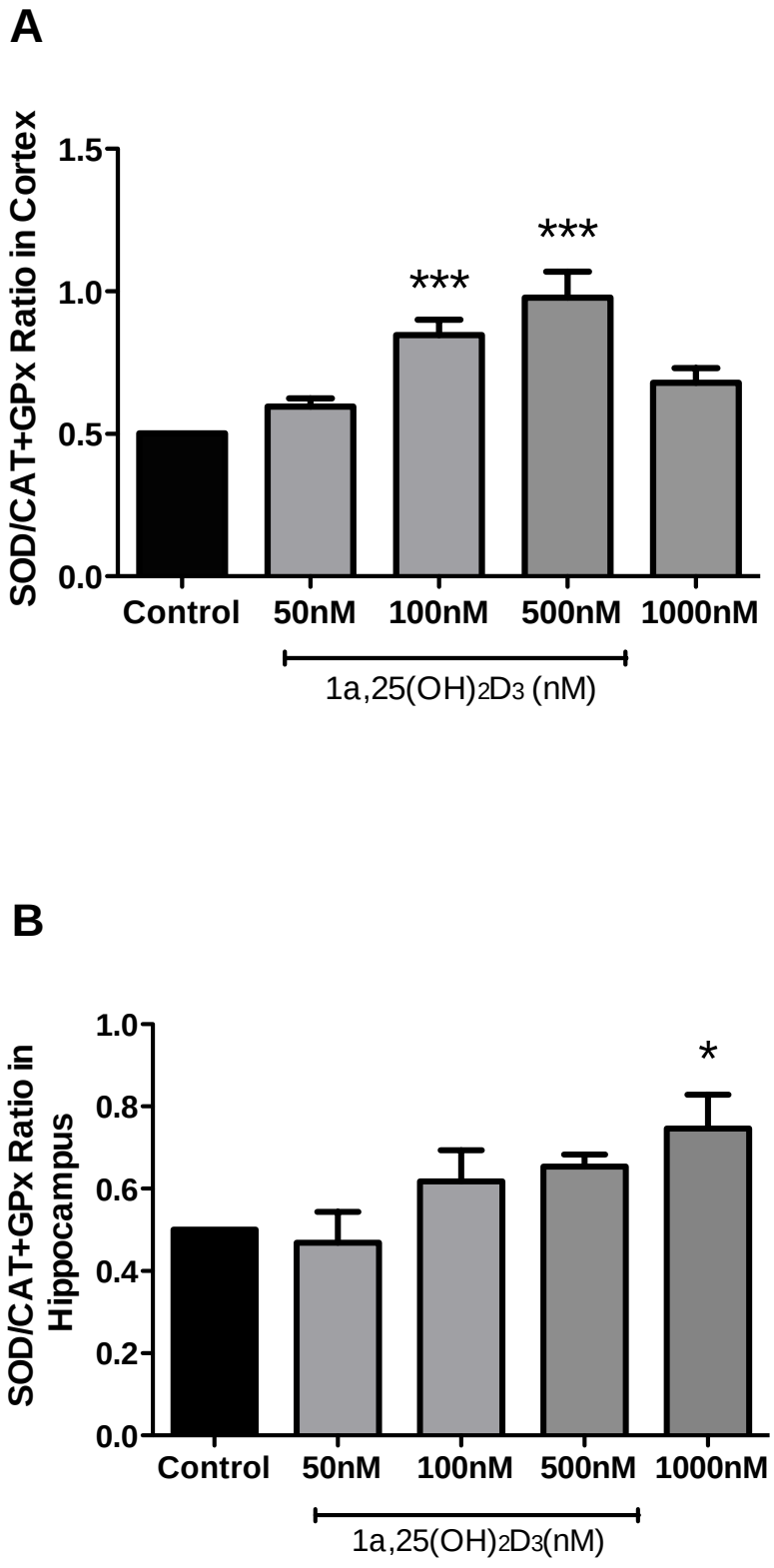

B
SOD/CAT+GPx Ratio in Liver
1.0
0.8
0.6
0.4
0.2
0.0
Control
50nM
100nM
500nM
1000nM
1a,25(OH)2D3 (nM)

Figure 2.


A
SOD/CAT+GPx Ratio in Cortex
1.5
1.0
0.5
0.0
***
***
Control
50nM
100nM
500nM
1000nM
1a,25(OH)2D3 (nM)
B
SOD/CAT+GPx Ratio in Hippocampus
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
50nM
100nM
500nM
1000nM
1a,25(OH)2D3(nM)

4. DISCUSSÃO

A vitamina D tem sido amplamente utilizada como suplemento alimentar, o que pode resultar em intoxicações quando em altas doses, com sinais e sintomas de hipercalcemia, hipercalciúria, calcificações em tecidos moles, bem como alterações metabólicas e cognitivas (Hathcock et al., 2007). Por outro lado, a hipovitaminose, que apresenta alta prevalência mundial, tem sido relacionada a uma série de condições patológicas, tais como arteriosclerose e o aumento do risco de desenvolvimento de problemas cardiovasculares (Artaza et al., 2009). Nesse sentido, um estudo que avalie possíveis alterações no estado redox celular em diferentes órgãos se faz necessário, a fim de definir a segurança de diferentes doses e concentrações da vitamina D e seus metabólitos ativos.

Inicialmente utilizamos uma abordagem *in vivo,* suplementando ratos adultos por 21 dias com vitamina D3, administrada via gavagem, em doses compatíveis com as utilizadas na clínica (dose terapêutica limítrofe: 5.000UI/Kg/dia e dose suprafisiológica: 30.000 UI/Kg/dia). Vinte e quatro horas após a última administração de vitamina D, os animais foram sacrificados por decapitação e os tecidos (coração, fígado, hipocampo e córtex cerebral) foram dissecados e utilizados nas medidas bioquímicas.

A vitamina D3 é uma molécula derivada do colesterol, e foi administrada aos animais diluída em óleo de girassol. A fim de avaliar se a suplementação de vitamina D altera o conteúdo lipídico hepático, realizamos a medição dos níveis de colesterol e triglicerídeos no fígado. A administração crônica de vitamina não alterou o conteúdo hepático de lipídios, bem como a evolução no peso corporal dos ratos durante o tratamento. Estes resultados

eliminam certos vieses, que poderiam interferir na interpretação dos dados obtidos nesse trabalho. Em termos de status redox, o fígado foi afetado pela administração de ambas as doses de vitamina, resultando no aumento da oxidação de proteínas (5.000 e 30.000 UI), medida pela carbonilação, e pela redução na atividade das enzimas CAT e GPx (30.000 UI). Verificamos aqui um possível prejuízo na eliminação de peróxido de hidrogênio, o que pode resultar na síntese de radical hidroxila, mais reativo que outras ERO de importância fisiológica (Halliwell e Gutteridge, 2006). Em adição, a vitamina D3 administrada é absorvida a nível intestinal e transportada ao fígado, onde é hidroxilada por um sistema dependente do citocromo P450 (Stokes et al., 2013; Hanukoglu, 2006), que pode ser fonte de ERO por meio da redução parcial do oxigênio molecular (Hanukoglu, 2006). Essas ERO geradas podem desencadear a oxidação de proteínas, resultando em carbonilação proteica (Levine, 2002). Cabe aqui destacar que o efeito danoso sobre as enzimas antioxidantes foi encontrado apenas na dose mais alta empregada, de 30.000 UI/Kg/dia.

Segundo Brondum-Jacobsen et al. (2012), existe uma correlação inversa entre os níveis de vitamina D e o risco de problemas cardiovasculares. Nossos dados mostram que a suplementação de vitamina D não afetou significativamente o estado redox cardíaco, mesmo em altas dosagens. Observamos apenas uma redução nos níveis de peroxidação lipídica, medidos pelo TBARS, em animais tratados com 30.000 UI/Kg/dia. Corroborando com nossos dados, foi relatada uma redução de produtos de peroxidação lipídica em pacientes recebendo altas doses de colecalciferol (Tarcin et al., 2009; Harbuzova, 2002). Estes efeitos são provavelmente

relacionados com a atividade antioxidante intrínseca do colecalciferol e seus metabólitos, e a capacidade de interagir e de estabilizar a membrana celular (Wiseman, 1993).

Poucos estudos têm avaliado o efeito da vitamina D e seus metabólitos no SNC, o qual possui reduzida proteção antioxidante e altas concentrações de moléculas pró- oxidativas, tais como ferro e dopamina, bem como alvos de oxidação representados pelos ácidos graxos poliinsaturados (Halliwell e Gutteridge, 2006). A passagem da vitamina D pela barreira hematoencefálica (BHE) ocorre naturalmente, existindo proteínas ligadoras de 1α,25$(OH)_2$D que permitem essa transferência (Wang et al., 2012). Após cruzar a BHE, a vitamina é distribuída entre as diferentes regiões cerebrais em quantidade semelhante. As células do cérebro, incluindo as células de Purkinje e outros neurônios no córtex cerebral e no hipocampo, expressam o VDR e 1α-OHase, que hidroxila 25(OH)D em 1α,25$(OH)_2$D (Zehnder et al., 1999), sugerindo que o calcitriol pode ser sintetizado localmente no SNC. A suplementação com dose terapêutica de vitamina D parece proteger os lipídios e proteínas de oxidação no córtex cerebral e hipocampo de ratos. Além disso, não foi observada nenhuma alteração no status antioxidante cerebral em ratos que receberam a dose de 5.000 UI/Kg/dia. Wiseman (1993) atribui à estrutura química da vitamina D e seus metabólitos, a capacidade de atuar como sequestrador de radicais livres e prevenir a oxidação lipídica, já que esses derivados do colesterol podem se integrar à bicamada lipídica de membranas celulares. Ainda, a administração de colecalciferol ou substâncias análogas à pacientes

promove redução em índices de oxidação lipídica e proteica em sangue (Song et al., 2012; Dogan et al., 2012; Izquierdo et al., 2012).

Quando administramos 30.000 UI de vitamina D3/Kg/dia, verificamos como consequência uma redução na oxidação de lipídeos e proteínas, similar ao observado na dose de 5.000 UI/Kg/dia, bem como uma diminuição na oxidação da 2',7'-diclorofluoresceína (DCFH) em córtex, o que concorda com os dados de oxidação em biomoléculas. Além disso, verificamos uma modulação das defesas antioxidantes enzimáticas e não enzimáticas tanto no córtex quanto no hipocampo. As atividades da CAT e da GPx no córtex cerebral encontraram-se reduzidas, enquanto a GSH estava aumentada, provavelmente em função da redução de uso pela GPx. Entre os produtos do metabolismo da vitamina D está o 24,25$(OH)_2$D, que possui a capacidade de se ligar e inibir a atividade da CAT (Khanal and Nemere, 2007; Larsson et al., 2006). Entretanto, a impossibilidade de medir os níveis de 24,25$(OH)_2$D em nosso modelo animal não nos permite definir se esse metabólito é responsável pela inibição da CAT em córtex. Além disso, em oposição aos dados em córtex, a atividade da CAT encontra-se aumentada em hipocampo. Esse efeito não é inédito, desde que Javanbakht et al. (2010) demonstraram um aumento na atividade da CAT em pacientes com dermatite atópica tratados com 1.600 UI de vitamina D/dia.

Considerando que a administração de vitamina D foi capaz de alterar o perfil redox dos tecidos estudados, nosso próximo objetivo foi verificar o envolvimento do metabólito mais ativo da vitamina D, 1α,25$(OH)_2$D, nas alterações verificadas *in* vivo, utilizando uma abordagem que permita a avaliação do efeito direto, *in vitro*, desse metabólito. Amostras frescas de

coração, fígado e cérebro de ratos sadios foram obtidas e incubadas em banho-maria metabólico a uma temperatura de 37ºC, durante uma hora, em seis grupos diferentes: grupo controle (tampão Dulbecco pH 7,4); grupo etanol controle (veículo do calcitriol, 10% de etanol em tampão Dulbecco pH 7,4); e 1α,25$(OH)_2$D nas concentrações finais de 50 nM, 100 nM, 500 nM e 1.000 nM.

No coração, a incubação com o 1α,25$(OH)_2$D promoveu um aumento da peroxidação lipídica, apesar dos demais parâmetros indicarem um efeito protetor sobre esse tecido. A produção de espécies reativas e os níveis de carbonilas proteicas foi reduzido pela incubação com 1.000 nM de calcitriol. Além disso, a incubação com 1.000 nM de 1α,25$(OH)_2$D modificou o status atividade, aumentando a atividade das enzimas SOD e GPx, bem como os níveis de GSH reduzida. Concentrações de calcitriol consideradas baixas e normais também melhoraram o status antioxidante, aumentando a atividade das enzimas SOD e CAT cardíacas. Em conjunto, nossos dados indicam que o calcitriol exerce um efeito cardioprotetor, reduzindo o dano oxidativo e aumentando a atividade da rede antioxidante formada por GSH e enzimas que detoxificam ERO, tais como superóxido e peróxido de hidrogênio. Nossos dados estão de acordo com estudos publicados, que relatam um aumento nos níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) em fígado de animais tratados com 25(OH)D, um precursor do calcitriol (Noyan et al. 2005). Em oposição, Izquierdo et al. (2012) recentemente mostraram que a administração de paricalcitol em 19 pacientes promoveu uma redução na lipoperoxidação e nos níveis de carbonilas, associada a um aumento na atividade das enzimas antioxidantes SOD, CAT e conteúdo de

GSH em sangue periférico, o que está de acordo com nossos dados obtidos em músculo cardíaco. Efeitos similares da administração de vitamina D foram obtidos em pacientes portadores de dermatite atópica (Javanbakht et al., 2010).

Apesar de nossos resultados mostrarem que o calcitriol na concentração de 1.000 nM possui um efeito direto sobre o coração, aumentando a capacidade antioxidante do tecido e reduzindo a produção de espécies reativas e a oxidação de proteínas; esses efeitos cardioprotetores não foram encontrados quando a vitamina D3 foi administrada via gavagem aos animais. Nesse contexto, comparando os dois modelos estudados, verificamos que a cardioproteção verificada nos estudos *in vitro* não se manteve no estudo *in vivo*, sugerindo que o efeito da administração sistêmica da vitamina D tem efeitos mediados não apenas pelo 1α,25$(OH)_2$D. Acreditamos que os efeitos benéficos ao sistema cardiovascular atribuídos à vitamina D (Salusky e Goodman 2002; Artaza et al. 2009) parecem não estar envolvidos com a modulação do estado redox celular, ao menos em resposta ao 1α,25$(OH)_2$D. Ainda, a administração de 30.000 UI de vitamina D/Kg/dia induziu uma redução nos níveis de peroxidação lipídica, medidos pelo TBARS, o que discorda com os dados de nosso estudo in vitro, quando a incubação de fatias de coração com 1.000 nM de 1α,25$(OH)_2$D promoveu um incremento na lipoperoxidação.

O fígado foi minimamente afetado, o sistema antioxidante enzimático apresentou uma redução na atividade da CAT, compensada pela ativação da GPx. Nenhum parâmetro de dano oxidativo ou produção de espécies reativas foi afetado nesse modelo *in vitro*. Apesar de o fígado ser um sítio

importante no metabolismo da vitamina D (Stokes et al., 2013; Hanukoglu, 2006), aqui utilizamos o metabólito ativo já hidroxilado na posição 25, o que possivelmente contribuiu para a ausência de efeitos oxidativos nesse tecido, tais como os verificados no modelo *in vivo*. A administração de calcitriol parece ter efeito protetor sobre o fígado de ratos tratados com toxina carcinogênica, prevenindo o dano ao DNA e modulando a atividade das enzimas antioxidantes, aumentando a atividade da SOD, glutationa-S-transferase e níveis de GSH.

A vitamina D é considerada um neuro-esteroide, com estrutura química altamente lipofílica, e capacidade de interagir com a membrana plasmática (Wiseman, 1993). Por outro lado, moléculas lipofílicas, como o calcitriol, podem diminuir a integridade das membranas de vesículas sinápticas, facilitando a liberação não especifica de dopamina para o citoplasma, um local bem menos ácido e que facilita a autooxidação de dopamina, produzindo ERO (Smythies e Galzigna 1998). Em fatias de córtex cerebral, a incubação com calcitriol promoveu um efeito neurotóxico bastante pronunciado, evidenciado pelo aumento na produção de espécies reativas, detectadas pela oxidação da DCFH, e aumento na oxidação lipídica, induzidos por concentração normo e hipovitamínicas, respectivamente. Existem indicativos de que a detoxificação de peróxido de hidrogênio também foi afetada, como demonstra a inibição das enzimas CAT (em altas concentração de calcitriol) e GPx (em todas as concentrações testadas). O aumento nos níveis de GSH em córtex podem ser resultado da redução na atividade da GPx, já que nesse modelo *in vitro*, com apenas 1 hora de incubação, é pouco provável que tenha ocorrido um aumento significativo na

síntese local desse antioxidante. Apesar disso, tem sido demonstrado que o 1α,25$(OH)_2$D regula a expressão e atividade de enzimas que atuam na síntese desse antioxidante, em cultura primária de astrócitos de ratos (Garcion et al., 1999) Já está bem estabelecido que um excesso de peróxido de hidrogênio pode ser utilizado, via reação de Fenton, para a síntese de radical hidroxila (Halliwell e Gutteridge, 2006), o que poderia estar associado aos efeitos prejudiciais verificados nessa estrutura cerebral.

O hipocampo também se mostrou sensível à incubação com 1α,25$(OH)_2$D, em relação ao desequilíbrio do ambiente redox. Observamos uma redução da oxidação da DCFH em todas as concentrações testadas, indicando uma redução de espécies reativas. A incubação com 50 e 100 nM decalcitriol parece ser protetora, não alterando a razão SOD/(CAT+GPX), apesar de 50 nM de calcitriol aumentar a atividade da CAT, e de 100 nM induzir um aumento da SOD e redução da GPx. Por outro lado, as concentrações mais altas de 1α,25$(OH)_2$D afetaram de forma mais intensa a atividade da GPx, resultando em desequilíbrio na razão das enzimas antioxidantes. A administração sistêmica de vitamina D, em doses consideradas baixas, atenuou o dano oxidativo causado pela administração de ferro no hipocampo de ratos, evidenciado pela redução no índice de peroxidação lipídica e no número de neurônios apoptóticos (Chen et al. 2003).

Nossos dados referentes ao estudo *in vivo* indicam que a dose de 5.000 UI/Kg/dia parece ser segura, ao menos quando consideramos a avaliação do estado redox tecidual. Por outro lado, a suplementação com 30.000 UI de vitamina D/Kg/dia promove efeitos prejudiciais no fígado e no

SNC, sugerindo o desenvolvimento de estresse oxidativo. Quando comparamos os resultados obtidos na avaliação do efeito do metabólito mais ativo da vitamina D, calcitriol, com os dados do experimento *in vivo* observamos diversas discrepâncias quanto aos efeitos sobre a oxidação de biomoléculas e a rede antioxidante, indicando que os efeitos verificados no trabalho *in vivo* não são devidos apenas ao calcitriol, mas a algum outro metabólito da vitamina D, ou à combinação deles.

5. CONCLUSÕES

A suplementação terapêutica (5.000 UI/Kg/dia) de vitamina D parece ser segura, ao menos no que tange o estado redox celular avaliado nesse estudo, demonstrando predominantemente um papel antioxidante. No entanto, quando uma dose alta (30.000 UI/kg/dia) foi administrada, verificaram-se efeitos perturbadores no sistema redox, principalmente sobre as enzimas antioxidantes no fígado e no córtex cerebral. De uma forma geral, nossos dados mostraram que:

- A administração de 30.000 UI de vitamina D3/Kg/dia promoveu uma redução no índice de peroxidação lipídica, TBARS, e não alterou os parâmetros antioxidantes, enquanto a administração de 5.000 UI não afetou nenhum dos parâmetros avaliados.
- O fígado foi afetado na menor dose, apresentando um aumento na oxidação de proteínas, bem como em animais que receberam a maior dose de vitamina, apresentando um aumento nas carbonilas proteicas e uma redução na atividade antioxidante da CAT e da GPx.
- O córtex e o hipocampo de ratos tratados com 5.000 ou 30.000 UI de vitamina/Kg/dia apresentaram uma redução nos parâmetros de dano celular. Enquanto apenas a CAT foi ativada no hipocampo, verificamos uma redução na atividade da CAT e da GPx em córtex de ratos tratados com a dose mais alta, o que pode comprometer o status redox celular.

A fim de verificar a contribuição do calcitriol nos efeitos redox ativos da vitamina D, realizamos um estudo avaliando o efeito *in vitro* desse metabólito. O presente trabalho forneceu a evidências de que o 1α,25$(OH)_2$D age de forma diferente de acordo com o tecido e a concentração estudadas,

podendo atuar como um antioxidante ou como um pró-oxidante. Nossos resultados mostraram que:

- A incubação de fatias de coração com 1.000 nM de calcitriol resultou em redução na produção de espécies reativas e na carbonilação de proteínas, enquanto a oxidação de lipídeos parece estar aumentada. O status antioxidante enzimático e os níveis de GSH apresentaram um incremento, indicando que mesmo níveis suprafisiológicos de 1α,25$(OH)_2$D parecem resultar em cardioproteção.
- O fígado foi minimamente afetado pela incubação com calcitriol, onde apenas concentrações de 1.000 nM inibiram a CAT e ativaram a GPx, como um possível efeito compensatório.
- O córtex cerebral e o hipocampo foram afetados de forma significativa pela incubação com calcitriol. De uma forma geral, a incubação com 1α,25$(OH)_2$D induziu lipoperoxidação, aumentou a produção de espécies reativas e inibiu as enzimas CAT e GPx, promovendo um desequilíbrio na detoxificação de ERO. O hipocampo também apresentou alterações na atividade das enzimas antioxidantes, entretanto, as espécies reativas detectadas pela oxidação do DCFH estavam reduzidas.

Avaliando o conjunto de dados obtidos nesse trabalho, podemos concluir que os efeitos verificados *in vivo*, por meio da administração da vitamina D3, são conflitantes quando comparados com os dados obtidos pela ação direta do metabólito mais ativo dessa vitamina, o calcitriol; indicando que os efeitos sistêmicos da vitamina D não são atribuídos apenas ao calcitriol, e podem ser o resultado de um efeito combinado dos metabólitos da vitamina D. Ainda, nossos achados indicam que a suplementação de

5.000 UI de vitamina D/Kg/dia é bem tolerada pelos animais, alterando o status redox para um estado antioxidante, enquanto que a suplementação com 30.000 UI de vitamina D/Kg/dia possui características pró-oxidantes. Nossos dados suportam a importância de novos estudos a fim de estabelecer doses seguras de vitamina D em humanos.

6. PERSPECTIVAS

Os resultados obtidos no presente trabalho abrem espaço para pesquisas mais apuradas no que diz respeito à variação dos parâmetros moleculares. É imprescindível investigar se os efeitos aqui encontrados são mediados pelos metabólitos da vitamina D (1α,25$(OH)_2$D; 24,25$(OH)_2$D e 25(OH)D), via ativação dos receptores nucleares e de membrana, das vias de sinalização mediadas por esses receptores, bem como avaliar os genes transcritos como resultado da ativação dessas vias de sinalização.

No futuro, pretendemos descrever a fundo os mecanismos envolvidos nas adaptações metabólicas observadas, tais como os efeitos oxidativos dos demais metabólitos da vitamina D, a quantificação por imunoconteúdo das enzimas de defesa antioxidantes e dos receptores de vitamina D nos tecidos estudados.

Também consideramos como importantes perspectivas verificar se existe morte celular nos tecidos afetados pela vitamina D e seus metabólitos, bem como caracterizar o tipo de morte celular.

Ainda, investigar as consequências e impactos das modulações moleculares e redox sobre o comportamento de animais submetidos à administração de vitamina D.

Por fim, avaliar se as modificações oxidativas mediadas pela vitamina D e seus metabólitos são transferidas às novas gerações, via programação metabólica.

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8. ANEXOS

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The manuscript should be typewritten, double-spaced, on paper 8.5 × 11 in. (or ISO A4). Page and line numbers should be used, beginning with the title page. For material that is to be set in italics, use an italic font; do not underline. Use capital letters only when the letters or words should appear in capitals.

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Report

Chief Medical Office. 2004. At least five a week: evidence of the impact of physical activity and its relationship to health. Department of Health, Waterloo, UK.

Book

Dishman, R.K., and Dunn, A.L. 1988. Exercise adherence: its impact on public health. Human Kinetics, Champaign, Ill.

Part of book

Healey, M.C. 1980. The ecology of juvenile salmon in Georgia Strait, Britsh Columbia.*In* Salmonid ecosystems of the North Pacific.*Edited by* W.J. McNeil and D.C. Himsworth. Oregon State University Press, Corvallis, Oreg. pp. 203–229.

Paper in conference proceedings

Kline, V.M., and McClintock, T. 1994. Effect of burning on a dry oak forest infested with woody exotics. *In* Proceedings of the 13th North American Prairie Conference: Spirit of the Land, Our Prairie Legacy, Windsor, Ont., 6–9 August 1992. *Edited by* R.G. Wickett, P.D. Lewis, A. Woodcliffe, and P. Pratt. Department of Parks and Recreation, Windsor, Ont. pp. 207–213.

Institutional publications and pamphlets

Dzikowski, P.A., Kirby, G., Read, G., and Richards, W.G. 1984. The climate for agriculture in Atlantic Canada.Available from the Atlantic Advisory Committee on Agrometeorology, Halifax, N.S. Publ. ACA 84-2-500. Agdex No. 070.

Thesis

Keller, C.P. 1987. The role of polysaccharidases in acid wall loosening of epidermal tissue from young*Phaseolus vulgaris* L. hypocotyls.M.Sc.thesis, Department of Botany, The University of British Columbia, Vancouver, B.C.

Electronic citation

Quinion, M.B. 1998. Citing online sources: advice on online citation formats [online]. Available from www.worldwidewords.org/articles/citation.htm [accessed 20 October 2005].

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Manuscript guidelines

Style guides

As a general guide for biological terms, *Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers* (7th ed., 2006) published by the Council of Science Editors, 12100 Sunset Hills Rd., Suite 130, Reston, VA 20190, USA, is recommended.

Abbreviations and acronyms

Acronyms should be defined when they are first mentioned in the text. Abbreviations and acronyms that are standard in the discipline need not be defined. Abbreviate terms denoting units of mass and measurement in the text only when they are preceded by numerals.

Units of measurement

SI units (Système international d'unités) should be used or SI equivalents should be given. This system is explained and other useful information is given in the *Metric Practice Guide* (2000), CAN/CSA-Z234.1-00, published by the Canadian Standards Association (5060 Spectrum Way, Mississauga, ON M9W 1R3, Canada). For practical reasons, some exceptions to SI units are allowed. Units such as kilocalorie and atmosphere may be used for the foreseeable future.

Statistical analyses

The assumptions and (or) the model underlying any statistical analysis should be clearly stated. Symbols such as * and **, denoting levels of significance, should **not** be used except in conjunction with the actual values of the associated test statistic; actual p values are preferred.

Nomenclature

Enzymes

For enzyme nomenclature, *Enzyme Nomenclature (1992): Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology* (Academic Press, San Diego, Calif.) should be followed.

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Trade names of drugs may be mentioned in parentheses in the first text reference to a drug, but generic names should be used in the text, tables, and figures. When a trade name is used, it should be capitalized; generic or chemical names are not capitalized. The chemical nature of new drugs must be given when it is known. The form of the drug used in calculations of doses (e.g., base or salt) should be indicated. When several drugs are used, it may save space to include a separate paragraph in Methods or a separate table listing relevant information about all drugs employed.

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Dates should be written in the sequence day–month–year without internal punctuation (On 9 October 1983 …).

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Submission of a manuscript will be held to imply that it contains original unpublished work and is not being submitted for publication elsewhere. In addition, the authors must respond to the questions related to the conflict of interest policy and scientific conduct and publication ethics.
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The editorial office reserves the right to reject manuscripts that do not comply with these requirements.

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All authors listed in a manuscript submitted to Nutrition Research must have contributed substantially to the work, participated in the writing of the manuscript, and seen and approved the submitted version. All individuals who have contributed to the writing of the manuscript must be listed as authors.

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Text must be in 12-point font (Times New Roman or Arial), double-spaced, with 1-inch margins. Consecutive line numbers must be included in the left margin, starting with the title page and ending with the reference section. Page numbers must be included in the bottom right-hand corner of each page. Text must be aligned to the left only and include 2 hard returns at the end of each paragraph, heading, and subheading.

Text should be clear and concise. Tables, figures and references must be cited in sequence in the text. Past tense should be used in reference to the work on which the paper is based, while present tense is normally limited to existing knowledge and prevailing concepts. Previous knowledge and new contributions should be clearly differentiated.

File Submission and Arrangement: Files must be uploaded in the following order: cover letter, revision letter (when applicable), checklist, text, tables, and figures. All manuscript files must be uploaded separately and in an editable format (cover letter, revision letter, and checklist may be in PDF form). PDF files are not accepted for the text, table, or figure files. After all the files are uploaded, a PDF file is generated automatically.

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Statement that the manuscript has not been submitted elsewhere for publication.

Signatures of all authors and statement that all authors have contributed to the work and agree to submit it for consideration to Nutrition Research.

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Document File • Title page - page 1

Title - single, declarative statement, stating the major finding of the work.

First name, Middle initial, and Last name of each author (no titles such as MD or PhD).

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Running heads, word counts, and any other information other than that stated above should not be included.

• Abbreviations page - page 2

Must include 1 abbreviation with meaning per line.

Abbreviations should be listed first followed by a semicolon and then the meaning.

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A single, double-spaced paragraph (250 word limit) that includes the hypothesis for the study, experimental design, use of the model for the study, major results, and conclusion.
Do not include subheadings in this section.
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List of at least 5 keywords/phrases taken from the medical subject headings of the Index Medicus (http://www.nlm.nih.gov/mesh/) below the abstract. The model used in the study must be included in the keywords.
• Manuscript text
• Main headings and subheadings must be numbered with Arabic numerals.
• Text must start on a new page and include the following main headings:
• Introduction - must state the hypothesis for the research and the supporting objectives to test the hypothesis. Must also state how this study advances human nutrition.
• Methods and materials - must explain the experimental design, control and treated groups; details of ingredient composition of diets should be presented in a table; all procedures and techniques must be explained and referenced; method of euthanasia for experimental animals must be stated; statistical analyses section must be complete with information on data presentation; must contain statistical tests and appropriate references; and must include an institutional statement of protocol approval for animal or human subjects (human consent is required).
• Results - must thoroughly describe the data presented in tables and figures.
• Discussion- should contain a specific description of the literature findings relevant to the results of the current investigation but not go beyond the data presented in the results. The limitations of the study should be included in this section.
• Acknowledgment (note spelling)
Technical or editorial assistance must be acknowledged.
Financial (grants or gifts) and other support as deemed as appropriate for the study must be indicated.
Do not include author contributions or individual titles (i.e., Dr., PhD, etc...) in this section.
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• References
Number consecutively in the order in which they are first mentioned in the text.
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References should appear as follows:

Journal articles
[1] Alzghoul MB, Gerrard D, Watkins BA, Hannon K. Ectopic expression of IGF-I and Shh by skeletal muscle inhibits disuse-mediated skeletal muscle atrophy and bone osteopenia in vivo. FASEB J 2004;18:221-3.
[2] Friedman AN, Moe SM, Perkins SM, Li Y, Watkins BA.Fish consumption

and omega-3 fatty acid status and determinants in long-term hemodialysis. Am J Kidney Dis 2006;47:1064-71.
[3] Gonzalez-Perez O, Gonzalez-Castaneda RE.Therapeutic perspectives on the combination of ?- lipoic acid and vitamin E. Nutr Res 2006;26:1-5.
Books
[4] Katz DL. Nutrition in clinical practice: a comprehensive, evidence-based manual for the practitioner. Philadelphia: Lippincott Williams & Wilkins; 2001.
Book chapters
[5] Hennig B, Toborek M, Ramadass P, Ludewig G, Robertson LW. Polychlorinated biphenyls, oxidative stress and diet. In: Preedy VR, Watson RR, editors. Reviews in food and nutrition toxicity. Vol. 3. Boca Raton: CRC Press; 2005. p. 93-128.

Note that a supplement on proper use of tables and figures is provided online via the EES website.

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Must be numbered consecutively with Arabic numerals.
Start each table on its own page.
Use minimal horizontal lines and no vertical lines.
Must have a description so that reader can understand the table without referring to the text.
Must have an explanation of the values and statistics used for analysis of the data and properly referenced.
Tables must be in an editable (word) file.

* All studies that include experimental diets must provide a table that lists the ingredients and enough detail for the nutrient content of those diets. Reference to established diets (such as AIN 93G) is appropriate when the major ingredients are listed and the premix levels are provided (actual details of each vitamin and mineral source listed is not necessary in this case). Diets that are developed with different lipid sources should provide a fatty acid compositional analysis of the lipids. In addition, studies that test a botanical or phytochemical ingredient should provide enough chemical compositional analysis as well as the amount of the active compounds.

Figures

Must be numbered consecutively with Arabic numerals.
Start each figure on its own page.
Provide clear axes labels and scale.
Use a simple space filling format (open, closed and hatched bars, etc.) for a clear and concise presentation of the data for easy interpretation.
Must have a description that the reader can understand without referring to the text.
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8.3. Lista de Figuras

8.4. Lista de Tabelas