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Investigação do mecanismo de indução de distúrbio comportamental em ratos Wistar adultos perante suplementação com vitamina A

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“Maybe it’s not too late to learn how to love and forget how to hate”

Ozzy Osbourne

Parte I

RESUMO

Fora o papel de micronutriente essencial à manutenção da fisiologia celular de mamíferos, a vitamina A é utilizada como fármaco no tratamento de patologias que vão desde distúrbios dermatológicos até certas formas de câncer. Embora a utilização de vitamina A na clínica venha sendo considerada segura, trabalhos mostram que a toxicidade exercida pela vitamina A atinge praticamente qualquer tecido, com conseqüências que podem comprometer a qualidade de vida do paciente. Além disso, o consumo inadvertido de vitamina A na forma de ésteres de retinol (palmitato de retinol, acetato de retinol) adicionados a alimentos colabora para a aquisição de mais vitamina A além daquilo recomendado para a vida saudável. Alguns estudos mostram que, a nível cognitivo, a ingestão excessiva de vitamina A pode levar à irritabilidade, distúrbios do sono, distúrbios alimentares, ansiedade, déficits de aprendizado e de memória, depressão e tentativa de suicídio. No entanto, embora estes efeitos sejam alarmantes e, mais preocupante, possam atingir indivíduos que não têm histórico de doença mental na família, não se tem uma clara visão acerca dos mecanismos envolvidos no desenvolvimento destes males. Então, decidimos investigar, neste trabalho, as conseqüências de uma suplementação com vitamina A na forma de palmitato de retinol nas doses de 1000, 2500, 4500 e 9000 UI/kg.dia^{-1} por 28 dias sobre os estados redox e bioenergético de regiões cerebrais envolvidas em disfunções neuropsiquiátricas como ansiedade e depressão (*substantia nigra*, estriado, hipocampo, hipotálamo, córtex frontal, córtex cerebral total e cerebelo) em ratos Wistar machos adultos (3 meses). Ainda, analisamos o imunoconteúdo de α- e β- sinucleínas, bem como o do receptor para produtos terminais de glicação avançada (RAGE) e do receptor dopaminérgico D2. Também avaliamos o efeito da vitamina A sobre a quantidade de fator de necrose tumoral-α (TNF-α) e o destino celular via ativação das enzimas pró-apoptóticas caspase-3 (via intrínseca) e caspase-8 (via extrínseca). Além destes parâmetros, observamos o comportamento dos animais em campo aberto e em três tarefas diferentes de abordagem de depressão. Encontramos disfunção mitocondrial considerando a cadeia transportadora de elétrons, aumento na quantidade de α-sinucleína e de RAGE, diminuição no nível de receptor D2 e aumento na atividade de caspase-3. A nível comportamental, a suplementação com vitamina A induziu ansiedade, mas não depressão neste modelo experimental. Assim, tais alterações, algumas vezes globais no sistema nervoso central (SNC) dos animais, podem ter um importante papel no desenvolvimento de distúrbio cognitivo, como ansiedade e, provavelmente, possa causar uma facilitação para o estabelecimento de futura neurodegeneração e declínio cognitivo.

ABSTRACT

In addition to the role of essential micronutrient to the maintenance of mammalian cells physiology, vitamin A has been utilized as an alternative drug during therapy of diseases that vary from dermatological disturbances to some types of cancer. Even thought the clinical utilization of vitamin A has been take as safe, some works demonstrate that the toxicity elicited by vitamin A affects virtually any tissue, leading to decreased life quality. Furthermore, inadvertent intake of vitamin A through vitamin A esters added to food facilitates the acquisition of excessive vitamin A. Some studies have shown that excessive vitamin A intake may induce cognitive decline, including irritability, sleep disturbances, metabolic impairments, anxiety, decreased capacities to learn and memory, depression, and suicide ideation. Nevertheless, even thought the alarming effects seen here and, more concerning, may affect subjects without prior clinical history to mental illness, the mechanisms around the development of such effects are not completely understood. Then, we decided to investigate here the consequences of vitamin A supplementation as retinol palmitate at 1000, 2500, 4500, or 9000 IU/kg.day-1 for 28 days on redox and bioenergetics states in cerebral regions that may be affected during neuropsychiatric dysfunctions, for instance anxiety and depression (*substantia nigra*, *striatum*, hippocampus, hypothalamus, frontal cortex, total cerebral cortex, and cerebellum), in adult male Wistar rats (90 days old). Moreover, we analyzed the amount of α- e β-synucleins, as well as that of receptor for advanced glycation end products (RAGE) and dopaminergic receptor D2. We also investigated the effect of vitamin A supplementation on tumor necrosis factor-α (TNF-α) levels and, regarding cell fate, we quantified caspase-3 (intrinsic pathway) and caspase-8 (extrinsic pathway) enzyme activity, which are pro-apoptotic enzymes. In addition to these molecular parameters, we have observed rat behavior in an open field apparatus and during three different tasks related to the study of depression. Overall, we found mitochondrial dysfunction regarding the mitochondrial transfer chain, increased amounts of α-synuclein and RAGE, decreased D2 levels, and increased caspase-3 enzyme activity. Regarding rat behavior, vitamin A supplementation induced anxiety-like behavior, but not depression-related behavior in this experimental model. Concluding, the effects elicited by vitamin A supplementation, which were observed to affect virtually any rat brain region investigated here, may take an important role during cognitive impairment, as for example anxiety, and it is plausible that this vitamin may induce a facilitation to future neurodegeneration and cognitive decline.

LISTA DE ABREVIATURAS

BHE – barreira hematoencefálica

CAT – catalase

CRABP – proteína ligadora de ácido retinóico citosólica, tipos I e II

CRBP – proteína ligadora de retinol citosólica

Cu/Zn-SOD – superóxido dismutase cobre/zinco

GPx – glutationa peroxidase

GR – glutationa redutase

GSH – glutationa reduzida

GST – glutationa S-transferase

Mn-SOD – superóxido dismutase manganês

RA – ácido retinóico

RAGE – receptor para produtos terminais de glicação avançada

RAR – receptor para ácidos retinóicos

RBP – proteína ligadora de retinol

RXR – receptor para retinóides

SNC – sistema nervoso central

TNF-α - fator de necrose tumoral

1. INTRODUÇÃO

A vitamina A foi descoberta em 1909 por Stepp em uma fração lipossolúvel da gema de ovo (Stepp, 1909). Desde então, muito tem se descoberto acerca dos papéis da vitamina A tanto biologicamente quanto como fármaco. A vitamina A é um micronutriente essencial ao ser humano, o que significa que deve ser ingerida, diariamente, numa pequena taxa, pois sua síntese não ocorre em células de mamíferos. A ingestão diária de vitamina A recomendada é de 600 μg (equivalendo a 2000 UI/dia) para mulheres e de 700 μg (o que equivale a 2333 UI/dia) para homens (Olson, 1996). A partir da vitamina A (também chamada retinol), são obtidos os retinóides, os quais medeiam parte dos fenômenos necessários tanto ao disparo de um evento biológico quanto à manutenção deste. Como exemplo, podemos citar a indução de diferenciação celular dependente de vitamina A que ocorre no sistema nervoso central (SNC) e a manutenção da homeostasia central via síntese de neurotransmissores e plasticidade neuronal, necessárias, também, aos processos de aprendizagem e de memória, os quais perduram ao longo de toda a vida do indivíduo (Mey e McCaffery, 2004; Lane e Bailey, 2005; Dräger, 2006). Considerando o SNC de mamíferos, estão confirmadas as presenças de transportadores de retinol e de retinóides na barreira hematoencefálica (MacDonald *et al*., 1990), de enzimas do metabolismo da vitamina A e de receptores nucleares em neurônios e células da glia (Zetterstrom *et al*., 1999); logo, o SNC é um potencial alvo tanto fisiológica quanto farmacologicamente da vitamina A e de seus derivados.

1.1. Metabolismo da vitamina A

A vitamina A pode ser obtida a partir da dieta como pré-vitamina A (alimentos de origem animal contendo palmitato de retinol ou acetato de retinol – dois ésteres de vitamina A) ou pró-vitamina A (alimentos de origem vegetal contendo carotenóides), e será armazenada no fígado, principalmente, sendo que alguns outros tipos celulares também podem estocar vitamina A após sua esterificação, o que garante sua estabilidade e posterior consumo durante diminuições na disponibilidade da vitamina às células de mamíferos (Napoli, 1999).

Os ésteres de retinil ingeridos são hidrolizados a retinol por hidrolases entéricas. O retinol e os carotenóides são absorvidos por células da mucosa intestinal. A partir dos carotenóides, pode-se obter retinóides por dois meios: primeiro, retinal pode ser sintetizado por clivagem oxidativa da ligação dupla central do carotenóide, seguido pela redução a retinol por uma retinal redutase microssomal; segundo, apo-carotenóides são formados através de clivagens excêntricas seguidas por transformação dos ácidos apo-carotenóides em ácidos retinóicos. Após retinal ser reduzido a retinol, este é re-esterificado, nas células da borda em escova do intestino, e embalado em partículas de quilomícrons. Os quilomícrons são, então, parcialmente degradados através da ação de lípases, em tecidos extra-hepáticos, gerando remanescentes de quilomícrons, que contêm praticamente, todo o complemento original de ésteres de retinil. Os ésteres são captados a partir daqueles remanescentes por células hepáticas e, enzimaticamente, clivados, em endossomos, fornecendo vitamina A livre. A vitamina A formada pode ser, novamente, esterificada, no retículo endoplasmático, e transferida às células estreladas hepáticas para estoque ou pode ser excretada da célula.

Em meios aquosos (como na circulação e no citoplasma, por exemplo), o retinol e os retinóides circulam após ligarem-se a proteínas específicas de transporte, as proteínas de ligação ao retinol (RBP), as quais regulam as ações destas moléculas hidrofóbicas, servindo de verdadeiros tampões de retinol e de retinóides. No plasma, a RBP medeia esta modulação, mas no citoplasma, há proteínas de ligação aos ácidos retinóicos (CRABP-I e –II) regulando a liberação e a solubilidade dos retinóides. Enquanto CRABP-I participa no metabolismo dos ácidos retinóicos, CRABP-II os transporta para o núcleo, onde se ligarão aos receptores nucleares. Ainda, a proteína celular de ligação ao retinol (CRBP) mantém retinol no citoplasma e promove ou metabolismo de retinol a retinaldeído ou esterificação a ésteres de retinol, necessários ao armazenamento do retinol (Napoli, 1999). Este mecanismo de transporte intra (CRABP-I, CRABP- II e CRBP) e extracelular (RBP) é importante para assegurar o destino desses retinóides. Atua tanto na proteção de diferentes estruturas, evitando a interação desses compostos com as membranas, assim como na manutenção das propriedades dos retinóides, impedindo-os de que sofram oxidação ou algum processo de isomerização. Além disso, a CRBP parece participar da regulação da captação de retinol. Quando a quantidade de retinol livre intracelular supera a de CRBP, tem-se um aumento da concentração de retinol intracelular. Uma possível explicação seria que, neste caso, a parte interna do transportador de membrana permanecesse ligada ao retinol (devido à menor quantidade de CRBP), bloqueando a continuidade da sua captação para o meio intracelular. Já caso a quantidade de CRBP exceda a de retinol, a sua captação estaria facilitada, uma vez que o transportador não estaria bloqueado, pois o retinol rapidamente formaria um complexo com a CRBP liberando o transportador (Napoli, 1999).

Após o retinol ser captado pela célula e ligar-se à CRBP, o complexo retinol-CRBP formado pode seguir diferentes caminhos conforme a necessidade da célula:

armazenamento na forma de éster de retinol, ou a formação de ácido retinóico. No primeiro caso o retinol-CRBP será diretamente esterificado sob ação da enzima lecitina:retinol aciltransferase. Já no segundo (síntese de ácido retinóico), o complexo retinol-CRBP sofre ação da enzima retinol desidrogenase para a formação de retinal (reação reversível). Em seguida, as retinal desidrogenases citosólicas catalisam a formação de RA a partir de retinal (reação termodinamicamente irreversível). O RA pode ser convertido a outros derivados de retinol, como 9-*cis*-RA e 13-*cis*-RA. Porém, caso o ácido retinóico ligue-se à CRABPII, ele será conduzido ao núcleo celular, onde exercerão seus efeitos via ligação aos seus receptores nucleares (Napoli, 1999).

A vitamina A e seus derivados induzem seus efeitos (pelo menos em parte) por meio de receptores nucleares para ácidos retinóicos (RAR) e para retinóides (RXR), os quais passam a agir como fatores de transcrição após ativação. Ainda, tem-se descrito que a vitamina A e os retinóides também disparam sinais não associados ao núcleo celular, como ocorre via ativação de RAR ou de RXR, agindo, portanto, por uma via não-genômica (também chamada de extra-nuclear). A via de sinalização vitamina A-dependente não-genômica pode ser estimulada via formação de radicais livres ou de espécies reativas do oxigênio, como veremos adiante.

É importante mencionar que trabalhos com animais abordando a biodisponibilidade de vitamina A e suas características farmacodinâmicas e farmacocinéticas nestes e as comparando com aquilo observado em humanos mostram que, nestes aspectos, a vitamina A é metabolizada muito similarmente, o que torna o uso de animais como ratos e camundongos uma excelente ferramenta na investigação de riscos associados à utilização de vitamina A e seus retinóides como suplemento alimentar e/ou fármaco, conforme mencionado na subseção abaixo (Kerr *et al*., 1982; Branzzell *et al*., 1983; Nulman *et al*.,

1998; Hendrix *et al*., 2004; Ferguson *et al*., 2006). Na verdade, nosso grupo (De Oliveira e Moreira, 2007; De Oliveira *et al*., 2007a,b; De Oliveira *et al*., 2008) e outros importantes grupos de pesquisa internacionais têm investigado os efeitos da suplementação com vitamina A/retinóides em animais baseando-se em doses que são recomendadas a humanos (Allen e Haskell, 2002; Myhre *et al*., 2003; Crandall *et al*., 2004; Lane e Bailey, 2005; Ferguson *et al*., 2006; O´Reilly *et* al., 2006), e isto tem sido bem aceito na literatura científica, pois, conforme mencionado acima, a distribuição tecidual destas moléculas é bastante similar considerando-se o grupo dos mamíferos.

1.2. Vitamina A e retinóides como fármacos

Além de modular morfológica e bioquimicamente diferentes tipos celulares, tais como células do sistema imune, células epiteliais, hepatócitos e neurônios, a vitamina A pode ser prescrita durante o tratamento de alguns estados patológicos. O uso terapêutico de vitamina A pode ocorrer na forma de palmitato de retinol ou de ácido retinóico 13-*cis*, e o tratamento pode ser co-adjuvante ou único, dependendo da situação. Na leucemia pró-mielocítica aguda, as doses de vitamina A prescritas chegam 150000 $UI.dia^{-1}$ para crianças (idades entre 7 e 15 anos) durante prazo indeterminado (Tsunati *et al*., 1990; Tsunati *et al*., 1991; Fenaux *et al*., 2001; Allen e Haskell, 2002; Myhre *et al*., 2003). Em tratamento objetivando-se ganho de peso em bebês prematuros, as doses utilizadas podem passar de 8500 $UI/kg.dia^{-1}$, também ocorrendo por período indeterminado, o qual pode durar alguns meses (Mactier e Weaver, 2005). Para tratamento de distúrbios dermatológicos, a vitamina A é administrada tanto quanto palmitato de retinol quanto como ácido retinóico 13-*cis*, sendo este último foco de inúmeros debates entre pesquisadores de saúde pública, visto que foram descritos efeitos colaterais bastante preocupantes quando do uso crônico (3-6 meses de tratamento diário) deste fármaco por indivíduos sofrendo de acne nodular (Allen e Haskell, 2002; O'Reilly *et al*., 2008).

1.3. Vitamina A e neurotoxicidade

Embora se tenha observado algumas conseqüências positivas que podem ser atribuídas à administração de vitamina A nos casos clínicos mencionados acima, também tem sido publicado um grande número de efeitos negativos, principalmente relacionados à

utilização crônica de vitamina A (neste caso, considera-se crônico o período compreendendo 3 meses ou mais). Também é notável a utilização de vitamina A como agente antioxidante, que vem ocorrendo, principalmente entre mulheres, no combate ao envelhecimento. No entanto, tal administração ocorre sem prescrição médica (a conhecida "self-administration"), e pode aumentar a quantidade de vitamina A ingerida em indivíduos não afetados por qualquer deficiência vitamínica (Croquet *et al*., 2000). Neste contexto, é útil salientar o papel que os alimentos enriquecidos (ou fortificados) com vitamina A tem em aumentar as chances de intoxicação aguda com vitamina A: pães, margarinas, farinhas, achocolatados, e mais um número de produtos que beira o infinito. Na verdade, a adição de palmitato de retinol (um precursor natural, mas também industrialmente produzido e comercializado da vitamina A) a gomas de mascar tem sido motivo de preocupação, pois já se evidenciou sua capacidade de induzir intoxicação alimentar em crianças (Lam *et al*., 2006). Por outro lado, na longa lista de efeitos crônicos indesejáveis, encontram-se irritabilidade, distúrbio de sono, ansiedade, depressão, incluindo tentativas de suicídio, considerando-se apenas o SNC (Hazen *et al*., 1983; Hull e D'Arcy, 2003; Myhre *et al*., 2003; McCaffery *et al*., 2005; O'Reilly *et al*., 2008). Nota-se, claramente, o quanto a vitamina A pode afetar a cognição de pacientes em diferentes idades quando cronicamente administrada, o que diminui, indubitavelmente, a qualidade de vida destes. Atrelado a isto, devem ser considerados os custos com a manutenção do tratamento de tais pacientes, visto que distúrbios neurológicos são de difícil acesso quanto ao correto diagnóstico (utilização de métodos avançados e de alto nível de tecnologia e manutenção técnica), e os fármacos administrados no tratamento de patologias neurodegenerativas como doença de Parkinson, doença de Alzheimer e mesmo na depressão, por exemplo, apresentam um valor bastante significativo considerando o sistema público de saúde brasileiro. Mais recentemente, uma

publicação bastante alarmante demonstrou que o uso diário de suplementos de vitamina A em doses baixas (previamente tidas como saudáveis, antioxidantes e que preveniriam o desenvolvimento de tumores) aumenta o risco de mortalidade entre seus usuários (Bjelakovic *et al*., 2007). Então, não só a qualidade de vida do paciente afetado psiquiatricamente pelo excesso de vitamina A, mas os custos que tal tratamento representam aos cofres públicos, devem ser considerados quando vitamina A é a escolha para tratar qualquer que seja a patologia que venha a atingir indivíduos de diferentes idades. Assim, todo estudo que tenha caráter preventivo quanto a evitar, portanto, a administração errônea de vitamina A, tem o valor de impedir o surgimento de conseqüências mais graves que venham, por vezes, a se tornar irreversíveis considerando-se o ciclo de vida neuronal e mesmo efeitos mais drásticos como o suicídio.

1.4. Ambiente redox

A manutenção de um equilíbrio entre o estado oxidado e reduzido de biomoléculas e da célula como um todo é praticamente impossível quando o organismo em análise se encontra em ambiente oxigenado. Não somente o oxigênio (O_2) é o causador do desequilíbrio redox, mas outras moléculas têm papel ativo em perturbar a homeostasia redox para o lado do aumento no dano a biomoléculas, situação conhecida como estresse redox, que pode ser do tipo oxidativo, nitrosativo e outros já conhecidos atualmente (Halliwell, 2006). Contra o ataque excessivo dos agentes pró-oxidantes, que podem ser ou não radicais livres (os quais apresentam elétrons desemparelhados em seus orbitais mais externos, em contraste às espécies reativas, que não contêm elétrons desemparelhados, mas

uma reatividade aumentada em relação a moléculas neutras), há um extenso número de defesas antioxidantes, classificadas entre enzimáticas e não-enzimáticas.

As enzimas antioxidantes mais estudadas são a superoxido dismutase (SOD) – existente em pelo menos três formas em mamíferos: Cu/Zn-SOD, Mn-SOD, e SOD extracelular –, a catalase (CAT), o par glutationa peroxidase (GPx) e glutationa redutase (GR) e as peroxirredoxinas. As enzimas SOD são responsáveis pela dismutação do radical ânion superóxido ($^{\bullet}O_2^-$), transformando duas moléculas de $^{\bullet}O_2^-$ em uma de peróxido de hidrogênio (H_2O_2). A CAT, por sua vez, leva o H_2O_2 à H_2O e O_2, eliminando, assim, o risco, por exemplo, de o H_2O_2 reagir com íons Fe^{2+} ou Cu^+ (metais de transição) – através da reação de Fenton –, ou via reação de H_2O_2 com $^{\bullet}O_2^-$ – via reação de Haber-Weiss – as quais levam à produção do radical livre mais instável e, portanto, mais reativo conhecido, o radical hidroxil ($^{\bullet}OH$). Sobre o H_2O_2, também age o par GPx e GR e as peroxirredoxinas, transformando em H_2O o excesso daquele (Halliwell, 2006).

No entanto, apenas defesas antioxidantes enzimáticas não garantem uma completa segurança contra a constante formação e ataques destas moléculas pró-oxidantes. São necessárias defesas antioxidantes não-enzimáticas, tais como a glutationa, algumas vitaminas, a tiorredoxina, e outros para uma adequada defesa contra a dinâmica e a instabilidade elevadas da formação e da reação das espécies reativas e dos radicais livres.

Então, devido a este forte aporte de defesas antioxidantes, momentos de estresse oxidativo, que seriam marcados por um aumento nas taxas de dano redox a biomoléculas, são constantemente amenizados, mas nem sempre completamente neutralizados, pois os hábitos diários dos seres humanos implicam em uma alteração bastante significativa da qualidade redox celular, e isto ocorre via ingestão de drogas, de alimentos com quantidade

calórica elevada, uso e abuso de suplementos alimentares, sedentarismo, e outros fatores exógenos, como poluição e raios ultravioleta.

1.5. Vitamina A e ambiente redox

Quimicamente, a vitamina A pode ser caracterizada como um agente redox-ativo, significando que pode atuar como molécula pró-oxidante ou antioxidante, dependendo de sua concentração no meio. Nosso grupo de pesquisa tem mostrado, ao longo dos anos, que a adição de retinol em concentrações pouco acima daquelas fisiológicas para diferentes tecidos animais pode acarretar em dano oxidativo em lipídios, proteínas e DNA *in vitro*, *ex vivo*, e *in vivo* (Klamt *et al*., 2000; Dal-Pizzol *et al*., 2000, 2001; Frota Jr. e*t al*., 2004, 2006; Klamt *et al*., 2005; Gelain *et al*., 2006; De Oliveira e Moreira, 2007; De Oliveira *et al*., 2007a,b; De Oliveira *et al*., 2008). Em parte, tais efeitos se devem a uma perturbação que o retinol induz na estrutura mitocondrial, diminuindo sua dinâmica funcional e levando a um aumento na produção de radical ânion superóxido ($^{\bullet}O_2^-$), o qual age *per se* como um radical livre levando a dano oxidativo a biomoléculas, mas também pode atuar como fonte, em ambientes ácidos, para o radical hidroperoxil ($HO_2^{\bullet}$), mais potente que o primeiro (Halliwell, 2006). Recentemente, demonstramos as conseqüências de uma suplementação com vitamina A (palmitato de retinol) por diferentes períodos (3, 7 e 28 dias) em doses terapêuticas (1000, 2500, 4500, e 9000 UI/kg.dia^{-1}) sobre o ambiente redox do fígado e de algumas regiões cerebrais de ratos Wistar. Mais especificamente, estudamos o papel de vitamina A sobre a homeostasia redox do fígado e, no sistema nervoso central (SNC), investigamos o córtex cerebral, cerebelo, hipocampo, substância negra e estriado (De

Oliveira e Moreira, 2007; De Oliveira *et al*., 2007a,b; De Oliveira *et al*., 2008). Em praticamente todos os tecidos mencionados, a suplementação diária com vitamina A foi capaz de induzir disfunção redox, levando a aumento nos níveis de marcadores de dano oxidativo e, por vezes, mesmo nitrosativo, o qual conta com a participação de peroxinitrito ($ONOO^-$), uma espécie reativa bastante instável e, portanto, muito reativa. Também encontramos disfunção mitocondrial significativa associada à suplementação com vitamina A por 28 dias em qualquer dose testada, mostrando que as mitocôndrias são também alvos *in vivo* da toxicidade induzida por vitamina A em excesso, levando a uma diminuição na redução completa de O_2 a H_2O, com conseqüente produção aumentada de $^{\bullet}O_2^-$ (De Oliveira e Moreira, 2007). Mais importante, ao analisarmos as conseqüências da administração diária de vitamina A sobre as capacidades de locomoção e de exploração (capacidades motoras) e aquelas cognitivas animais, observamos diminuição nas capacidades de locomoção e de exploração e indução de comportamento tipo-ansiedade (mas não depressão) entre os animais tratados com vitamina A em qualquer dose testada por 28 dias (De Oliveira *et al*., 2007a). No entanto, não é sabido qual o papel da perturbação do equilíbrio redox induzido por vitamina A no aparecimento de declínio cognitivo naqueles animais.

1.6. Disfunção mitocondrial na neurodegeneração

A mitocôndria é a organela onde se dá a manutenção do estado energético em praticamente todas as células de mamíferos. A localização estratégica da cadeia transportadora de elétrons permite o acoplamento entre bombeamento de prótons para o

espaço intermembranas e o retorno dos mesmos via ATP sintase, resultando em síntese de ATP. No entanto, o fluxo de elétrons através de cada componente da cadeia transportadora (complexos I-IV) não é perfeito, e pode ocorrer redução parcial de O_2 (aceptor final de elétrons neste sistema) ao final da cadeia (complexo IV), resultando na produção de $^{\bullet}O_2^-$, principalmente, nos complexos I e III (sendo que o ciclo Q, que ocorre no complexo III, é um facilitador deste processo redox). Cerca de 2-5% do O_2 consumido pela cadeia transportadora de elétrons mitocondrial resulta na produção de $^{\bullet}O_2^-$, e este radical livre tem importante papel na disfunção redox que se origina de disfunção mitocondrial, por exemplo (Halliwell, 2006).

Já está claro que disfunção mitocondrial tem papel central em muitos processos neurodegenerativos, tanto participando da produção aumentada de espécies reativas de oxigênio e de nitrogênio quanto rendendo menos ATP para a manutenção do estado bioenergético. Além disso, é sabido que a via apoptótica dependente de liberação mitocondrial de citocromo c (via intrínseca) pode favorecer perda neuronal em processos de neurotoxicidade ou de pré-disposição genética (patologias herdadas) (Moreira *et al*., 2010).

Então, o estudo da função mitocondrial, bem como da qualidade de seus componentes – neste caso, qualidade redox – é importante para melhor compreender os mecanismos mediando processos de declínio cognitivo e de neurotoxicidade.

1.7. α- e β-Sinucleínas e receptor dopaminérgico D2: fisiologia e neurodegeneração

Muito tem se debatido acerca das proteínas α- e β-sinucleínas, as quais tem importante papel no eixo dopaminérgico. No entanto, pouco se sabe sobre a função exata da α-sinucleína, a não ser de que sua posição é pré-sináptica e pode ter papel no controle de neurotransmissores na fenda sináptica (Goedert, 2001; Halliwell, 2006). Já a β-sinucleína é um agente regulador da conformação e da expressão da α-sinucleína, agindo como uma chaperona desta (Goedert, 2001). Portanto, uma diminuição na quantidade de β-sinucleína pode favorecer um acúmulo e posterior agregação de α-sinucleína. A agregação citosólica de α-sinucleína ocorre tanto por mutação quanto por oxidação desta molécula, e tem sido alvo de intensos estudos em pesquisas que lidam com os mecanismos por trás da neurodegeneração observada nas doenças de Parkinson e de Lewy, onde estes agregados protéicos afetam negativamente a fisiologia neuronal (Maries *et* al., 2003; Halliwell, 2006; Kim e Lee, 2008).

Já o receptor D2 participa da regulação da liberação de dopamina, pois inibe a liberação deste neurotransmissor através de um mecanismo de retroação negativa ("feedback" negativo), evitando, assim, que um excesso desta molécula reativa se acumule tanto na fenda sináptica (Bozzi e Borrelli, 2006). A dopamina apresenta elevada reatividade em ambiente alcalino, transformando-se em semi-quinona após sofrer auto-oxidação, processo que também produz $^{\bullet}O_2^-$, outra molécula reativa e pró-oxidante (Graham, 1978; Maker *et al*., 1981; Lotharius e Brundin, 2002). Assim, a manutenção dos níveis de dopamina dentro daquilo fisiologicamente seguro é importante para assegurar a homeostasia redox do tecido nervoso.

1.8. Receptor para produtos terminais de glicação avançada (RAGE)

Um desequilíbrio oxidativo não culmina apenas na produção de moléculas danificas e estáticas, mas forma, também, produtos intermediários que podem induzir alterações conformacionais deletérias em biomoléculas vizinhas, e mesmo um sinal que pode fazer com que o desequilíbrio redox se perpetue. Neste contexto, o RAGE tem um papel central na manutenção, no desenvolvimento do desequilíbrio gerado por disfunção redox, sendo que tanto espécies reativas (e os radicais livres são espécies reativas) quanto produtos de dano oxidativo/nitrosativo podem ativar este receptor, o qual dispara cascatas de sinalização que participam do ambiente redox, levando a uma série de eventos deletérios à célula (Bierhaus *et al*., 2005). Além disso, já se sabe que a ativação de RAGE torna mais fácil o transporte do peptídeo β-amilóide através da barreira hematoencefálica (BHE), o que pode culminar em doença de Alzheimer (Takuma *et al*., 2009). A ativação de RAGE induz um estado pró-inflamatório via ativação de NFkB, o que pode levar a um estado pró-oxidante potencializado, então, que pode ser celular ou tecidual (Bierhaus *et al*., 2005). Assim, a análise da quantidade de RAGE em neurônios alvo da vitamina A é crucial para o entendimento dos mecanismos que compreendem sua neurotoxicidade.

2. OBJETIVOS DO TRABALHO

Considerando o exposto na Introdução desta tese, bem como dados anteriores de nosso grupo de pesquisa, os quais demonstram que vários parâmetros associados a dano oxidativo tanto *in vitro* quanto *in vivo* são conseqüência da suplementação com vitamina A, este trabalho tem como objetivo elucidar possíveis alterações bioquímicas relacionadas às disfunções cognitivas previamente observadas, analisando, então, a *substantia nigra*, o estriado, o hipocampo, o hipotálamo, o córtex frontal, o córtex cerebral total e o cerebelo de animais tratados diariamente com vitamina A na forma de palmitato de retinol (Arovit®) nas doses de 1000, 2500, 4500 e 9000 UI/kg por 28 dias.

2.1. Objetivos específicos

1) Avaliar o estado redox de membranas mitocondriais isoladas a partir daquelas regiões cerebrais;
2) Quantificar a atividade de complexos da cadeia transportadora de elétrons mitocondrial;
3) Quantificar os níveis de α- e β-sinucleínas, de receptor para produtos terminais de glicação avançada (RAGE), do receptor dopaminérgico D2, do fator de necrose tumoral-α (TNF-α) e do conteúdo de 3-nitrotirosina;
4) Analisar a variação na atividade das enzimas caspase-3 e caspase-8;
5) Observar alterações comportamentais compreendendo comportamento tipo-ansiedade e comportamento relacionado à depressão.

Parte II

3. MATERIAIS E MÉTODOS E RESULTADOS

Os resultados desta tese serão apresentados na forma de artigos científicos. No corpo principal da tese, foram adicionados os seis artigos diretamente associados aos efeitos da suplementação com vitamina A sobre o sistema nervoso central dos animais experimentais, sendo os demais colocados ao final da tese como anexo. Brevemente, o desenho experimental se baseia em uma suplementação com vitamina A diária via gavagem (forma de administração intragástrica) nas doses de 1000, 2500, 4500 e 9000 UI/kg a ratos machos Wistar adultos (3 meses de idade no começo do tratamento). A aplicação do tratamento se dava à noite, tendo em vista que o metabolismo destes animais é acelerado na fase noturna. Ao final dos tratamentos, os animais eram submetidos a análises comportamentais visando avaliar comportamento tipo-ansiedade e comportamento relacionado à depressão. Após estas tarefas comportamentais, os animais eram sacrificados e as regiões cerebrais da *substantia nigra*, estriado, hipocampo, hipotálamo, córtex frontal, córtex cerebral total e cerebelo foram removidas cirurgicamente e homogeneizadas em cada tampão específico a cada técnica. As amostras foram armazenadas a –80ºC até o momento das análises.

Capítulo I

"Evaluation of the effects of vitamin A supplementation on adult rat *substantia nigra* and *striatum* redox and bioenergetics states: Mitochondrial impairment, increased 3-nitrotyrosine and α-synuclein, but decreased D receptor contents"

Marcos Roberto de Oliveira, Max William Soares Oliveira, Guilherme Antônio Behr, Mariana Leivas Muller Hoff, Ricardo Fagundes da Rocha, José Cláudio Fonseca Moreira

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Evaluation of the effects of vitamin A supplementation on adult rat *substantia nigra* and *striatum* redox and bioenergetic states: Mitochondrial impairment, increased 3-nitrotyrosine and α-synuclein, but decreased D2 receptor contents

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ABSTRACT

Vitamin A at moderate to high doses is applied in the treatment of some life threatening pathological conditions, for instance cancers. Additionally, vitamin A at low concentrations is a known antioxidant molecule. However, by increasing vitamin A (or its derivatives) concentrations, there is an increase in the levels of oxidative stress markers in several experimental models. Furthermore, it was reported that vitamin A therapy at high doses might induce cognitive decline among the patients, which may become anxious or depressive, for example, depending on vitamin A levels intake. We have previously reported increased levels of oxidative stress markers in rat *substantia nigra* and *striatum*. However, the mechanism by which this vitamin altered the redox environment in such rat brain regions remains to be elucidated. In the herein presented work, we have investigated the effects of vitamin A supplementation at clinical doses (1000–9000 IU/kg day^{-1}) for 28 days on rat *substantia nigra* and *striatum* mitochondrial electron transfer chain (METC) activity, which may produce superoxide anion radical ($O_2^{\bullet-}$) when impaired. Additionally, the levels of non-enzymatic antioxidant defenses were evaluated, as well as 3-nitrotyrosine, α- and β-synucleins and TNF-α levels through ELISA assay. We observed impaired METC in both rat brain regions. Moreover, we found increased $O_2^{\bullet-}$ production and nitrotyrosine content in the nigrostriatal axis of vitamin A-treated rats, suggesting that the use of vitamin A at therapeutic doses may be rethought due to this toxic effects found here.

1. Introduction

Traditionally, vitamin A and its derivatives – the retinoids – have been viewed as antioxidant compounds (Palace et al., 1999). However, it has been demonstrated that these molecules are able to induce a pro-oxidant state in several experiments, from in vitro assays to in vivo experimental models, as discussed below. Then, vitamin A and retinoids have been called redox-active molecules, exerting either antioxidant or pro-oxidant effects depending mainly on its concentration. We have reported that vitamin A (also referred as retinol) affects the redox environment in cultured Sertoli cells, where retinol (or its derivatives, the retinoids) induced oxidative damage to lipids, proteins, and DNA, at least in part, through a mechanism involving increased superoxide anion radical ($O_2^{\bullet-}$) production in dysfunctional mitochondria (Dal-Pizzol et al., 2000, 2001; Frota et al., 2006; De Oliveira and Moreira, 2007, 2008). Indeed, retinol and some retinoic acids were demonstrated to induce swelling in mitochondria isolated from rat liver, consequently facilitating cytochrome *c* release from the organelle (Rigobello et al., 1999; Klamt et al., 2005). Mitochondrial leakage of cytochrome *c* culminates in increased free radicals production by the mitochondrial electron transfer chain (METC), since less oxygen is completely reduced to water, originating the partially reduced oxygen molecule called $O_2^{\bullet-}$, which give rise to a more potent pro-oxidant agent, namely hydroperoxyl radical, in acidic environments — thereby, increasing the risk of oxidative damage to mitochondrial membranes, an event that would maintain the pro-oxidant state, and more free radicals would be produced in a chain reaction, as reviewed elegantly by (Halliwell, 2006).

Functional mitochondria produces $O_2^{\bullet-}$ physiologically, but it was demonstrated that in some neurodegenerative processes, Alzheimer's disease (AD) and Parkinson's disease (PD) for example, free radicals production, including other more unstable species and, consequently,

Abbreviation: AD, Alzheimer's disease; CNS, central nervous system; D2R, dopamine receptor 2; GSH, reduced glutathione; GST, glutathione-S-transferase; METC, mitochondrial electron transfer chain; $NO^{\bullet}$, nitric oxide; $^{\bullet}NO_2$, nitrogen dioxide radical; $O_2^{\bullet-}$, superoxide anion radical; $^{\bullet}OH$, hydroxyl radical; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; PD, Parkinson's disease; SDH, succinate dehydrogenase; SMP, submitochondrial particles; TAR, total antioxidant reactivity; TNF-α, tumor necrosis factor-α; TRAP, total radical-trapping antioxidant parameter.

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potentially more pro-oxidants than $O_2^{-\bullet}$, is increased several fold (Halliwell, 2006). Furthermore, it was shown that under certain diets, for instance high fat diets, there is a significantly decrease in life quality due to increased rates of cognitive decline among the population, showing the importance of maintaining a nutritionally balanced food ingestion (Tanskanen et al., 2001; Song et al., 2003). In this regard, vitamin supplements use has increased among non-deficient subjects, an event that have lead supplements users to suffer from hepatotoxicity, and, more drastically, from central nervous system (CNS)-associated disturbances, for example headache, pseudotumor cerebri, and confusion, as well as cognitive impairments as irritability, anxiety, and depression (Myhre et al., 2003). Recently, it was reported that vitamin supplements use increases mortality rates among healthy subjects that have ingested daily unnecessary vitamin supplements for a long-term period (Bjelakovic et al., 2007). Among the vitamins that induce high rates of mortality it was found vitamin A, which not only induces an oxidative insult to biomolecules, but also is able to modulate negatively CNS-related functions, for instance cognition. Indeed, we have found that vitamin A supplementation at therapeutic doses induced anxiety-like behavior in adult rats submitted to a 28-day period treatment (De Oliveira et al., 2007a,b, 2008a,b). Additionally, we have observed decreased locomotory and exploratory activities performed by vitamin A-treated rats in an open field apparatus (De Oliveira and Moreira, 2007).

Since there is a lack of studies showing the in vivo effects of vitamin A at clinical doses on the CNS environment, we have decided to investigate the consequences of vitamin A supplementation at doses used in the treatment of pathologies in the field of oncology and dermatology on adult rat *substantia nigra* and *striatum* regarding METC activity, $O_2^{-\bullet}$ production in submitochondrial particles (SMP), and non-enzymatic antioxidant defenses capacity. Furthermore, we analyzed 3-nitrotyrosine and α- and β-synucleins content in the nigrostriatalaxis of vitamin A-treated rats. The retinol palmitate doses utilized in the herein presented work belong to a therapeutic range usually recommended to patients suffering from some life-threatening conditions. For example, it is prescribed long-term retinol palmitate treatment at doses exceeding 150,000 IU day^{-1} to subjects suffering from leukemia and other types of cancer (Allen and Haskell, 2002). Additionally, very-low-weight-preterm infants receive vitamin A at 8500 IU/kg day^{-1} during undetermined periods during weight gain treatment (Mactier and Weaver, 2005).

2. Materials and methods

2.1. Animals

Adult male Wistar rats (280–300 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (7:00–19:00 h), at a temperature-controlled colony room (23±1 °C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

2.2. Drugs and reagents

Arovit® (retinol palmitate, a water-soluble form of vitamin A) was purchased from Roche, Sao Paulo, SP, Brazil. Polyclonal antibody to 3-nitrotyrosine was obtained from Calbiochem, USA. Polyclonal antibodies to α- and β-synucleins and D2 receptor were purchased from Chemicon International, USA. TNF-α assay kit was obtained from BD Biosciences, San Diego, CA, USA. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A treatment was prepared daily and it occurred by protecting from light.

2.3. Treatment

The animals were treated once a day for 28 days with a gavage. The treatments were carried out at night (*i.e.* when the animals are more active and take a greater amount of food) in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. The animals were treated with vehicle (0.15 M saline; $N=10$ animals), 1000 ($N=10$ animals), 2500 ($N=10$ animals), 4500 ($N=10$ animals), or 9000 ($N=10$ animals) IU/kg of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0.6 mL. Adequate measures were taken to minimize pain or discomfort.

2.4. Mitochondrial electron transfer chain (METC) activity

To obtain SMP from *substantia nigra* and *striatum* in order to assess METC activity, we have homogenized each tissue in 250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4, and 50 IU/mL heparin buffer. The samples were centrifuged 1000 ×*g* and the supernatants were colleted. Then, the samples were frozen and thawed three times, and METC activity detection was performed as described below.

2.4.1. Complex I-CoQ-III activity

Complex I-CoQ-III activity was determined by following the increase in absorbance due to reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\in=19.1\ mM^{-10}\ cm^{-1}$). The reaction buffer contained 20 mM potassium phosphate, pH 8.0, 2.0 mM KCN, 10 μM EDTA, 50 μM cytochrome *c*, and 20–45 μg supernatant protein. The reaction started by addition of 25 μM NADH and was monitored at 30 °C for 3 min before the addition of 10 μM rotenone, after the which the activity was monitored for an additional 3 min. Complex I–III activity was the rotenone-sensitive NADH:cytochrome *c* oxidoreductase activity (Shapira et al., 1990).

2.4.2. Complex II and succinate dehydrogenase (SDH) activities

Complex II (succinate–DCPIP–oxidoreductase) activity was measured by following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCPIP) at 600 nm with 700 nm as reference wavelength ($\in=19.1\ mM^{-1}\ cm^{-1}$). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8.0 μM DCPIP was preincubated with 48–80 μg supernatant protein at 30 °C for 20 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction was started by addition of 40 μM DCPIP and was monitored for 5 min at 30 °C. Succinate dehydrogenase (SDH) activity was assessed by adding 1 mM phenazine methasulphate to the reaction mixture. Then, SDH activity was monitored for 5 min at 30 °C at 600 nm with 700 nm as reference wavelength (Fischer et al., 1985).

2.4.3. Complex II-CoQ-III activity

Complex II-CoQ-III activity was measured by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength ($\in=21\ mM^{-1}\ cm^{-1}$). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate was preincubated with 50–100 μg supernatant protein at 30 °C for 30 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction started by the addition of 0.6 μg/mL cytochrome *c* and monitored for 5 min at 30 °C (Fischer et al., 1985).

2.4.4. Complex IV activity

Complex IV activity was measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\in=19.15\ mM^{-1}\ cm^{-1}$). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM

n-dodecyl-β-D-maltoside, 2–4 μg supernatant protein and the reaction was started with addition of 0.7 μg reduced cytochrome *c*. The activity of complex IV was measured at 25 °C for 10 min (Rustin et al., 1994).

2.5. Oxidative parameters in submitochondrial particles

Briefly, to obtain submitochondrial particles (SMP), *substantia nigra* and *striatum* were dissected and homogenized in 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl and 1 mM EDTA (pH 7.4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free SMP. The SMP solution was also washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) to ensure Mn-SOD release from mitochondria. To quantify superoxide ($O_2^{\bullet-}$) production, SMP was incubated in reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 μM catalase, and 1 mM epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32 °C, as previously described (Poderoso et al., 1996; De Oliveira and Moreira, 2007). As an index of lipid peroxidation, we used the formation of TBARS during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described (Draper and Hadley, 1990). Briefly, the samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm. Results are expressed as TBARS/mg protein.

2.6. Total radical-trapping antioxidant parameter (TRAP assay)

The non-enzymatic antioxidant cellular defenses were estimated by the total radical-trapping antioxidant parameter (TRAP), which determines the non-enzymatic antioxidant potential of the sample, as previously described (Wayner et al., 1985). Briefly, the reaction was initiated by injecting luminol and AAPH (2,2′-Azobis[2-methylpropionamidine]dihydrochloride) – a free radical source that produces peroxyl radical at a constant rate – in glycine buffer (0.1 M, pH 8.6), resulting in a steady luminescent emission. Nigral or striatal samples (100 μg of protein) were mixed in glycine buffer in the reaction vial and the decrease in luminescence monitored in a liquid scintillation counter for 60 min after the addition of the sample homogenates. The area under the curve obtained of the chemiluminescence values were transformed to percentage values and compared against the control values.

2.7. Glutathione S-transferase enzyme activity

Glutathione-S-transferase (GST) activity was determined spectrophotometrically according to the method of Habig et al., 1974. GST activity was quantified in nigral or striatal homogenates in a reaction mixture containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 1 mM glutathione as substrates in 0.1 M sodium phosphate buffer, pH 6.5, at 37 °C. Enzyme activity was calculated by the change in the absorbance value from the slope of the initial linear portion of the absorbance time curve at 340 nm for 5 min. Enzyme activity was expressed as nmol of CDNB conjugated with glutathione/min mg^{-1} protein.

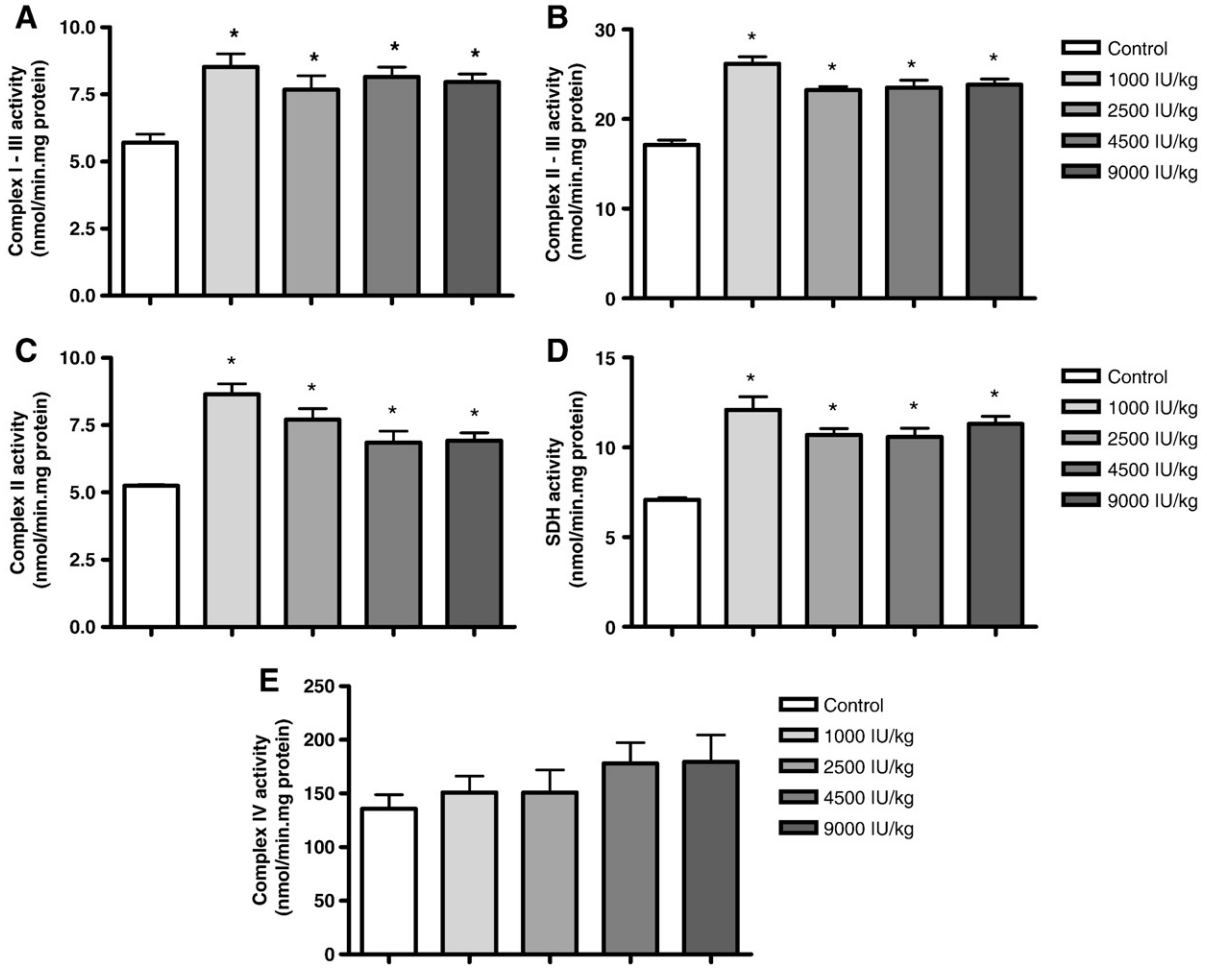

Fig. 1. Effects of vitamin A supplementation on complex I–III (A), complex II–III (B), complex II (C), succinate dehydrogenase (SDH) (D), and Complex IV (E) enzyme activities of rat *substantia nigra*. Data are mean ± S.E.M. of 10 animals per group performed in triplicate. *$p<0.05$ (one-way ANOVA followed by Tukey's test).

2.8. Enzyme-linked immunosorbent assay (ELISA) to 3-nitrotyrosine, α- and β-synucleins, and dopamine receptor 2

Indirect ELISA assay was performed to analyze changes in the content of nitrotyrosine by utilizing a polyclonal antibody to nitrotyrosine (Calbiochem) diluted 1:2000 in phosphate-buffered saline (PBS) pH 7.4 with 5% albumin. The polyclonal antibodies to α- and β-synucleins (Chemicon) were diluted 1:1000 in PBS with 5% albumin. Dopamine D2R polyclonal antibody (Chemicon) was diluted 1:40,000 in PBS with 5% albumin. Briefly, microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in PBS with 5% albumin. Plates were then washed four times with wash buffer (PBS with 0.05% Tween-20), and the specific antibodies were added to the plates for 2 h at room temperature. After washing (four times), a second incubation with anti-rabbit antibody peroxidase conjugated (diluted 1:1000) for 1 h at room temperature was carried out. After addition of substrates (hydrogen peroxide and 3, 3′, 5, 5′-tetramethylbenzidine 1:1 v:v), the samples were read at 450 nm in a plate spectrophotometer. Results are expressed as changes in percentage among the groups.

2.9. Caspase-3 activity

Caspase-3 activity was determined in the nigrostriatal axis through a fluorimetric commercial kit according manufacturer's instructions (Sigma). Briefly, the samples were homogenized in lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT), and centrifuged at 10,000 ×*g* for 15 min at 4 °C. The supernatants were used to determine caspase-3 assay in a microplate fluorimeter at 360 nm excitation and 460 nm emission for 180 min at 25 °C. Results are expressed as nmol 7-amino-4-methylcoumarin (AMC) produced/min mg^{-1} protein.

2.10. TNF-α quantification

We have measured TNF-α through commercial kit for sandwich enzyme-linked immunosorbent assay (ELISA) according manufacturer's instructions (BD Biosciences). Briefly, tissue samples were collected and suspended in lysis buffer containing protease inhibitors. Following cell lysis, the homogenate was centrifuged, and a portion of the supernatant was reserved for protein concentration measurement, and the remaining was stored at −80 °C for posterior TNF-α levels quantification. The samples were read in a microplate spectrophotometer at 450 nm.

2.11. Statistical analysis

Data are expressed as means ± standard error of the mean (S.E.M.); p values were considered significant when $p<0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test whenever necessary.

3. Results

3.1. Mitochondrial electron transfer chain (METC) enzyme activities

Vitamin A supplementation at any dose tested induced an increase in complex I–III ($p<0.05$; Fig. 1A), complex II–III ($p<0.05$; Fig. 1B), complex

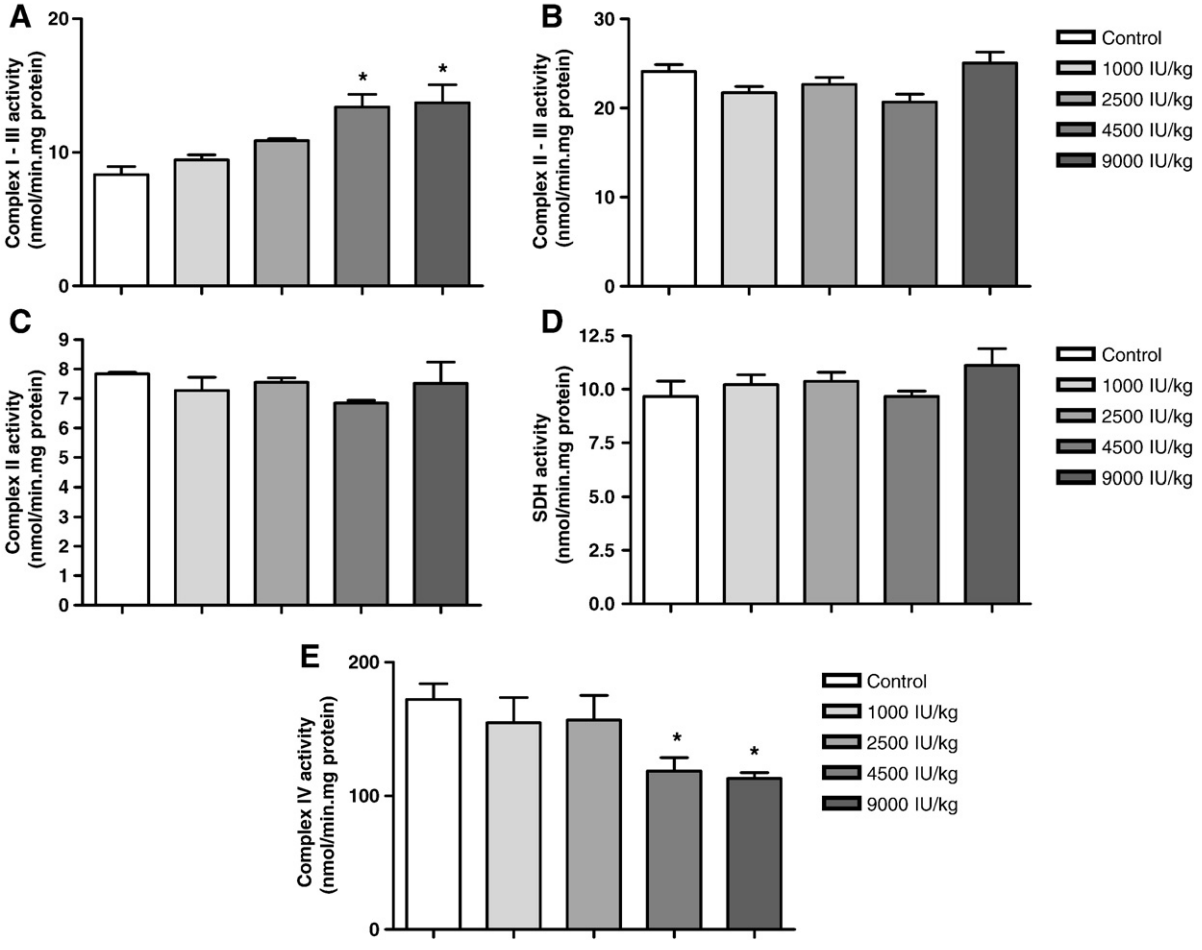

Fig. 2. Effects of vitamin A supplementation on complex I–III (A), complex II–III (B), complex II (C), succinate dehydrogenase (SDH) (D), and Complex IV (E) enzyme activities of rat *striatum*. Data are mean ± S.E.M. of 10 animals per group performed in triplicate. *$p<0.05$ (one-way ANOVA followed by Tukey's test).

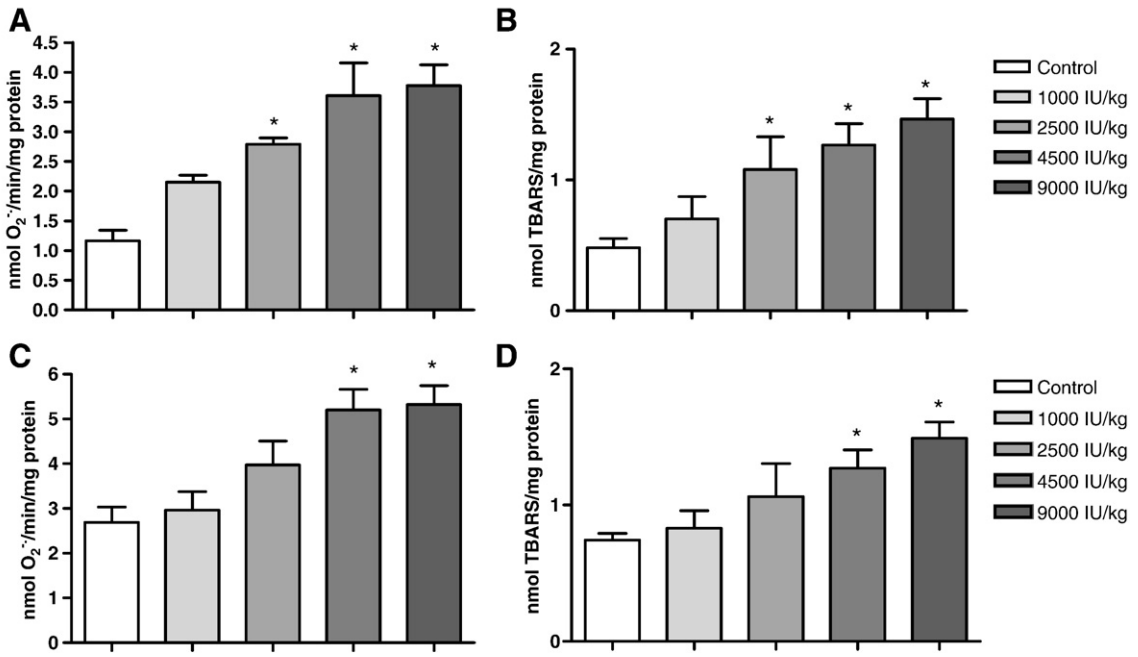

Fig. 3. Effects of vitamin A supplementation on superoxide anion radical production in rat *substantia nigra* (A) and *striatum* (C), and lipid peroxidation in submitochondrial particles isolated from rat *substantia nigra* (B) and *striatum* (D). Data are mean ± S.E.M. of 10 animals per group performed in triplicate. *$p<0.05$ (one-way ANOVA followed by Tukey's test).

II ($p<0.05$; Fig. 1C), and succinate dehydrogenase (SDH) ($p<0.05$; Fig. 1D) enzyme activities in rat *substantia nigra*. However, nigral complex IV enzyme activity did not change in this experimental model (Fig. 1E). As depicted in Fig. 2A, vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} did increase complex I–III enzyme activity in rat *striatum*. However, complex II–III as well as complex II and SDH enzyme activities did not change in the *striatum* of vitamin A-treated rats (Fig. 2B, C, and D, respectively). Surprisingly, vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} did induce a decrease in complex IV enzyme activity in adult rat *striatum* ($p<0.05$; Fig. 2E).

3.2. Superoxide anion radical ($O_2^{\cdot-}$) production and lipid peroxidation levels in mitochondrial membranes

As demonstrated in Fig. 3A, vitamin A supplementation at 2500, 4500, or 9000 IU/kg day^{-1} induced an increase in $O_2^{\cdot-}$ production in nigral submitochondrial particles (SMP) ($p<0.05$). Moreover, vitamin A supplementation at 2500, 4500, or 9000 IU/kg day^{-1} did increase the levels of mitochondrial membranes lipid peroxidation in rat *substantia nigra* ($p<0.05$; Fig. 3B). Striatal $O_2^{\cdot-}$ production, as well as SMP lipid peroxidation levels, were observed increased in the rats that received

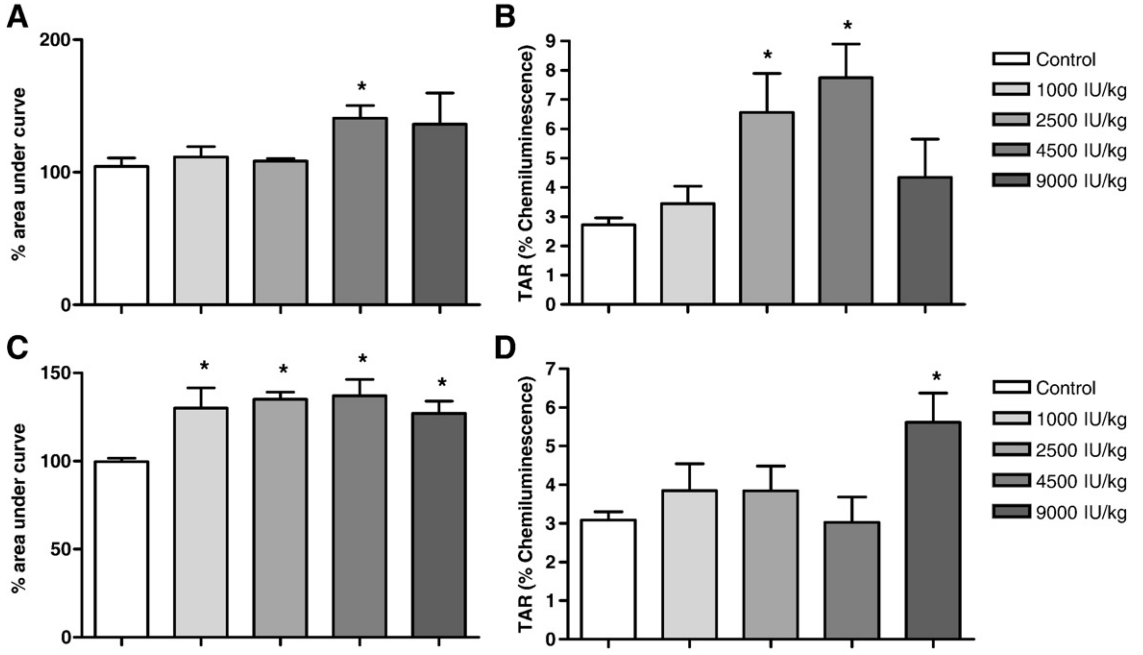

Fig. 4. Effects of vitamin A supplementation on the nigrostriatalnon-enzymatic antioxidant defenses. TRAP area under curve values are shown in (A) for *substantia nigra* samples and in (C) for striatal samples. The total antioxidant reactivity (TAR) of *substantia nigra* is shown in (B), and striatal TAR values are shown in (D). Data are mean ± S.E.M. of 10 animals per group performed in duplicate. *$p<0.05$ (one-way ANOVA followed by Tukey's test).

vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Fig. 3C and D, respectively).

3.3. Non-enzymatic antioxidant defenses

The TRAP assay revealed that vitamin A supplementation at 4500 IU/kg day^{-1} was pro-oxidant to the nigral environment regarding non-enzymatic antioxidant defenses ($p<0.05$; Fig. 4A). TAR values, which represent sample reactivity (*i.e.* higher TAR values indicate more pro-oxidant samples), were found higher than control in the nigral samples of the rats that received vitamin A supplementation at 2500 or 4500 IU/kg day^{-1} ($p<0.05$; Fig. 4B).

Area under curve values, which were obtained from the TRAP assay, were found higher than control samples in the *striatum* of the rats that received vitamin A supplementation at any dose tested ($p<0.05$; Fig. 4C). TAR values were found higher only in the *striatum* of the rats that were treated with vitamin A supplementation at 9000 IU/kg day^{-1} ($p<0.05$; Fig. 4D).

3.4. Glutathione S-transferase (GST) enzyme activity

According to Fig. 5A, nigral GST enzyme activity was found increased in the rats that were administrated vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$). Also, we observed increased GST enzyme activity in the *striatum* of the rats that received vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Fig. 5B).

3.5. 3-Nitrotyrosine

We have observed increased 3-nitrotyrosine content in the *substantia nigra* and *striatum* of the rats that received vitamin A at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Fig. 6A and B, respectively).

3.6. α- and β-synucleins

Vitamin A supplementation at 9000 IU/kg day^{-1} increased α-synuclein content in rat *substantia nigra* ($p<0.05$; Fig. 7A). Striatal α-synuclein content was increased in the rats that received vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Fig. 7B). β-synuclein content did not change in this experimental model (Fig. 8).

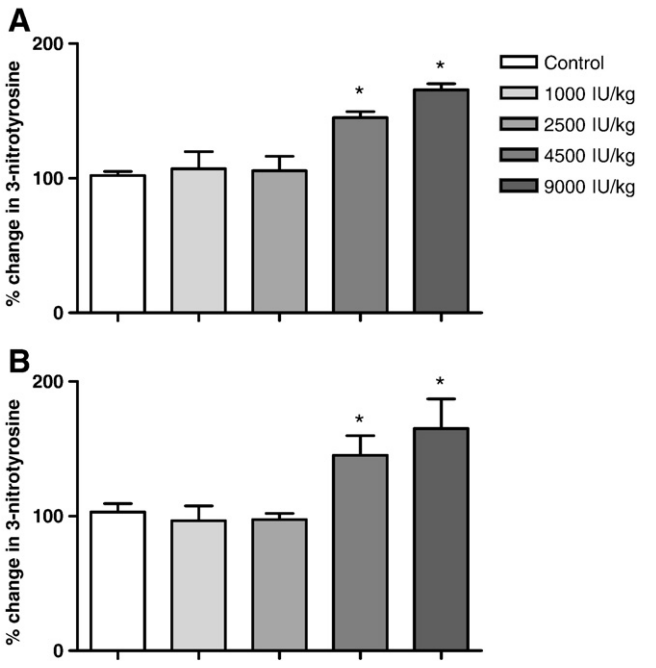

Fig. 6. Effect of vitamin A supplementation on 3-nitrotyrosine content in adult rat *substantia nigra* (A) and *striatum* (B). Data are percentage change of 10 animals per group performed in duplicate. *$p<0.05$ (one-way ANOVA followed by Tukey's test).

3.7. Dopamine receptor 2 (D2R)

D2R content did not change in rat *substantia nigra* in this study (Fig. 9A). However, we observed decreased D2R content in the *striatum* of the rat that were administrated with vitamin A supplementation at 2500, 4500, or 9000 IU/kg day^{-1} ($p<0.05$; Fig. 9B).

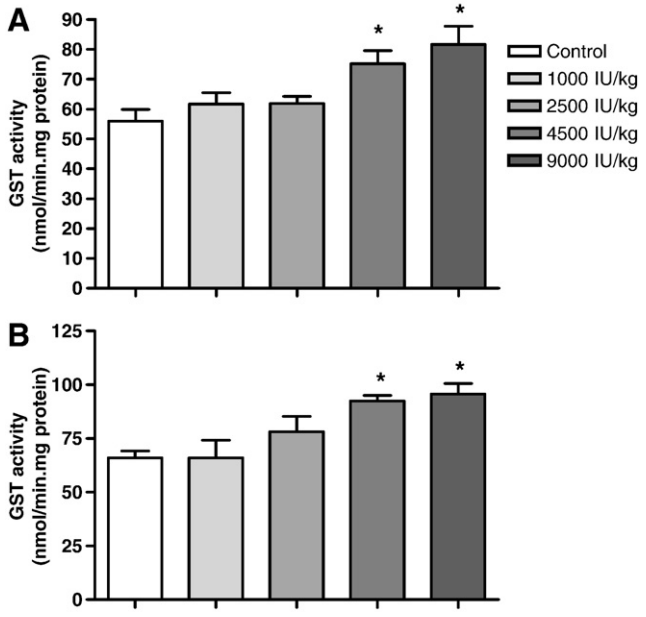

Fig. 5. Effect of vitamin A supplementation on glutathione S-transferase (GST) in rat *substantia nigra* (A) and *striatum* (B). Data are mean±S.E.M. of 10 animals per group performed in triplicate. *$p<0.05$ (one-way ANOVA followed by Tukey's test).

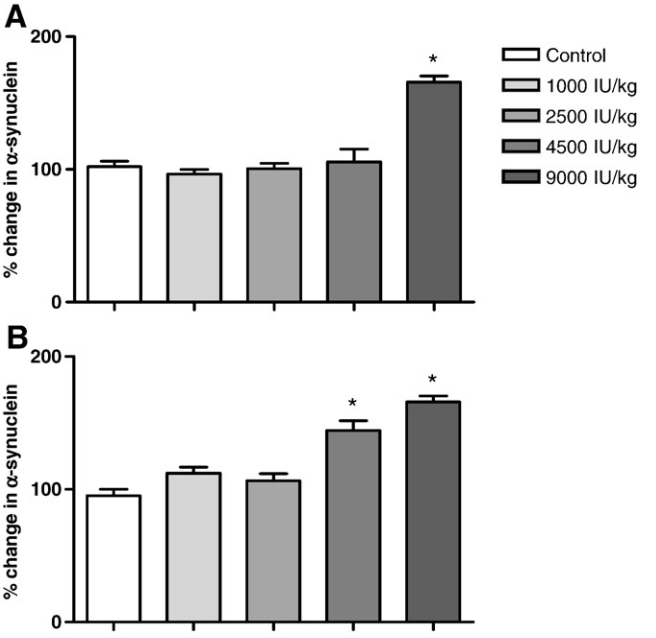

Fig. 7. Effect of vitamin A supplementation on α-synuclein content in adult rat *substantia nigra* (A) and *striatum* (B). Data are mean±S.E.M. of 10 animals per group performed in duplicate. *$p<0.05$ (one-way ANOVA followed by Tukey's test).

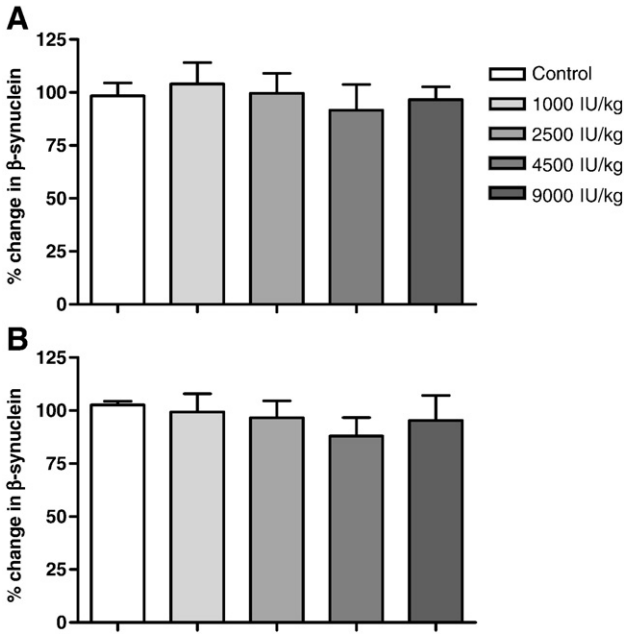

Fig. 8. Effect of vitamin A supplementation on β-synuclein content in adult rat *substantia nigra* (A) and *striatum* (B). Data are mean±S.E.M. of 10 animals per group performed in duplicate.

3.8. Caspase-3 enzyme activity and TNF-α levels

Vitamin A supplementation did alter neither caspase-3 enzyme activity nor TNF-α levels in this experimental model in rat *substantia nigra* and *striatum* (Fig. 10A–B, C–D, respectively).

4. Discussion

In the herein presented work, we have investigated the effects of vitamin A supplementation at clinical doses on the rat nigrostriatal axis redox and bioenergetics states. We observed impaired mitochondrial electron transfer chain (METC) activity in both rat brain regions (Figs. 1 and 2). Moreover, vitamin A supplementation increased the production of mitochondrial superoxide anion radical ($O_2^{\bullet-}$), leading to a pro-oxidative event in such rat brain regions, as represented by increased mitochondrial lipid peroxidation levels and 3-nitrotyrosine content in the nigrostriatal axis (Figs. 3–6). Importantly, vitamin A supplementation did alter neither food consumption nor rat body weight in this experimental model (data not shown), suggesting that the data presented here is a consequence of vitamin A supplementation and not an indirect effect of this drug on rat nigrostriatalaxis redox and energetic states due to altered rat metabolism.

We have previously reported that vitamin A supplementation (as retinol palmitate) induced a pro-oxidant state in some rat brain regions, including *substantia nigra* and *striatum* (De Oliveira et al., 2007a, 2008a). However, the mechanism by which oral vitamin A increased the levels of oxidative stress markers in such regions was not demonstrated. Here, we found that vitamin A supplementation at clinical doses (1000–9000 IU/kg day^{-1}) increased METC activity (complex I–III, II–III, II, and SDH enzyme activity) in rat *substantia nigra* (Fig. 1 A–D). However, nigral complex IV enzyme activity did not change in this experimental model (Fig. 1E). These data suggest that vitamin A supplementation induced an impairment in the nigral METC activity, therefore leading to an increased mitochondrial $O_2^{\bullet-}$ production, as observed (Fig. 3A and B). In this case, unaltered complex IV enzyme activity may result in partial reduction of O_2 in such complex. Consequently, $O_2^{\bullet-}$ is produced from such partial reduction of O_2 (Halliwell, 2006). On the other hand, only vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} modulated METC activity in rat *striatum* (Fig. 2). It was observed increased complex I–III enzyme activity (Fig. 2A), but decreased complex IV enzyme activity (Fig. 2E) in the *striatum* of the rats that received vitamin A supplementation at 4500 or 9000 IU/kg day^{-1}. Such alteration in METC activity could thereby result in increased $O_2^{\bullet-}$ production, as demonstrated in Fig. 3C, due to partial reduction of O_2 to water, as mentioned above. Increased electron flux in the mitochondrial complexes, but decreased complex IV enzyme activity is very likely to result in increased $O_2^{\bullet-}$ production due to electron leakage or simply as a result of increased rates of electron transfer between the complexes. Also, it is important to mention that it has been shown that increased activity of mitochondrial complexes may take a role during stressful conditions, since more ATP is needed in detoxifying events, for example, as reviewed (Manoli et al., 2007). However, more accurately analyses would be very useful to elucidate the mechanism by which vitamin A supplementation modulates nigrostriatal METC activity, since either complex I–III or complex II–III activity is very likely to produce $O_2^{\bullet-}$ during normal metabolism and pathological conditions (Kudin et al., 2004; Halliwell, 2006).

We also demonstrated in this work that vitamin A supplementation induced a decrease in the non-enzymatic antioxidant defenses in both rat *substantia nigra* and *striatum* (Fig. 4). The TRAP assay, which is showed here as "area under curve" indicates the kinetics of non-enzymatic antioxidant defenses in the rat samples. Higher area under curve values indicates more pro-oxidant samples. It also indicates how much time the samples resist to a pro-oxidant moment, as that triggered in the TRAP assay. Based on our results, we suggest that the tissue samples of the rats that received vitamin A supplementation demonstrate decreased non-enzymatic antioxidant defenses, which may favor the maintenance of a pro-oxidant state in such rat brain regions. Furthermore, TAR values indicate how fast the samples react with the pro-oxidant environment of the TRAP assay (Lissi et al., 1995). The data showed here reinforce the result obtained from the TRAP assay, suggesting decreased non-enzymatic antioxidant defenses and increased production of oxidant species in the *substantia nigra* and *striatum* of the vitamin A-treated rats.

GST enzyme is an important detoxifying system in a wide range of mammalian cells (Habig et al., 1974). This enzyme condenses reduced glutathione (GSH) to apolar molecules in order to increase its solubility

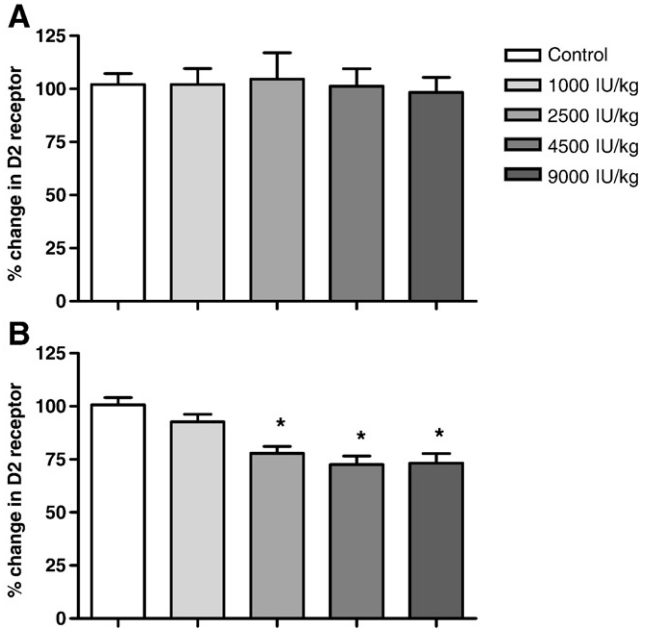

Fig. 9. Effect of vitamin A supplementation on D2 receptor content in adult rat *substantia nigra* (A) and *striatum* (B). Data are mean±S.E.M. of 10 animals per group performed in duplicate. *$p<0.05$ (one-way ANOVA followed by Tukey's test).

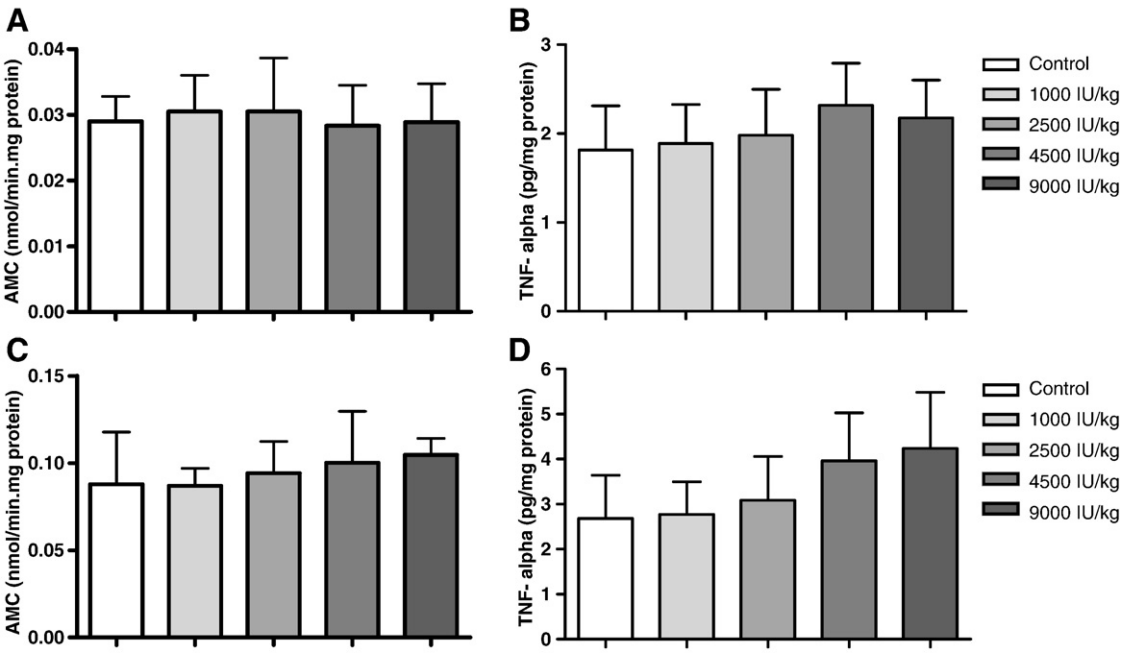

Fig. 10. Effects of vitamin A supplementation on caspase-3 enzyme activity in adult rat *substantia nigra* (A) and *striatum* (C). TNF-α levels were quantified in *substantia nigra* (B) and *striatum* (D) of vitamin A-treated rats. Data are mean ± S.E.M. of 10 animals per group performed in triplicate.

in aqueous environments, increasing the rate of excretion of such molecules. On the other hand, GST consumes an important non-enzymatic antioxidant in such process, which may lead to decreased levels of GSH. We observed here increased GST enzyme activity in the nigrostriatal axis of the rats that received vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} (Fig. 5), suggesting a deleterious effect of such vitamin A on rat *substantia nigra* and *striatum*.

Increased 3-nitrotyrosine content indicates that peroxynitrite ($ONOO^-$) production may be increased in the nigrostriatal axis of vitamin A-treated rats. This reactive specie is produced from nitric oxide ($NO^{\bullet}$) and $O_2^{\bullet-}$ when the levels of these two radicals increase in the cellular environment. Peroxynitrite is a non-radical specie, but plays an important role in several pathologies, including rheumatoid arthritis, endotoxic shock, motor neuron disease and Alzheimer's disease (Halliwell et al., 1999; Halliwell, 2006). Peroxynitrite may give rise to peroxynitrous acid (ONOOH) under physiological conditions, which may generates toxic products with increased reactivities, for instance nitryl cation (NO_2^+), nitrogen dioxide radical ($^{\bullet}NO_2$), and hydroxyl radical ($^{\bullet}OH$) through undergoing homolytic fission (Alvarez and Radi, 2003; Halliwell, 2006; Calabrese et al., 2007), thereby amplifying a pro-oxidant effect in biological systems. The results presented here indicate that vitamin A supplementation increases $ONOO^-$ production in adult rat *substantia nigra* and *striatum*, which may oxidize and nitrate biomolecules directly or indirectly.

Interestingly, vitamin A supplementation increased the content of α-synuclein in the nigrostriatal axis (Fig. 7). α-synuclein is a pre-synaptic protein whose function is not completely understood. However, increased levels of α-synuclein are observed in Parkinson's disease (PD) forming the Lewy's bodies, proteinaceous inclusions present in the *substantia nigra* of PD patients (Goedert, 2001). The consequences of α-synuclein accumulation are still on debate, but it seems that increased levels of such dysfunctional protein may facilitate neuronal death through either necrosis or apoptosis (Cookson and van der Brug, 2008). The mechanism by which α-synuclein accumulates inside neurons involves, at least in part, oxidative stress (Maguire-Zeiss et al., 2005). It has been demonstrated that a vicious cycle is maintained when dysfunctional α-synuclein aggregates and inhibits proteasomal activity, which would be responsible for unfolded proteins degradation (Lindersson et al., 2004). Then, mitochondrial impairment may result, for example, from such accumulation due to perturbation of the mitochondrial membranes induced by α-synuclein aggregates (Hsu et al., 2000; Elkon et al., 2002). Indeed, we have demonstrated here decreased complex IV activity in the *striatum* of the rats that received vitamin A at 4500 or 9000 IU/kg day^{-1} (Fig. 2E). Vitamin A at those same doses increased α-synuclein content in the rat *striatum* (Fig. 7B). Whether there is some association between decreased striatal complex IV activity and increased α-synuclein content it is not known, but more investigations would be useful in this regard. Surprisingly, we did not find any change in β-synuclein content in the nigrostriatal axis (Fig. 8). β-synuclein is thought to participate in the maintenance of folded α-synuclein and is also involved in other signaling pathways that control cell survival (Hashimoto et al., 2004; Snyder et al., 2005; Fan et al., 2006). Then, it is very likely that the accumulation of α-synuclein without any change in β-synuclein content may take an important role in the cognitive decline observed in adult Wistar rats, which showed increased levels of anxiety-like behavior and decreased locomotion in and exploration of an open field apparatus, as previously reported by our research group (De Oliveira et al., 2007a,b, 2008a,b).

Surprisingly, vitamin A supplementation at clinical doses (2500–9000 IU/kg day^{-1}) induced a decrease in D2 receptor content in the rat *striatum* (Fig. 9B). It was shown that some retinoids might increase D2 receptor expression in vitro (Samad et al., 1997). However, depending on the concentration, vitamin A – and its derivatives the retinoids – may become toxic in vitro or in vivo, as mentioned in the "Introduction" section. Here, we demonstrated that vitamin A decreased D2 receptor content in rat *striatum*, which may took an important role in the neurotoxic effect induced by vitamin A at the doses tested here, since decreased D2 receptor content in found in some neurodegenerative processes, as previously reported (Bonci and Hopf, 2005). Additionally, we have analyzed here the effects of oral vitamin A supplementation in vivo, which gives rise to a myriad of retinoids in the different rat tissues, and not only a certain retinoid, as investigated by Samad and colleagues (Samad et al., 1997). Then, it is not a surprise to find different results in a very different experimental model.

Even under a pro-oxidant state and with an impaired METC activity, we did find any change neither in caspase-3 enzyme activity nor in TNF-α levels in the *substantia nigra* and *striatum* of the rats that were treated with vitamin A (Fig. 10), suggesting that apoptotic cell death or

inflammation – which might result, at least in part, from necrotic cell death – are not a consequence from vitamin A supplementation at clinical doses. Caspase-3 activation would result from cytochrome *c* release from mitochondria (Hengartner, 2000; Newmeyer and Ferguson-Miller, 2003). Then, we suggest that the decrease observed in striatal complex IV enzyme activity did not result from cytochrome *c* leakage from mitochondria. Furthermore, the increased $O_2^{\cdot}$ production seen here may not be attributed to TNF-α – which is able to increase $O_2^{\cdot}$ production in some experimental models – since we did not observe any change in such parameter.

Vitamin A administration at moderate to high doses is applied in the treatment of some life-threatening pathologies, for instance some types of cancer (Fenaux et al., 2001; Allen and Haskell, 2002; Myhre et al., 2003). It is administrated vitamin A (as retinol palmitate) at doses exceeding 150,000 IU day^{-1} to infants and children suffering from leukemia (Allen and Haskell, 2002). Furthermore, very-low-weight-birth preterm infants receive daily vitamin A at doses as high as 8500 IU/kg (Mactier and Weaver, 2005). We have previously demonstrated that vitamin A supplementation induced anxiety-like behavior in adult Wistar rats under a 28-day period treatment (De Oliveira et al., 2007b). However, it was not observed depression-related behavior in such animals, as recently published (De Oliveira et al., 2008b). Indeed, vitamin A at moderate to high levels (even when therapeutically administrated) may induce cognitive decline in patients at different age, from infants to elderly (Myhre et al., 2003; O'Reilly et al., 2008). The mechanism by which oral vitamin A therapy may result in cognitive disturbance is still on debate, but a role to decreased METC activity, for example, could not be discarded due to its importance in the energetic homeostasis of the cells. Moreover, the consequences of increased oxidative damage to biomolecules, as showed here as 3-nitrotyrosine content, seem to play a pivotal role in neurodegenerative diseases (Halliwell, 2006). It is important to note that some nutritional supplements, as for example *Gingko biloba*, were not effective in preventing oxidative damage in some studies, suggesting that the antioxidant capacity of a molecule would depend on other aspects that not only the nature of the molecule (DeKosky et al., 2008; Schneider, 2008; Kelley and Knopman, 2008). Vitamin A and retinoids are considered antioxidants; however, it would depend on its concentration and, importantly, on some conditions associated to the health of the patient. We did not perform retinoids quantification in rat plasma due to the low reliability of such measurement, since plasma retinol levels, for example, were found at normal levels even in cases of hypervitaminosis A (Ellis et al., 1986; Croquet et al., 2000; Mills and Tanumihardjo, 2006). Additionally, it is very difficult to affirm that certain retinoid is exerting the deleterious effects seem here only because its levels were found high, since there is a wide range of retinoids that may be originated from oral vitamin A intake and some of these retinoids would decompose very fast due to its higher reactivity than other retinoids, as elegantly reviewed by Napoli (Napoli, 1999), *i.e.* the effects of some retinoids would be seen, but the presence of the retinoid would be very difficult to be detected. The use of vitamin A supplementation at clinical doses needs extra caution regarding the deleterious consequences that may arise from such therapy, which may decrease significantly the patients' life quality. Furthermore, increased levels of α-synuclein in such rat brain region may take an important role in cognitive decline, as previously reported by our group.

Acknowledgements

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Capítulo II

“Vitamin A supplementation at clinical doses induces a dysfunction in the redox and bioenergetics states, but did change either caspases activities not TNF-α levels in the frontal cortex of adult Wistar rats”

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Vitamin A supplementation at clinical doses induces a dysfunction in the redox and bioenergetics states, but did change neither caspases activities nor TNF-α levels in the frontal cortex of adult Wistar rats

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ABSTRACT

Vitamin A and its derivatives, the retinoids, exert modulatory roles on central nervous system (CNS) function. However, the clinical use of vitamin A at moderate to high doses induces serious side effects, including dysfunctional brain metabolism and mood disorders. Then, we have investigated in this work the effects of vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day for 28 days on redox and bioenergetics parameters in adult rat frontal cortex. Additionally, we have measured caspase-3 and caspase-8 activities to analyze whether vitamin A supplementation as retinol palmitate induces neuronal death in such brain area. The levels of the pro-inflammatory cytokine TNF-α were also quantified. We have found increased rates of $O_2^{\cdot-}$ production and increased levels of markers of oxidative insult in frontal cortex and also in mitochondrial membranes. Superoxide dismutase (SOD) enzyme activity was increased, and catalase (CAT) enzyme activity did not change in this experimental model. Surprisingly, we observed increased mitochondrial electron transfer chain (METC) activity. Caspase-3 and caspase-8 activities and TNF-α levels did not change in this experimental model. Finally, vitamin A supplementation did not induce depression in adult rats after 28 days of treatment. However, exploration in the center of an open field was decreased and time spent in freezing behavior was increased in vitamin A treated rats.

1. Introduction

Vitamin A and its derivatives – the retinoids – exert a regulatory role in the maintenance of the central nervous system (CNS) function (Lane and Bailey, 2005; McCaferry et al., 2005). Nevertheless, several neurological disturbances, including cognitive impairments, have been attributed to excessive vitamin A intake, which may occur through either therapeutic or inadvertently use (Fenaux et al., 2001; Allen and Haskell, 2002; Myhre et al., 2003). Indeed, it was experimentally demonstrated that retinoids commonly applied in the treatment of acne might induce hippocampal cell loss and, consequently, decrease the abilities to learning and memory in adult mice (Crandall et al., 2004). Alternatively, it was found that those therapeutic retinoids might induce mood disorders in patients during their acne treatment, including depression and increased suicide attempts (Byrne and Hnatko, 1995; Josefson, 1998; Jick et al., 2000).

Besides its role in CNS function maintenance, vitamin A has redox properties that could be associated to some of the effects that were observed either in *in vitro* or *in vivo* investigations. For example, vitamin A at low doses is a known antioxidant (Halliwell, 2006). Indeed, it was proposed that cortical β-amyloid deposites that were found in vitamin A-deficient animals occurred due to decreased antioxidant capacities in those animals, suggesting a protective effect of the vitamin (Tafti and Ghyselinck, 2007). Indeed, the adult mammalian CNS is able to transport and metabolize retinoid molecules, since it contains the molecular apparatus that is responsible for the production and maintenance of all-*trans*-retinoic acid in neurons, *i.e.* retinal dehydrogenases and cellular retinoid binding proteins (MacDonald et al., 1990; Zetterstrom et al., 1994; Duester, 2000). Then, vitamin A and its retinoids easily penetrate the blood–brain barrier (BBB) and may rapidly increase their concentrations in the CNS.

We have recently demonstrated that vitamin A supplementation at therapeutic doses (1000–9000 IU/kg/day) induces cognitive impairments in adult Wistar rats, for instance anxiety-like behavior and decreased exploratory activities. Furthermore, a pro-oxidant effect was found in some rat brain regions (De Oliveira et al., 2007a; De Oliveira et al., 2007b; De Oliveira et al., 2008).

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Additionally, retinol and all-*trans*-retinoic acid have been demonstrated to be able to induce several redox perturbations in some experimental models. For example, we have demonstrated that retinol treatment induces increased rates of superoxide anion ($O_2^{\cdot-}$) production in rat liver mitochondria (Klamt et al., 2005), in cultured Sertoli cells (Dal-Pizzol et al., 2001), and in submitochondrial particles isolated from rat cerebral cortex and cerebellum (De Oliveira and Moreira, 2007). In addition, it was found increased lipid peroxidation and protein carbonylation levels in some experimental models, indicating a potential pro-oxidant effect of retinoids in biological systems in concentrations slightly above the physiologic levels (Moreira et al., 1997; Dal-Pizzol et al., 2001; Frota et al., 2004; De Oliveira et al., 2005; Zanotto-Filho et al., 2008).

Among the possible toxic effects that occur due to increased vitamin A intake, there is a great concern regarding the recent reports showing increased risk of death among vitamin A supplements users (Watkins et al., 2000; Bjelakovic et al., 2007). Vitamin A supplementation is a cause of concern among public health researchers due to the relatively easy distribution of this medication in drug stores. Furthermore, there are evidences appointing to a possible decreased metabolism rate in some brain regions of patients chronically treated with retinoids (Bremner et al., 2005), which could indicate a potential effect of vitamin A (or its derivatives) on the neuronal bioenergetics status. However, the exact mechanism responsible for these effects remains to be established.

In the herein presented work, we have investigated the effects of vitamin A supplementation at therapeutic doses on the redox environment of adult rat frontal cortex. In addition, we have assessed mitochondrial electron transfer chain (METC) activity in such brain area. Also, the activities of caspase-3 and caspase-8 were determined, since we have previously reported an anxiety-like behavior in adult rats receiving vitamin A supplementation and neuronal death may be implicated in cognitive impairments. We have also observed rat behavior regarding mood disorders as assessed through sucrose consumption test, forced swimming test, tail suspension test, and open field apparatus. Importantly, the retinol palmitate doses tested here are within a therapeutic range commonly applied in cancer treatment in both children and adult humans (Fenaux et al., 2001; Allen and Haskell, 2002; Myhre et al., 2003). For example, it is prescribed retinol palmitate at doses exceeding 150,000 IU/day to patients (including infants and children) under leukemia treatment during an undetermined period (Allen and Haskell, 2002). Additionally, very-low-weight-preterm infants receive vitamin A at a maximum daily dose of 8500 IU/kg during weight gain treatment, as reviewed by Mactier and Weaver (2005).

2. Experimental procedures

2.1. Animals

Adult male Wistar rats (290–320 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12 h light–dark cycle (7:00–19:00 h), at a temperature-controlled colony room (23 ± 1 °C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80–23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

2.2. Drugs and reagents

Arovit® (retinol palmitate, a water-soluble form of vitamin A) was purchased from Roche, Sao Paulo, SP, Brazil. Caspase-8 activity assay kit was purchased from Biotium, Inc., Hayward, CA, USA. TNF-α assay kit was obtained from BD Biosciences, San Diego, CA, USA. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A treatment was prepared daily and it occurred by protecting from light.

2.3. Treatment

The animals were treated once a day for 28 days with a gavage. The treatments were carried out at night (*i.e.* when the animals are more active and take a greater amount of food) in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. The animals were treated with vehicle (0.15 M saline), 1000, 2500, 4500, or 9000 IU/kg of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0.8 mL. Adequate measures were taken to minimize pain or discomfort.

2.4. Oxidative stress and antioxidant enzyme activities analyses

Before sacrifice, the animals were anesthetized with ketamine plus xylazine (100 mg/kg and 14 mg/kg, respectively). The animals were sacrificed by decapitation at 24 h after the last vitamin A administration. The frontal cortices were dissected out in ice immediately after the rat was sacrificed and stored at −80 °C for posterior oxidative stress analyses. The homogenates were centrifuged (700 g, 5 min) to remove cellular debris. Supernatants were used to all biochemical assays described herein. All the results were normalized by the protein content using bovine albumin as standard (Lowry and Rosebrough, 1951).

2.4.1. Thiobarbituric acid reactive species (TBARS)

As an index of lipid peroxidation, we used the formation of TBARS during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described (Draper and Hadley, 1990). Briefly, the samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm. Results are expressed as TBARS/mg protein.

2.4.2. Measurement of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) as previously described (Levine et al., 1990). Briefly, proteins were precipitated by the addition of 20% TCA and redissolved in DNPH and the absorbance read in a spectrophotometer at 370 nm. Results are expressed as nmol carbonyl/mg protein.

2.4.3. Measurement of protein thiol content

Other assay that serves to analyze oxidative alterations in proteins is to measure the level of protein thiol content in samples. Briefly, an aliquot was diluted in SDS 0.1% and 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB) in ethanol was added and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min (Ellman, 1959). Results are expressed as μmol SH/mg protein.

2.4.4. Antioxidant enzyme activities estimations

Superoxide dismutase activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described (Misra and

Fridovich, 1972), and the results are expressed as U SOD/mg protein. Catalase (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm (Aebi, 1984), and the results are expressed as U CAT/mg protein. A ratio between SOD and CAT activities (SOD/CAT) were applied to better understand the effect of vitamin A supplementation upon these two oxidant-detoxifying enzymes that work in sequence converting superoxide anion to water (Halliwell and Gutteridge, 1999).

2.5. Oxidative parameters in submitochondrial particles

Briefly, to obtain submitochondrial particles (SMP), frontal cortices were dissected and homogenized in 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl and 1 mM EDTA (pH 7.4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free SMP. The SMP solution was also washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) to ensure Mn–SOD release from mitochondria. To quantify superoxide ($O_2^{-\cdot}$) production, SMP was incubated in reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 10 mM HEPES–KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 μM catalase, and 1 mM epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32 °C, as previously described (Poderoso et al., 1996; De Oliveira and Moreira, 2007). As a marker of lipid peroxidation, we measured the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, as previously described (Draper and Hadley, 1990). The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine (DNPH) as previously described above (Levine et al., 1990). Protein thiol content in frontal cortex SMP samples were determined as described above. Briefly, an aliquot was diluted in SDS 0.1% and 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB) in ethanol was added and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min (Ellman, 1959).

2.6. Mitochondrial electron transfer chain (METC) activity

To obtain SMP from frontal cortex in order to assess METC activity, we have homogenized the tissue in 250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4, and 50 IU/mL heparin buffer. The samples were centrifuged 1000 *g* and the supernatants were colleted. Then, the samples were frozen and thawed three times, and METC activity detection was performed as described below.

2.6.1. Complex I–CoQ–III activity

Complex I–CoQ–III activity was determined by following the increase in absorbance due to reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength (ϵ = 19.1 mM^{-1} cm^{-1}). The reaction buffer contained 20 mM potassium phosphate, pH 8.0, 2.0 mM KCN, 10 μM EDTA, 50 μM cytochrome *c*, and 20–45 μg supernatant protein. The reaction started by addition of 25 μM NADH and was monitored at 30 °C for 3 min before the addition of 10 μM rotenone, after the which the activity was monitored for an additional 3 min. Complex I–III activity was the rotenone-sensitive NADH:cytochrome *c* oxidoreductase activity (Shapira et al., 1990).

2.6.2. Complex II and succinate dehydrogenase (SDH) activities

Complex II (succinate–DCPIP–oxidoreductase) activity was measured by following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCPIP) at 600 nm with 700 nm as reference wavelength (ϵ = 19.1 mM^{-1} cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8.0 μM DCPIP was preincubated with 48–80 μg supernatant protein at 30 °C for 20 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction was started by addition of 40 μM DCPIP and was monitored for 5 min at 30 °C. Succinate dehydrogenase (SDH) activity was assessed by adding 1 mM phenazine methasulphate to the reaction mixture. Then, SDH activity was monitored for 5 min at 30 °C at 600 nm with 700 nm as reference wavelength (Fisher et al., 1985).

2.6.3. Complex II–CoQ–III activity

Complex II–CoQ–III activity was measured by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength (ϵ = 21 mM^{-1} cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate was preincubated with 50–100 μg supernatant protein at 30 °C for 30 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction started by the addition of 0.6 μg/mL cytochrome *c* and monitored for 5 min at 30 °C (Fisher et al., 1985).

2.6.4. Complex IV activity

Complex IV activity was measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength (ϵ = 19.15 mM^{-1} cm^{-1}). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg supernatant protein and the reaction was started with addition of 0.7 μg reduced cytochrome *c*. The activity of complex IV was measured at 25 °C for 10 min (Rustin et al., 1994).

2.7. Caspase-3. activity

Caspase-3 activity was determined through a fluorimetric commercial kit according manufacturer's instructions (Sigma). Briefly, the samples were homogenized in lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT), and centrifuged at 10000 *g* for 15 min at 4 °C. The supernatants were used to determine caspase-3 assay in a microplate fluorimeter at 360 nm excitation and 460 nm emissions for 180 min at 25 °C. Results are expressed as nmol AMC produced/min.mg^{-1} protein.

2.8. Caspase-8. activity

Caspase-8 activity was determined through a colorimetric commercial kit according manufacturer's instructions (Biotium). The samples were prepared as described to investigate caspase-3 activity. However, caspase-8 activity was monitored in a microplate spectrophotometer at 495 nm for 180 min at 25 °C. Results are expressed as nmol R110 produced/min mg^{-1} protein.

2.9. TNF-α quantification

We have measured TNF-α through commercial kit for enzyme-linked immunosorbent assay (ELISA) according manufacturer's instructions (BD Biosciences). Briefly, tissue samples were collected and suspended in lysis buffer containing protease inhibitors. Following cell lysis, the homogenate was centrifuged, and a portion of the supernatant was reserved for protein concentration measurement, and the remaining was stored at −80 °C for posterior TNF-α levels quantification. The samples were read in a microplate spectrophotometer at 450 nm.

2.10. Behavioral assessment

2.10.1. Sucrose drinking test

Anhedonia was assessed according to Simen and colleagues (2006). At the end of the period of treatment with vitamin A, adult

Wistar rats were allowed to unrestricted access to 1% sucrose solution for 2 days before testing. On 2 consecutive days, after periods of 4 and 14 h of water deprivation, respectively, 1% sucrose solution was presented for 1 h and consumption was measured by weighing the solution. In each behavioral analysis, rats were used only once. At the end of the test, the animals were killed for brain analyses.

2.10.2. Forced swimming test

The test was performed between 10:00 and 13:00 h at the end of the treatment with vitamin A, according to Porsolt et al., 1977. Before the animals really were tested, a pre-test session was performed. The pre-test session consisted of a forced swim during 15 min in a cylinder (height 120 cm; diameter: 50 cm) containing 90 cm of water at 25 °C. They were removed from the cylinder and were allowed to dry for 20 min in a cage placed below an infrared lamp. Twenty-four hours after the pre-test session, the rats were again placed in the same cylinder for 5 min (test session), and an observer blind to the treatment have recorded their behavior. The duration of the following behavior was recorded: immobility (*i.e.,* floating and making only those movements necessary to keep nose above the water level); swimming (*i.e.,* active motion including diving); climbing (*i.e.*, when rats move their forepaws in and out the water, usually against the walls). At the end of this session, the animals were killed for brain analyses.

2.10.3. Tail suspension test

The rats were suspended by the tail on a horizontal beam 80 cm high. Rats were taped to the bar using adhesives placed approximately 3 cm from the tip of the tail. An observer blind to the treatments recorded immobility time during a 5 min test period. At the end of the test, the animals were killed for brain analyses.

2.11. Open field apparatus

The behavioral task occurred after the last vitamin A treatment, and was performed between 10:00 and 13:00 h. The open field task was carried out in 60 cm × 40 cm open field surrounded by 50 cm high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. The animals were placed on the same initial rectangle and were left to freely explore the arena for 5 min. Then, we observed: (1) latency time (*i.e.*, time spent to the animals move to the center of the open field for the first time), (2) number of entries into the center, (3) time spent if freezing behavior, (4) number of crossings (locomotion), and (5) number of faecal boli excreted in the open field. At the end of the test, the animals were killed for brain analyses.

2.12. Statistical analyses

Both biochemical and behavioral results are expressed as means ± standard error of the mean (S.E.M.); p values were considered significant when $p < 0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test whenever necessary.

3. Results

3.1. Oxidative damage levels

We have found increased lipid peroxidation levels in the frontal cortex of the animals that received vitamin A supplementation at 4500 and 9000 IU/kg/day ($p < 0.05$; Fig. 1A). Similarly, vitamin A supplementation at 4500 and 9000 IU/kg/day induced a 1.5–1.7-fold increase in protein carbonylation levels in rat frontal cortex ($p < 0.05$; Fig. 1B). Protein sulfhydryl levels were found decreased after vitamin A supplementation at 9000 IU/kg/day in frontal cortex of rats ($p < 0.05$; Fig. 1C).

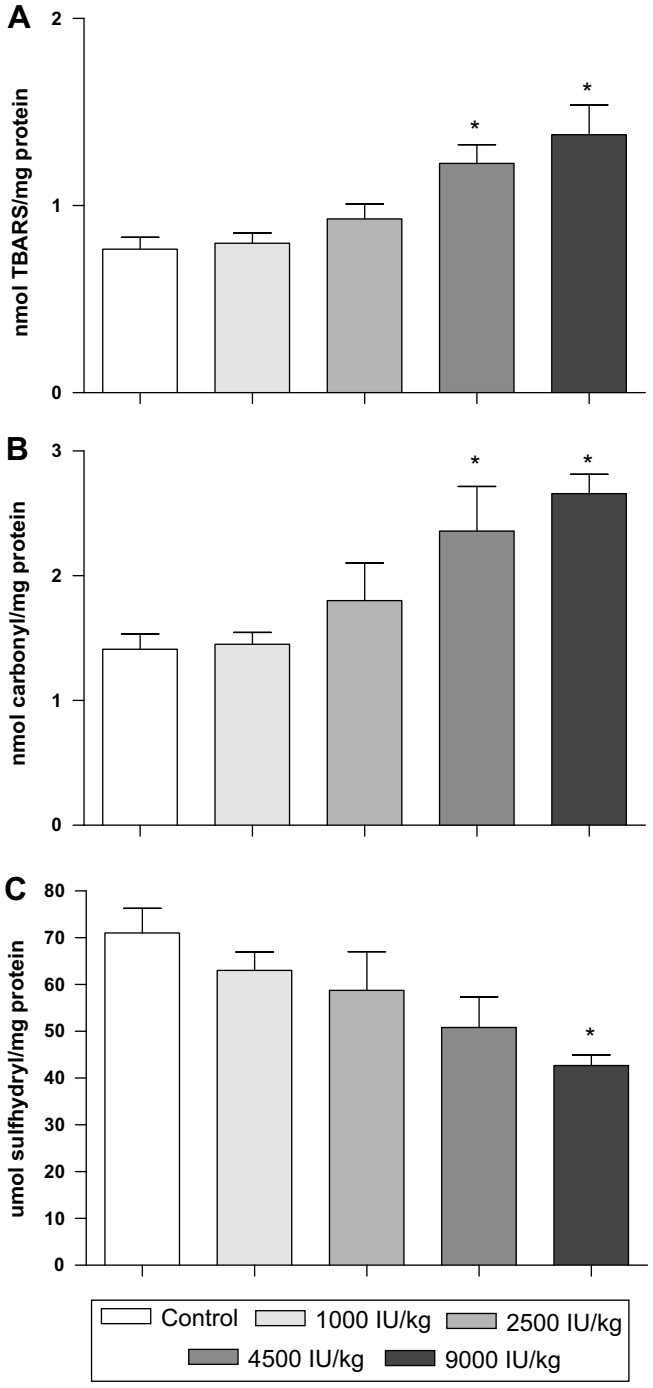

Fig. 1. Effects of vitamin A supplementation on lipid peroxidation (A), and protein carbonylation levels (B), and protein sulfhydryl content (C) in the rat frontal cortex. Data are mean ± S.E.M. (n = 8–10 per group). $^{*}p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

3.2. Antioxidant enzymes activities

Vitamin A supplementation induced a 1.8-fold increase of SOD activity in frontal cortex of the rats that were treated with 9000 IU/kg/day vitamin A ($p < 0.05$; Fig. 2A). Interestingly, CAT

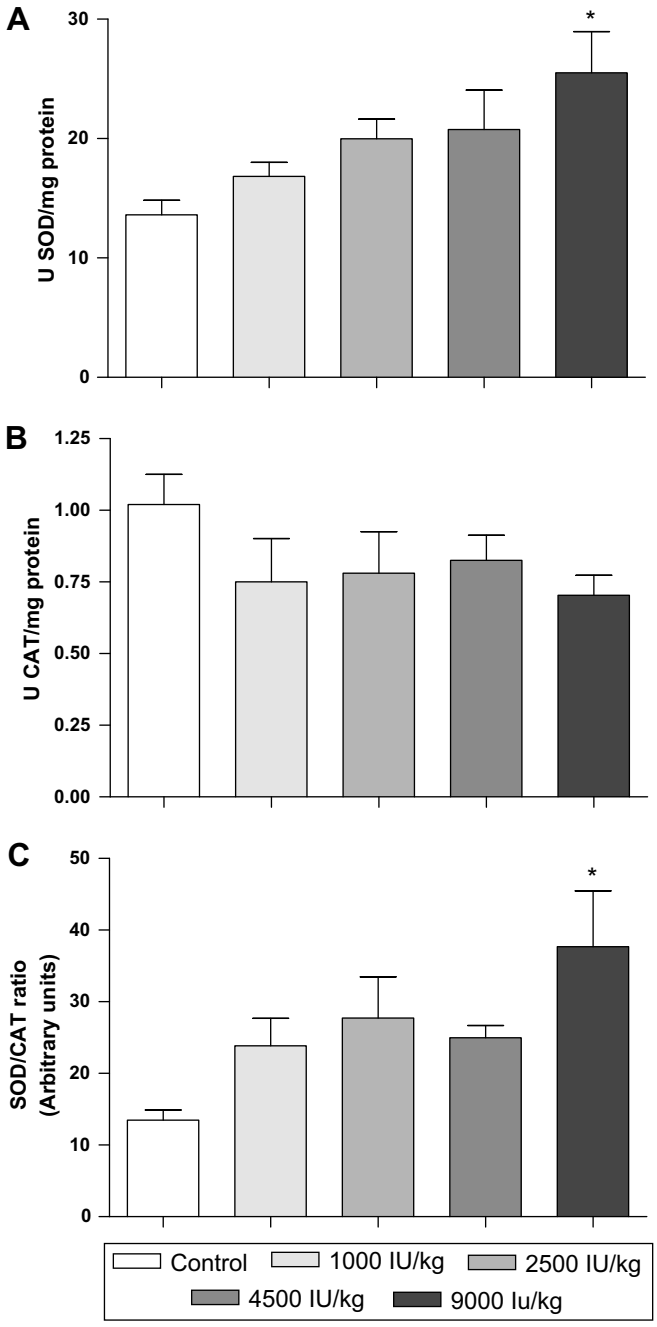

Fig. 2. Effects of vitamin A supplementation on SOD (A) and CAT (B) enzymes activities in the adult rat frontal cortex. SOD/CAT ratio is depicted in (C). Data are mean ± S.E.M. (n = 8–10 per group). $^{*}p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

activity did not change in the animals that were treated with vitamin A (Fig. 2B). The SOD/CAT ratio, which may be useful in indicating hydrogen peroxide production and cleaning (Halliwell, 2006), was found higher than control group only in the animals that received vitamin A at 9000 IU/kg/day ($p < 0.05$; Fig. 2C).

3.3. Oxidative parameters in submitochondrial particles

We have found that vitamin A supplementation induced a 1.7–1.9-fold increase of superoxide anion production in SMP isolated from frontal cortex of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/kg/day ($p < 0.01$; Fig. 3A). However, lipid peroxidation and protein carbonylation levels were found higher than control group levels only in the frontal cortex SMP isolated from the animals that received vitamin A supplementation at 4500 and 9000 IU/kg/day ($p < 0.01$; Fig. 3B and C, respectively). Protein sulfhydryl levels did not change in frontal cortex SMP of vitamin A treated rats when compared to control group (Fig. 3D).

3.4. Mitochondrial electron transfer chain (METC) activity

Complex I–CoQ–III activity was found increased (1.4–1.7-fold) in the frontal cortex of the animals that were treated with vitamin A supplementation at 4500 and 9000 IU/kg/day ($p < 0.05$; Fig. 4A). However, complex II and succinate dehydrogenase (SDH) activities did not change when analyzed alone (Fig. 4B and C, respectively). Similarly, complex II–CoQ–III activity did not change (Fig. 4D). Additionally, vitamin A supplementation did not alter frontal cortex complex IV activity (Fig. 4E).

3.5. Caspase-3. and caspase-8 activities

As depicted in Fig. 5A and B, vitamin A supplementation did alter neither caspase-3 nor caspase-8 enzymes activities in rat frontal cortex, respectively.

3.6. TNF-α levels

TNF-α, a pro-inflammatory cytokine, did not change in the rats that received vitamin A supplementation at the doses tested here (Fig. 6).

3.7. Behavioral assessment

As shown in Fig. 7A, chronic vitamin A supplementation did not induce anhedonia in adult Wistar rats at the doses tested here. Sucrose consumption did change neither after a 4 h nor after a 14 h period of water deprivation. As depicted in Fig. 7B, the time spent immobile in the forced swimming test did not differ among the experimental groups in the herein presented work. Similarly to the results obtained in the other behavioral tests described above, we did not find any change in rat behavior in the tail suspension test regarding immobility time (Fig. 7C). However, we observed increased latency time (time spent to move to the center of the open field for the first time) and decreased number of entries into the center of the open field in the rats that were treated with vitamin A at any dose tested (Table 1). Furthermore, the animals that received vitamin A supplementation at any dose tested spent more time in freezing behavior than control animals. Accordingly, the number of crossings in an open field apparatus was observed decreased in the animals that received vitamin A supplementation at any dose tested. The animals that were treated with vitamin A supplementation at any dose tested excreted more faecal boli than control animals in the open field apparatus (Table 1).

4. Discussion

In an attempt to investigate the mechanism by which vitamin A supplementation induces cognitive impairments in rats, we have analyzed, in the present work, biochemical and behavioral parameters that have been shown to be associated with mood disorders, for instance anxiety and depression (Duman, 2004; Burroughs and French, 2007). In the herein presented work, we analyzed redox and bioenergetics parameters in frontal cortex of vitamin A treated adult rats. In addition, we have investigated whether vitamin A induces cell death in the rat frontal cortex, since increased rates of

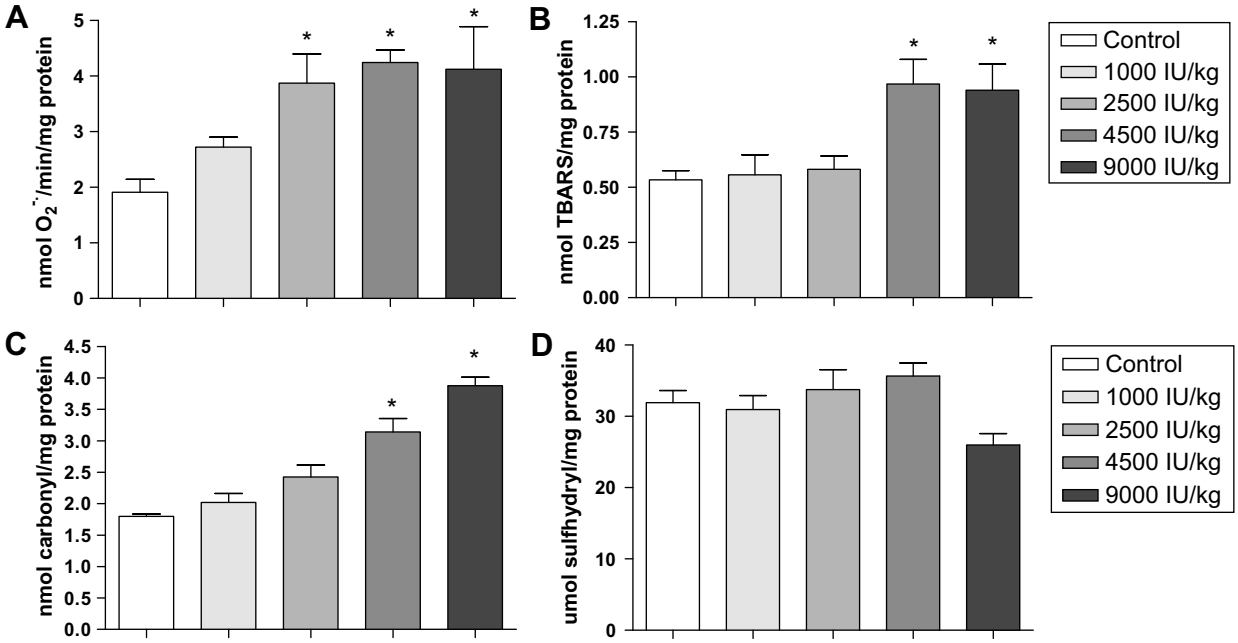

Fig. 3. Effects of vitamin A supplementation on $O_2^{\cdot-}$ production (A), lipid peroxidation (B), protein carbonylation (C), and protein sulfhydryl levels (D) in frontal submitochondrial particles. Data are mean ± S.E.M. (n = 8–10 per group). $^{*}p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

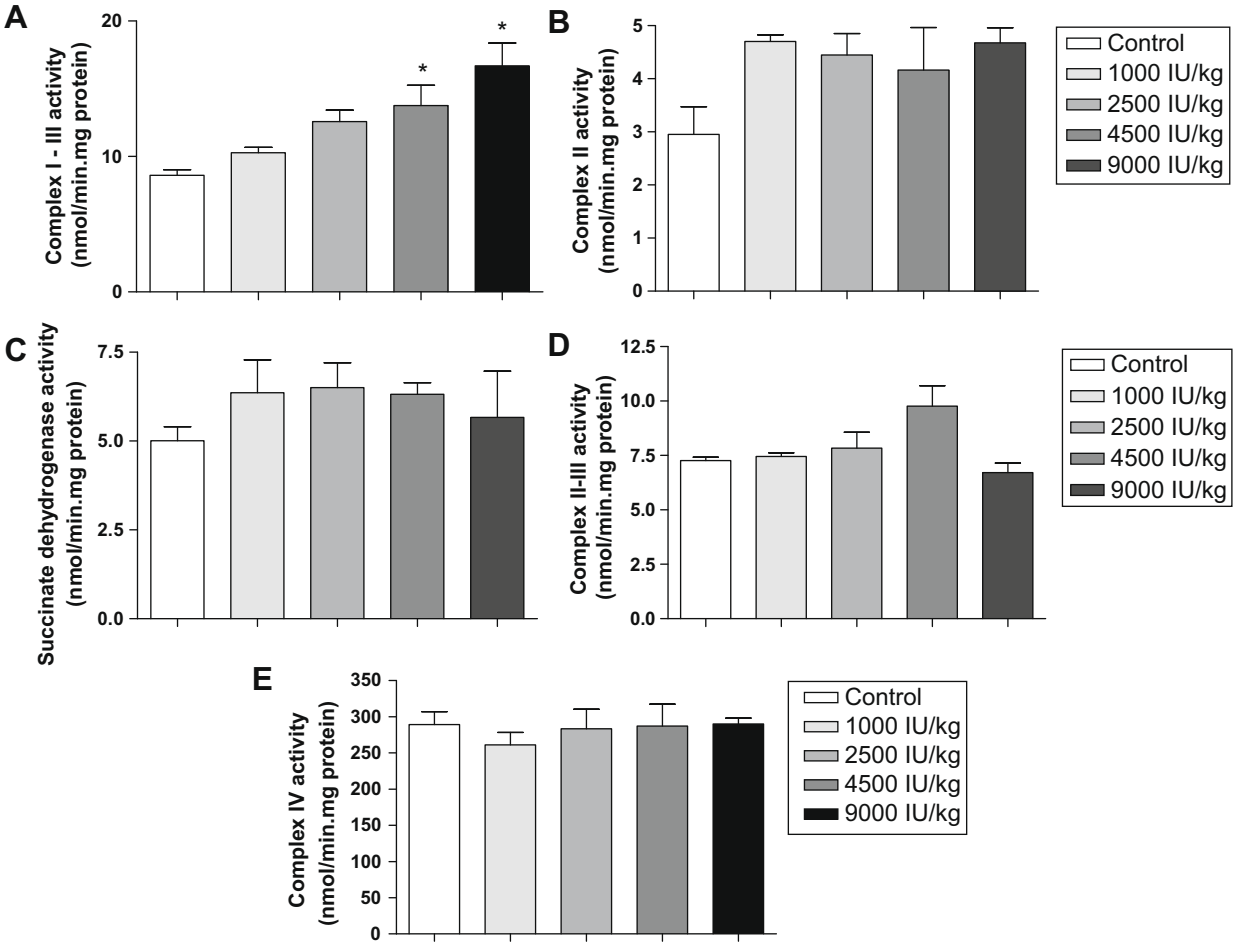

Fig. 4. Effects of vitamin A supplementation on Complex I–III (A), Complex II (B), succinate dehydrogenase (SDH) (C), Complex II–III (D), and Complex IV (E) activities in adult rat frontal cortex. Data are mean ± S.E.M. (n = 8–10 per group). $^{*}p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

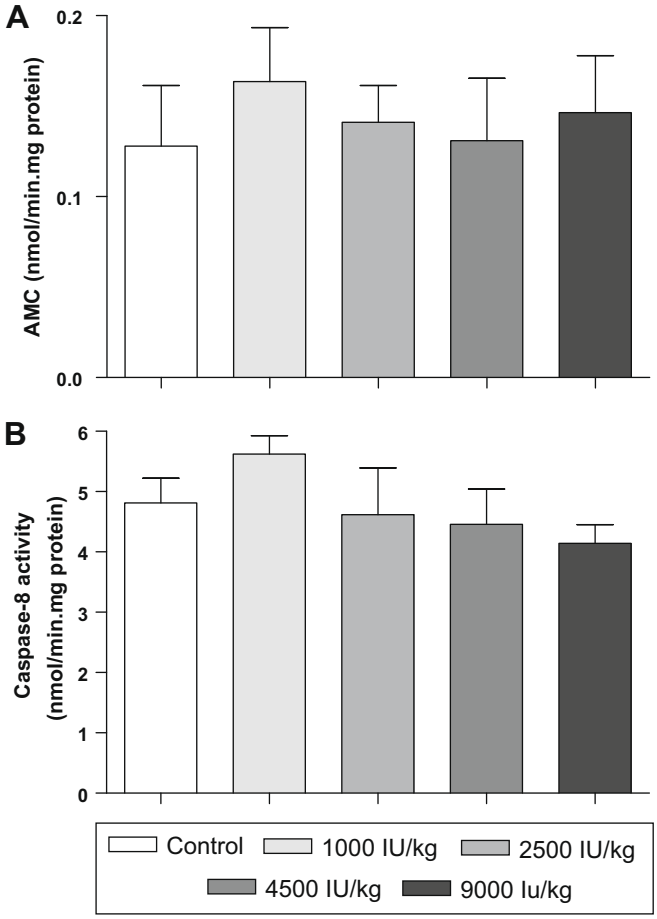

Fig. 5. Effects of vitamin A supplementation on caspase-3 (A) and caspase-8 (B) enzymes activities in the adult rat frontal cortex. Data are mean ± S.E.M. (n = 8–10 per group).

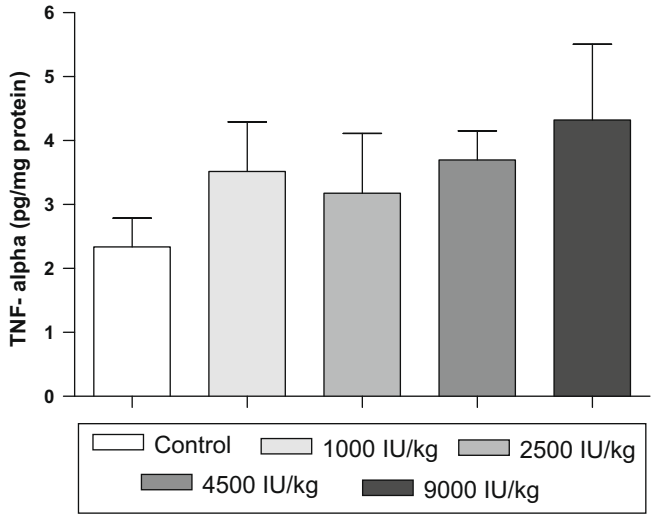

Fig. 6. Effects of vitamin A supplementation on TNF-α levels in the adult rat frontal cortex. Data are mean ± S.E.M. (n = 8–10 per group).

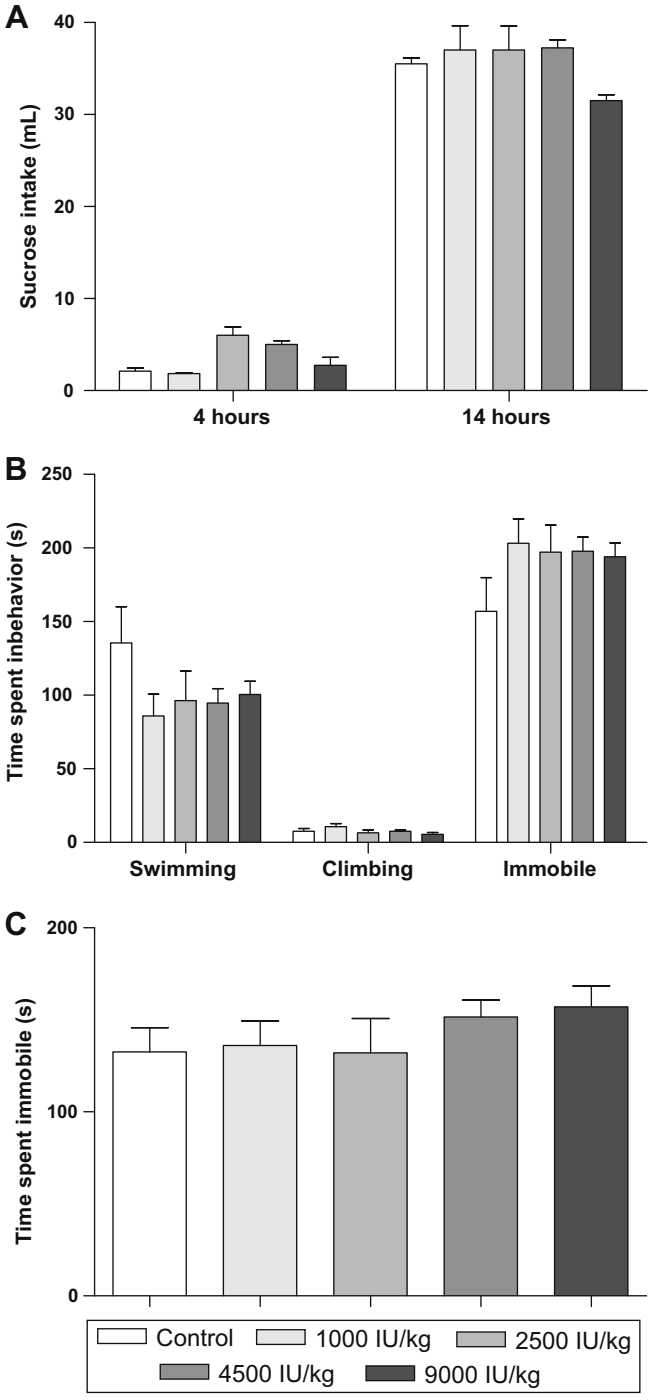

Fig. 7. Effects of vitamin A supplementation on the consumption of 1% sucrose drinking solution by adult rats (A), on rat behavior in the forced swimming test (B), and on time spent immobile in the tail suspension test (C). Data are mean ± S.E.M. (n = 10 per group).

apoptosis and/or necrosis in this brain area may be implicated in the mechanism of mood disorders, including depression, as mentioned above. Surprisingly, we have detected moderated oxidative stress markers levels in the frontal cortex when compared to other rat brain regions previously published (De Oliveira and Moreira, 2007; De Oliveira et al., 2007a,b; De Oliveira et al., 2008). In addition, we have found increased complex I–III activity, a result that was not expected, since oxidative damage levels are, commonly, inversely associated with METC activity. However, the increase in complex I–III activity was not accompanied by a similar increase in complex IV, which may give rise to an increase in superoxide anion ($O_2^{-\cdot}$) production. Even though the presence of a pro-oxidant state, we have not observed any change in caspases activity, which suggests that cell death is not occurring, at least in part, by those ways in frontal cortex. Finally, depression-like behavior was not

Table 1
Effects of vitamin A supplementation on rat behavior in an open field.

Group	Latency time	Entries into center (times)	Time spent in freezing behavior (s)	Number of crossings	Faecal boli (number)
Control	68.3 ± 4.1	7.5 ± 0.6	10.2 ± 2.3	125.0 ± 6.1	1.22 ± 0.43
1000 IU/kg day^{-1}	124.4 ± 16.0*	3.0 ± 0.4*	61.5 ± 5.5*	77.0 ± 4.6*	5.22 ± 0.32*
2500 IU/kg day^{-1}	132.3 ± 11.6*	3.5 ± 0.6*	44.0 ± 7.9*	65.6 ± 5.7*	4.77 ± 0.66*
4500 IU/kg day^{-1}	139.6 ± 12.4*	3.3 ± 0.5*	62.2 ± 8.1*	65.0 ± 9.1*	5.00 ± 0.85*
9000 IU/kg day^{-1}	146.3 ± 14.4*	2.0 ± 0.7*	50.5 ± 6.2*	64.2 ± 7.8*	5.67 ± 0.7*

Values are means ± S.E.M. of 10 animals per group.
* $p < 0.05$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

observed, but some parameters that may suggest anxiety-like behavior were found.

To the best of our knowledge, this is the first work demonstrating *in vivo* cell fate in frontal cortex of vitamin A treated rats. Even thought we have not observed any change in cell death rates in frontal cortex, this is an important finding in the search by the mechanism by which vitamin A induces cognitive impairments in mammals, showing that other cellular signaling pathways could be involved in the onset of cognitive impairments triggered by vitamin A supplementation at clinical doses. We have recently demonstrated that vitamin A supplementation induced anxiety-like behavior in adult Wistar rats. In addition, we have shown that that vitamin A treatment decreased the exploratory activity of rats in an open field apparatus (De Oliveira et al., 2007b). Now, we have investigated other behavioral parameters commonly associated with mood disorder. Surprisingly, we did observe neither anhedonia nor increased immobility time in behavioral tests widely adopted to assess depressive behavior in animals. However, we observed here that vitamin A treated rats spent more time to move for the first time to the center of an open field than control animals. Additionally, vitamin A treated rats spent more time in freezing behavior and excreted more faecal boli than the animals that receive saline daily (Table 1), reinforcing our previously published data showing anxiety-like behavior in vitamin A treated rats (De Oliveira et al., 2007b).

Regarding $O_2^{-\cdot}$ production and METC activity, it is plausible that $O_2^{-\cdot}$ production is increased, since we have found increase complex I–III activity, but complex IV, the fate of cytochrome *c* (cyt *c*) in the electron transfer chain (ETC), had its activity found unaltered (Fig. 4E). It was previously demonstrated that when there is cyt *c* leakage from the ETC, there is an increase in $O_2^{-\cdot}$ production (Klamt et al., 2005). On the other hand, retinol metabolism *per se* is able to induce an increase in NADH production in the cytosol through its metabolism by the enzymes responsible for the retinoid metabolism, namely alcohol dehydrogenase (Napoli, 1999). This excess in NADH can be carried through electron shuttles into the mitochondria, increasing the activity of complex I–III. Then, we could detect an increased complex I–III activity, an unaltered complex IV activity, and an increase rate of $O_2^{-\cdot}$ production, as seen here. The hypotheses that cyt *c* has been released by mitochondrial damage is not as strong as that of increased NADH production through retinoid metabolism in such brain area, since we have not detected increased caspase-3 activity, which is a next step after cyt *c* leakage from mitochondria. This would result in increased apoptosis rates, a process where both caspase-3 and caspase-8 may be involved (Hengartner, 2000; Newmeyer and Ferguson-Miller, 2003). Additionally, we have not detected any change in TNF-α levels, a pro-inflammatory cytokine that participates in the induction of cell death by an extrinsic pathway where caspase-8 activity is increased after TNF-α signaling (Griffin, 2006). An explanation to the unaltered caspases activity would be that about cysteine oxidation in the active center of the enzyme, since the reduced cysteine residue presented there is crucial to the caspase activity (Hengartner, 2000). However, we did not observe any change in the protein sulfhydryl redox status in the frontal cortex, indicating that redox-dependent inhibition of caspases is not an effect of the treatment in this experimental model.

Other authors have found depressive behavior in animals that were treated for 28 days with 13-*cis* retinoic acid, a vitamin A derivative used in the treatment of acne. However, the drug used in the present work is retinol palmitate, an ester of vitamin A commonly found in food of animal origin, as widely described (Napoli, 1999). In addition, depending on animal species and strain, the results are very likely to be different. It is a fact that vitamin A supplementation induces anxiety-like behavior in adult Wistar rats (De Oliveira et al., 2007b), and also in humans, as demonstrated by Myhre et al., 2003. It is also known that anxiety is a preliminary step to depression in several experimental models and was also observed in humans (Burroughs and French, 2007). Currently, vitamin A (retinol palmitate) at doses exceeding 150,000 IU/day is applied in the treatment of infants and children with cancer during a long period (Allen and Haskell, 2002). However, it remains to be tested whether this long term treatment would induce cognitive disturbances in such patients. Unfortunately, it is almost impossible to indicate the vitamin A metabolite responsible for the observed effects, given the vast number of vitamin A metabolites existing (Napoli, 1999). Also, case reports of vitamin A toxicity have shown serum retinol concentrations within normal limits (Ellis et al., 1986; Croquet et al., 2000; Mills and Tanumihardjo, 2006), indicating that serum retinol is not a good measure of vitamin A status during toxicity.

Here, we have investigated the effects of a 28 days period treatment with retinol palmitate on rat behavior and biochemical parameters. Then, it is plausible that a longer period of treatment with retinol palmitate, as that applied in patients suffering from leukemia and dermatological disturbances would result in depressive behavior in rats, since here we have found a trend to this effect. Additionally, it is now well accepted that there is a population of patients, for example, that is more sensitive to the effects elicited by vitamin A treatment regarding mood disorders and other cognitive impairments (O'Reilly et al., 2008). More investigations are needed to accurately elucidate the mechanism by which vitamin A induces anxiety-like behavior in adult rats and, more importantly, whether there is a chance to this treatment induce other cognitive impairments in mammals, including human under long term vitamin A treatment.

Conflict of interest

Authors state that they have no conflicts of interest.

Contributors

Author M.R. De Oliveira and J.C.F. Moreira designed the study and wrote the protocol. Author M.R. De Oliveira performed gavage procedures. Authors M.W.S. Oliveira and G.A. Behr performed the homogenization of the samples in lysis buffer immediately after their isolation from rat brain. M.R. De Oliveira performed the

biochemical analyses and M.R. De Oliveira and J.C.F. Moreira undertook the statistical analysis. M.R. De Oliveira wrote the first draft of the manuscript, which was revised by J.C.F. Moreira. All authors contributed to and have approved the final manuscript.

Role of funding sources

Funding for this study was provided by CNPq. This Institution has no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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Capítulo III

“Vitamin A supplementation at pharmacological doses induces nitrosative stress on the hypothalamus of adult Wistar rats”

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Vitamin A supplementation at pharmacological doses induces nitrosative stress on the hypothalamus of adult Wistar rats

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ABSTRACT

Vitamin A is a micronutrient involved in the regulation of a normal mammalian brain function. In spite of this, it has been demonstrated that vitamin A exerts a wide range of deleterious effects regarding neuronal homeostasis, for instance impairing brain metabolism and suppressing neurogenesis, to cite a few. In addition, vitamin A is a redox active molecule, *i.e.* it is both anti- and pro-oxidant, depending on its concentration. In the herein presented work, we performed some experiments aiming to investigate the effects of clinically applied doses of vitamin A (1000–9000 IU/kg/day during 28 days) on rat hypothalamic redox state and mitochondrial electron transfer chain (METC) activity, as well as on hypothalamic α-synuclein and D2 receptor (dopamine receptor) contents. Additionally, we quantified caspase-3 activity and tumor necrosis factor-α (TNF-α) levels to assess either neuronal death or an inflammatory state in such brain area. We found that vitamin A supplementation increased free radical production, as well as oxidative and nitrosative stress, in rat hypothalamus. Also, we observed increased complex I-III activity, but decreased complex IV activity in the hypothalamus of vitamin A-treated rats, which may give rise to the increased superoxide anion ($O_2^{\bullet -}$) production found here. Other parameters investigated here, *i.e.* α-synuclein and D2 receptor contents did not change. Even though we did not observe signs of increased cell death or inflammation in the rat hypothalamus, more attention is needed when vitamin A is the choice of treatment in certain pathologies.

1. Introduction

The mammalian brain needs vitamin A during all life, from embryonic to adult stages [1–3]. The importance of this necessity may be observed in works reporting morphologic, functional, and cognitive abnormalities in vitamin A deprived animals [4–6]. Nevertheless, there is considerable attention to the effects of vitamin A (retinol palmitate) on the central nervous system integrity and function, since a great body of evidence indicates a potential neurotoxic capacity of this vitamin on both animal and human brains [7–10]. It was shown that vitamin A, whose daily intake varies among individuals and is also strongly associated with the use of the vitamin as a therapeutic, induces necrotizing vasculitis, hepatotoxicity, headache, irritability, confusion, and even depression in patients under treatment of leukemia and/or dermatological disturbance [10–12]. Regarding retinol palmitate (vitamin A), which is commonly commercialized at drug stores, there is a lack of information about the consequences of its use at clinical doses, although it is currently applied in the treatment of some pathologies, as mentioned above. However, we have recently shown that retinol palmitate induces oxidative stress in some rat brain regions and, more importantly anxiety-like behavior in adult rats [13–16].

Actually, vitamin A is a redox active molecule, *i.e.* it has an ability to protect biological systems against the pro-oxidant potential of some molecules [17]. However, it was also reported that vitamin A induces pro-oxidant effects in several experimental models (please see below). We have demonstrated that vitamin A treatment (retinol) is able to increase the production of reactive oxygen species (ROS) and to modulate antioxidant enzyme activities *in vitro* and *in vivo* [13–16,18–23]. In addition, it was demonstrated that rat liver mitochondria incubated with retinol produces more superoxide anion radical ($O_2^{\bullet -}$) and, consequently, presented increased lipid peroxidation levels, which may favor a mitochondrial swelling with concomitant cytochrome *c* release from damaged mitochondria, an early pro-apoptotic step, consequently leading to caspase-3 enzyme activation by positively regulating caspase-9 enzyme activity [24,25]. As mentioned above, we have also observed a pro-oxidant effect of vitamin A in *in vivo* experimental models—acute and chronic vitamin A supplementation

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increased the levels of molecular markers of oxidative stress in rat hippocampus, striatum, *substantia nigra*, cerebral cortex and cerebellum [13–16]. Even though free radicals' production may be induced in the cellular environment through exposition to some pro-oxidant compounds, the mitochondrial electron transfer chain (METC) activity is responsible for about 20% of total $O_2^{\bullet -}$ production physiologically. Mitochondrial dysfunction, as that attributed to vitamin A, consequently increases the rate of free radicals' production to dangerous levels, which may culminate in oxidative damage to biomolecules and loss of function of proteins, for example [17].

In the herein presented work, we have analyzed the effects of vitamin A supplementation at clinical doses on both redox status and METC activity of adult rat hypothalamus. In addition, we have investigated the effects of such treatment on hypothalamic α-synuclein, D2 receptor, and receptor for advanced glycation endproducts (RAGE) contents. Furthermore, based on the previously reported findings that retinoids modulate cell fate [24,25], we quantified here hypothalamic caspase-3 enzyme activity and tumor necrosis factor-α (TNF-α) levels. Given that the mammalian hypothalamus plays a pivotal role in regulating food intake [26], we have chosen to investigate this brain region because there are reports describing both loss of appetite and weight loss in patients under vitamin A treatment [10]. The retinol palmitate (vitamin A) doses tested here are commonly applied in cancer treatment in both children and adult humans [9,10,12]. Therefore, it is above the nutritional requirement of the vitamin to humans. An example is the administration of vitamin A (as retinol palmitate) at doses exceeding 150,000 IU/kg/day to infants and children suffering from some types of cancer [9]. Additionally, very-low-weight-preterm infants receive vitamin A at a maximum daily dose of 8500 IU/kg during weight gain treatment, which occurs for an undetermined period, as reviewed in [27].

2. Materials and methods

2.1. Animals

Adult male Wistar rats (270–300 g; 90 days old) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12 h light–dark cycle (7:00–19:00 h), at a temperature-controlled colony room (23 ± 1 °C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

2.2. Drugs and reagents

Arovit® (retinol palmitate, a water-soluble form of vitamin A) was purchased from Roche, Sao Paulo, SP, Brazil. Antibodies to α-synuclein and D2 receptor were obtained from Chemicon, USA. Antibody to RAGE was purchased from Calbiochem, USA. TNF-α assay kit was obtained from BD Biosciences, San Diego, CA, USA. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A treatment was prepared daily and it occurred by protecting from light.

2.3. Treatment

The animals were treated once a day for 28 days with a gavage. Treatments were carried out at night (*i.e.* when the animals are more active and take a greater amount of food) in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. The animals were treated daily with vehicle (0.15 M saline), 1000, 2500, 4500, or 9000 IU/kg of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0.6 mL. Adequate measures were taken to minimize pain or discomfort.

2.4. Oxidative stress and antioxidant enzyme activity analyses

Before sacrifice, the animals were anesthetized with ketamine plus xylazine (100 and 14 mg/kg, respectively). The animals were sacrificed by decapitation at 24 h after the last vitamin A administration. Rat hypothalamus was dissected out in ice immediately after the rat was sacrificed and stored at −80 °C for posterior oxidative stress analyses. The homogenates were centrifuged (700 × *g*, 5 min) to remove cellular debris. Supernatants were used for all biochemical assays described herein. All the results were normalized by the protein content using bovine albumin as standard [28].

2.4.1. Thiobarbituric acid reactive species (TBARS)

As an index of lipid peroxidation, we used the formation of TBARS during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described [29]. Briefly, the samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm. Results are expressed as TBARS/mg protein.

2.4.2. Indirect enzyme-linked immunosorbent assay (ELISA) to 3-nitrotyrosine

Indirect ELISA assay was performed to analyze changes in the content of 3-nitrotyrosine by utilizing a polyclonal antibody to 3-nitrotyrosine diluted 1:5000 in phosphate-buffered saline (PBS) pH 7.4 with 5% albumin. Briefly, microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:5 in PBS with 5% albumin. Plates were then washed four times with wash buffer (PBS with 0.05% Tween-20), and the specific antibodies were added to the plates for 2 h at room temperature. After washing (four times), a second incubation with anti-rabbit antibody peroxidase conjugated (diluted 1:1000) for 1 h at room temperature was carried out. After addition of substrates (hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine, 1:1, v/v), the samples were read at 450 nm in a plate spectrophotometer. Results are expressed as changes in percentage among the groups.

2.4.3. Measurement of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH) as previously described [30]. Briefly, proteins were precipitated by the addition of 20% TCA and redissolved in DNPH and the absorbance read in a spectrophotometer at 370 nm. Results are expressed as nmol carbonyl/mg protein.

2.4.4. Antioxidant enzyme activity estimations

Superoxide dismutase activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described [31], and the results are expressed as U SOD/mg protein. Catalase (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm [32], and the results are expressed as U CAT/mg protein.

2.5. Oxidative parameters in submitochondrial particles

To obtain submitochondrial particles (SMP), hypothalamus was dissected and homogenized in 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl and 1 mM EDTA (pH 7.4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free SMP. The SMP solution was also washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) to ensure Mn-SOD release from mitochondria. To quantify superoxide ($O_2^{\bullet -}$) production, SMP was incubated in reaction medium consisting of 230 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 μM catalase, and 1 mM epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32 °C, as previously described [13,33]. As a marker of lipid peroxidation, we measured the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, as previously described [29].

2.6. METC activity

To obtain SMP from hypothalamus in order to assess METC activity, we have homogenized the tissue in 250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4, and 50 IU/mL heparin buffer. The samples were centrifuged 1000 × *g* and the supernatants were colleted. Then, the samples were frozen and thawed three times, and METC activity detection was performed as described below.

2.6.1. Complex I-CoQ-III activity

Complex I-CoQ-III activity was determined by following the increase in absorbance due to reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\in$ = 19.1 mM^{-1} cm^{-1}). The reaction buffer contained 20 mM potassium phosphate, pH 8.0, 2.0 mM KCN, 10 μM EDTA, 50 μM cytochrome *c*, and 20–45 μg supernatant protein. The reaction was started by addition of 25 μM NADH and was monitored at 30 °C for 3 min before the addition of 10 μM rotenone, after which the activity was monitored for an additional 3 min. Complex I-III activity was the rotenone-sensitive NADH:cytochrome *c* oxidoreductase activity [34].

2.6.2. Complex II and succinate dehydrogenase (SDH) activities

Complex II (succinate–DCPIP–oxidoreductase) activity was measured by following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCPIP) at 600 nm with 700 nm as reference wavelength ($\in$ = 19.1 mM^{-1} cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8.0 μM DCPIP was preincubated with 48–80 μg supernatant protein at 30 °C for 20 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction was started by addition of 40 μM DCPIP and was monitored for 5 min at 30 °C. Succinate dehydrogenase (SDH) activity was assessed by adding 1 mM phenazine methasulphate to the reaction mixture. Then, SDH activity was monitored for 5 min at 30 °C at 600 nm with 700 nm as reference wavelength [35].

2.6.3. Complex II-CoQ-III activity

Complex II-CoQ-III activity was measured by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength ($\in$ = 21 mM^{-1} cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate was preincubated with 50–100 μg supernatant protein at 30 °C for 30 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction was started by the addition of 0.6 μg/mL cytochrome *c* and monitored for 5 min at 30 °C [35].

2.6.4. Complex IV activity

Complex IV activity was measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\in$ = 19.15 mM^{-1} cm^{-1}). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg supernatant protein and the reaction was started with addition of 0.7 μg reduced cytochrome *c*. The activity of complex IV was measured at 25 °C for 10 min [36].

2.7. Caspase-3 activity

Caspase-3 activity was determined through a fluorimetric commercial kit according to manufacturer's instructions (Sigma). Briefly, the samples were homogenized in lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT), and centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatants were used to determine caspase-3 assay in a microplate fluorimeter at 360 nm excitation and 460 nm emission for 180 min at 25 °C. Results are expressed as nmol AMC produced/min/mg protein.

2.8. TNF-α levels quantification

We have measured TNF-α through commercial kit for enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (BD Biosciences). Briefly, tissue samples were collected and suspended in lysis buffer containing protease inhibitors. Following cell lysis, the homogenate was centrifuged, and a portion of the supernatant was reserved for protein concentration measurement, and the remaining was stored at −80 °C for posterior TNF-α levels quantification.

2.9. Statistical analyses

Results are expressed as means ± standard error of the mean (S.E.M.); *p* values were considered significant when $p < 0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test whenever necessary.

3. Results

3.1. Oxidative damage levels and antioxidant enzyme activities

Vitamin A supplementation at 4500 or 9000 IU/kg/day induced an increase in lipid peroxidation levels in adult rat hypothalamus ($p < 0.05$; Fig. 1A). The levels of 3-nitrotyrosine were observed increased in the hypothalamus of the rats that were treated with vitamin A supplementation at 9000 IU/kg/day ($p < 0.05$; Fig. 1B). Also, we found that vitamin A at 4500 or 9000 IU/kg/day induced an increase in protein carbonylation levels ($p < 0.05$; Fig. 1C). SOD activity was found increased only in the hypothalamus of the rats that received vitamin A at 9000 IU/kg/day ($p < 0.05$; Fig. 1D). Vitamin A supplementation at 4500 or 9000 IU/kg/day increased hypothalamic CAT activity ($p < 0.05$; Fig. 1E). Nevertheless, the SOD/CAT ratio did not change in the hypothalamus of vitamin A-treated rats (Fig. 1F).

3.2. Oxidative parameters in submitochondrial particles

We found a 1.6–1.8-fold increase of lipid peroxidation levels in SMP isolated from the hypothalamus of the rats that received vitamin A supplementation at 4500 and 9000 IU/kg/day ($p < 0.05$; Fig. 2A). Interestingly, we found that vitamin A supplementation at 2500, 4500 or 9000 IU/kg/day induced a 1.6–2.4-fold increase of

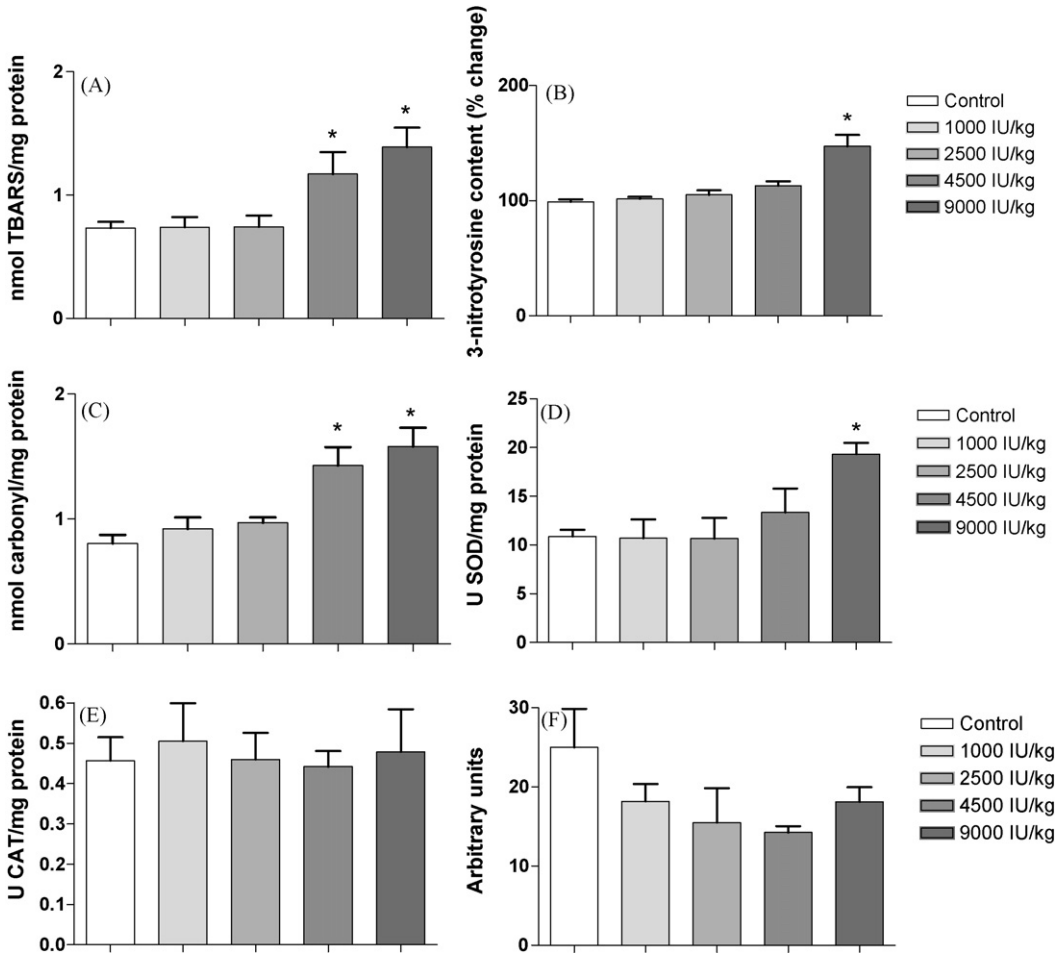

Fig. 1. Effects of vitamin A supplementation on lipid peroxidation (A), 3-nitrotyrosine content (B), protein carbonylation levels (C), SOD (D) and CAT activity (E) in the rat hypothalamus. SOD/CAT ratio is represented in (F). Data are mean ± S.E.M. ($n = 8–10$ per group). $^{*}p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

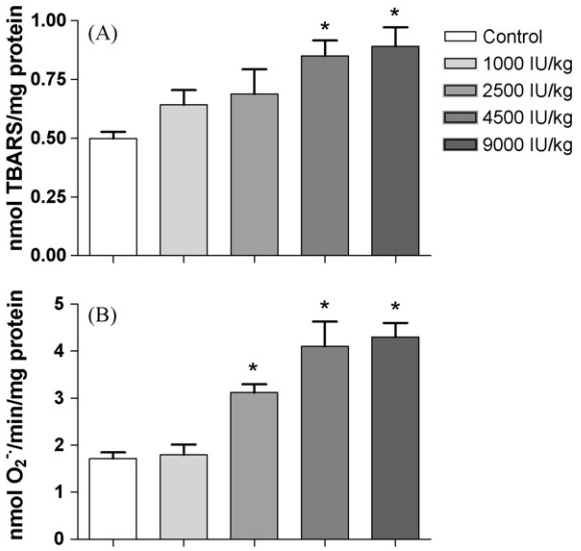

Fig. 2. Effects of vitamin A supplementation on lipid peroxidation (A) and on $O_2^{\bullet -}$ production (B) in hypothalamic SMP. Data are mean ± S.E.M. ($n = 8–10$ per group). $^{*}p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

superoxide anion radical ($O_2^{\bullet -}$) production in hypothalamic SMP ($p < 0.05$; Fig. 2B).

3.3. *METC activity*

Vitamin A supplementation at 4500 and 9000 IU/kg/day induced an increase in hypothalamic complex I-III activity ($p < 0.05$; Fig. 3A). However, we did not find any change in complex II or SDH activity in the hypothalamus of the rats that received vitamin A supplementation at any dose tested (Fig. 3B and C, respectively). Accordingly, complex II-III activity did not change in this experimental model (Fig. 3D). Complex IV activity was decreased in the hypothalamus of the rats that were treated with vitamin A supplementation at 4500 and 9000 IU/kg/day ($p < 0.05$; Fig. 3E).

3.4. *α-Synuclein, D2 receptor and RAGE contents*

As depicted in Fig. 4, vitamin A supplementation did not alter hypothalamic α-synuclein, D2 receptor and RAGE contents (Fig. 4A, B, and C, respectively).

3.5. *Caspase-3 activity and TNF-α levels*

We did not find any change regarding caspase-3 activity, as well as TNF-α levels, in the hypothalamus of vitamin A-treated rats (Fig. 5A and B, respectively).

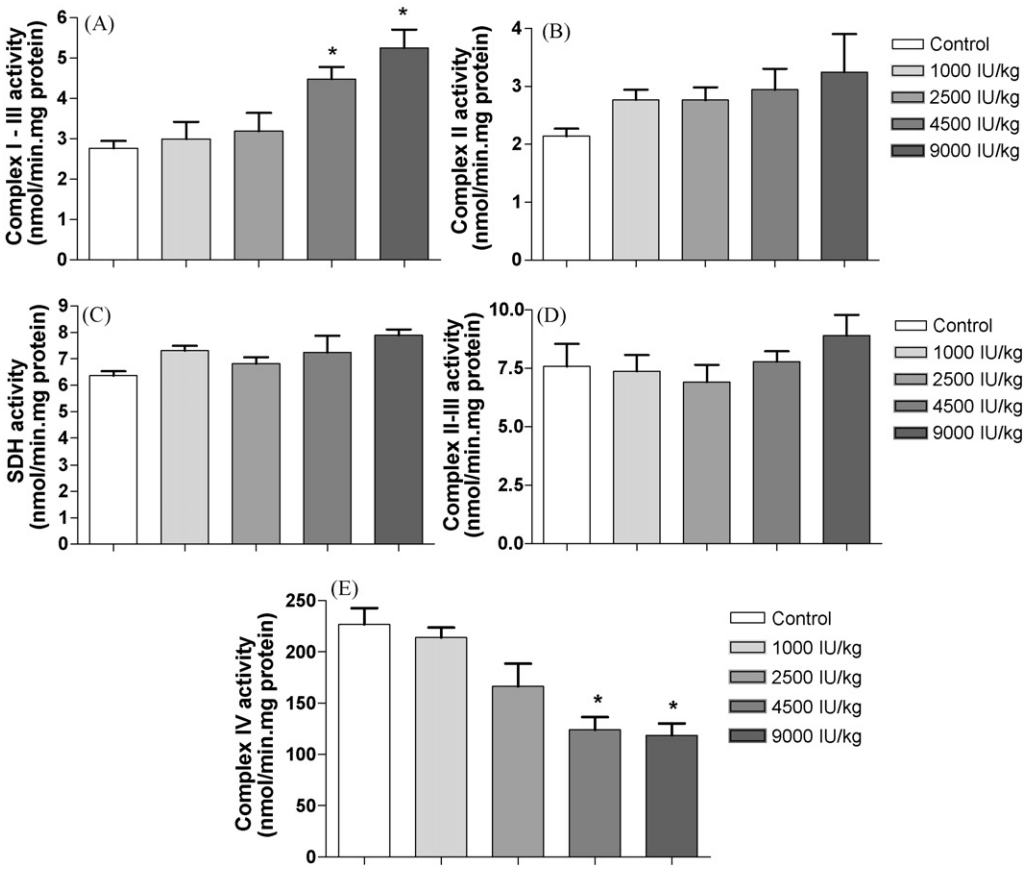

Fig. 3. Effects of vitamin A supplementation on complex I-III (A), complex II (B), SDH (C), complex II-III (D), and complex IV (E) activites in adult rat hypothalamus. Data are mean ± S.E.M. (n = 8–10 per group). $^{*}p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

4. Discussion

In this work, we have found, for the first time, that chronic vitamin A supplementation at doses commonly used therapeutically [9,10,12,37] induces hypothalamic nitrosative stress and an imbalanced increase in the METC activity, since we have observed increased complex I-III activity, but decreased complex IV activity, which is very likely to result in increased $O_2^{\bullet -}$ production due to electron leakage and partial reduction of O_2 by cytochrome oxidase enzyme (complex IV). In SMP isolated from rat hypothalamus we also detected increased lipid peroxidation levels. Additionally, we have found increase in both SOD and CAT enzymes activity in hypothalamic samples, suggesting increased reactive species production in this experimental model. Food ingestion during the period of treatment did not change among the groups, since we have not observed any change regarding rat body weight (data not shown), suggesting that animals have eaten normally during vitamin A exposition and, more importantly, the effects seen here are not an indirect consequence of changes in rat metabolism due to vitamin A intake, but a direct effect of the treatment on the redox and bioenergetics states on rat hypothalamus.

3-Nitrotyrosine is originated in tyrosine residues of proteins that have reacted with peroxynitrite ($ONOO^-$), which is produced by the reaction of $O_2^{\bullet -}$ with nitric oxide ($NO^{\bullet}$) [17]. However, even detecting increased oxidative and nitrosative stress in proteins, we did not observe any change regarding α-synuclein content here (Fig. 4A). Also, D2 receptor content was not modulated in the present work (Fig. 4B). It was previously reported that retinoids increased D2 receptor expression *in vitro* in an experimental model utilizing striatal neurons [38]. However, we did not observe such effect here. It may be attributed to differences between hypothalamic and striatal vitamin A metabolism, as well as to the different experimental models applied to investigate the effects of vitamin A and retinoids on neuronal cells. Interestingly, we also did not find altered RAGE content here (Fig. 4C). RAGE is modulated, at least in part, by increased rates of oxidative/nitrosative stress, as previously postulated [39]. Then, it is very plausible to conclude that this receptor did not take a role in the pro-oxidant effect elicited here by vitamin A, since RAGE is also responsible for the maintenance of pro-oxidative events [39,40].

Vitamin A at high levels is a choice of treatment to patients suffering from maladies such as dermatological disturbances and some types of cancer, mainly leukemia [9,10]. However, toxicity resulting from such treatment is not well understood. Unfortunately, even quantifying retinoids in rat hypothalamus would not render conclusions about the retinoid that is associated with the results seen here, since there are a great number of derivatives that would originate from oral vitamin A administration [41].

Overall, the results showed in this work indicate that vitamin A induces oxidative and nitrosative stress in adult rat hypothalamus by a mechanism that, at least in part, involves increased, but unbalanced, electron flux through METC in hypothalamic SMP. As previously postulated, increased respiratory chain activity is a common feature of cells under stressful conditions—to produce more ATP in an attempt to decrease stress consequences [42]. Importantly, we have not detected any change in caspase-3 activity, suggesting that the intrinsic apoptotic way is not activated in hypothalamic neurons. Also, TNF-α levels were not found altered in the hypothalamus of vitamin A-treated rats. This finding suggests that the increased superoxide anion production observed here is not associated to TNF-α levels, a cytokine that may induce free radical production, as previously observed [17]. Even though it is very

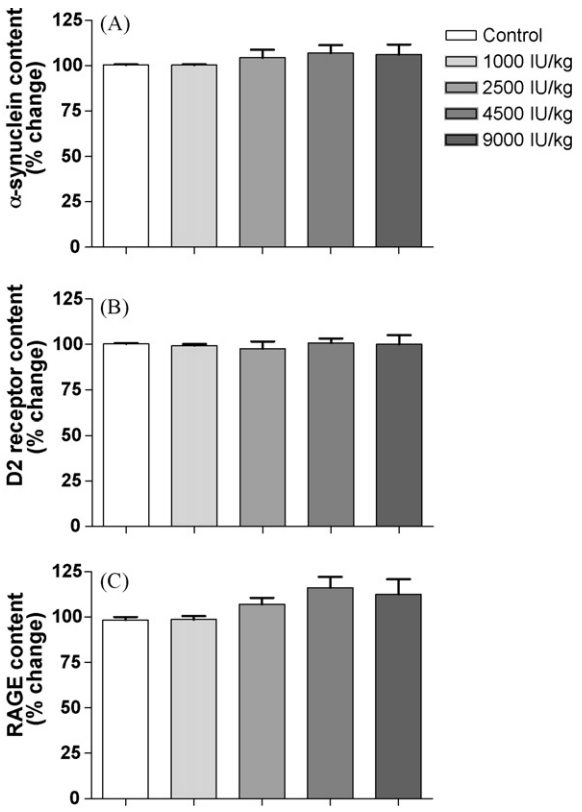

Fig. 4. Effects of vitamin A supplementation on α-synuclein (A), D2 receptor (B), and RAGE (C) contents in rat hypothalamus. Data are mean ± S.E.M. (n = 8–10 per group).

difficult to extrapolate the results presented here to humans, more attention is needed when vitamin A is the choice in treatment of pathologies as cancer, in which retinol palmitate is administrated at high doses during a long-term period even to children, as mentioned above. Additionally, the mechanism of the loss of appetite and weight loss observed in patients under retinol palmitate treatment must be accurately investigated in order to avoid decreased life quality among those subjects.

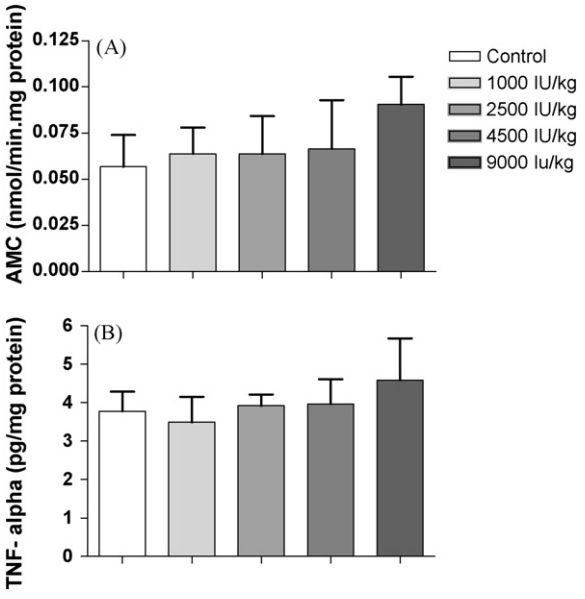

Fig. 5. Effects of vitamin A supplementation on hypothalamic caspase-3 activity (A) and TNF-α levels. (B). Data are mean ± S.E.M. (n = 8–10 per group).

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Capítulo IV

“Increased receptor for advanced glycation endproducts immunocontent in the cerebral cortex of vitamin A-treated rats”

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ORIGINAL PAPER

Increased Receptor for Advanced Glycation Endproducts Immunocontent in the Cerebral Cortex of Vitamin A-Treated Rats

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Abstract Vitamin A, beyond its biological role, is an alternative choice in treating some life threatening pathologies, for instance leukemia and immunodeficiency. On the other hand, vitamin A therapy at moderate to high doses has caused concern among public health researchers due to the toxicological aspect resulting from such habit. It has been described hepatotoxicity, cognitive disturbances and increased mortality rates among subjects ingesting increased levels of vitamin A daily. Then, based on the previously reported data, we investigated here receptor for advanced glycation endproducts (RAGE) immunocontent and oxidative damage levels in cerebral cortex of vitamin A-treated rats at clinical doses (1,000–9,000 IU/kg day^{-1}). RAGE immunocontent, as well as oxidative damage levels, were observed increased in cerebral cortex of vitamin A-treated rats. Whether increased RAGE levels exert negative effects during vitamin A supplementation it remains to be investigated, but it is very likely that deleterious consequences may arise from such alteration.

Keywords Vitamin A · Cerebral cortex · RAGE · Oxidative stress

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Introduction

The role of vitamin A, and its derivatives—the retinoids, as antioxidant molecules have raised interest as potential therapeutic agents in preventing or combating pro-oxidant events in a wide-range of life-threatening conditions [1]. Additionally, vitamin A therapy (mainly as retinol palmitate) at moderate to high doses (30,000–300,000 IU/day) is applied to patients suffering from dermatological disturbances to some types of cancer. For example, it has been administrated vitamin A at doses exceeding 150,000 IU/day to infants, children, and young adults during leukemia treatment [2–4]. Moreover, vitamin A supplementation at 8,500 IU/kg day^{-1} is utilized during weight gain treatment in very-low-weight-birth preterm infants [5]. HIV-exposed children also receive mega doses of vitamin A as an alternative choice to increase immune function [6–8]. However, there are controversy data regarding vitamin A effectiveness in preventing HIV contamination in such subjects [9]. Furthermore, Myhre and colleagues have demonstrated that vitamin A supplementation exert deleterious effects even when applied therapeutically at low doses regarding hepatic and central nervous system (CNS)-related functions [4]. Indeed, vitamin A and retinoids have been demonstrated to induce general toxicity and cognitive impairment in both humans and experimental animals [2–4, 10].

We have demonstrated that vitamin A supplementation at clinical doses induced anxiety-like behavior and decreased both locomotory and exploratory activities in adult Wistar rats under a 28-day treatment [11–15]. Interestingly, vitamin A induced a pro-oxidant effect in some rat brain regions, for instance hippocampus and striatum [12, 13]. Even though it is known that vitamin A and retinoids are redox-active molecules, which has been demonstrated as a pro-oxidant agent in vitro and in vivo

experiments [14–23], the exact relationship between oxidative insult and cognitive impairment associated to vitamin A supplementation is not clear. Vitamin A induces mitochondrial dysfunction, leading to increased pro-oxidant molecules production, as for example superoxide anion radical (O_2^-) [19].

The receptor for advanced glycation endproducts (RAGE) is a multiligand signal transduction receptor, which may be up-regulated in pro-oxidative events, participating in a oxidative cycle that maintain the production of free radicals increased. Oxidatively altered biomolecules may activate RAGE, and this process is thought to play an important role during the onset of neurodegenerative disturbances, for instance in Alzheimer's disease (AD) [24–27]. It was demonstrated that advanced glycation endproducts (AGE), whose levels are increased in diabetes mellitus, by up-regulating RAGE, favor the transport of β-amyloid peptide through the blood–brain barrier, which may lead to symptoms observed in AD patients [28]. Then, oxidative stress and RAGE are closely related, and the consequences of increased RAGE levels include, at least in part, the maintenance of a pro-oxidant cycle and, consequently, neurodegeneration in this case.

On the basis that vitamin A supplementation at clinical levels impaired the redox environment in several experimental models, and that there are a few reports showing the consequences of vitamin A therapy at moderate to high levels regarding the central nervous system (CNS) homeostasis, we performed here an investigation aiming to observe the effect of vitamin A supplementation at 1,000–9,000 IU/kg day^{-1} for 28 days on oxidative stress parameters and RAGE immunocontent in adult rat cerebral cortex. The doses investigated here belong to a therapeutic range usually applied in the treatment of pathologies from dermatological to oncology fields, as mentioned above.

Experimental Procedure

Animals

Adult male Wistar rats (280–300 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (7:00–19:00 h), at a temperature-controlled colony room (23 ± 1°C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80–23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

Drugs and Reagents

Arovit® (retinol palmitate, a water-soluble form of vitamin A) was purchased from Roche, Sao Paulo, SP, Brazil. Caspase-8 activity assay kit was purchased from Biotium, Inc., Hayward, CA, USA. TNF-α assay kit was obtained from BD Biosciences, San Diego, CA, USA. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A treatment was prepared daily and it occurred by protecting from light.

Treatment

The animals were treated once a day for 28 days with a gavage. The treatments were carried out at night (i.e., when the animals are more active and take a greater amount of food) in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. The animals were treated with vehicle (0.15 M saline; $n = 10$ animals), 1,000 ($n = 10$), 2,500 ($n = 10$), 4,500 ($n = 10$), or 9,000 IU/kg ($n = 10$) of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0.6 ml. Adequate measures were taken to minimize pain or discomfort.

Measurement of Protein Carbonyls

Before sacrifice, the animals were anesthetized with ketamine plus xylazine (100 and 14 mg/kg, respectively). The animals were sacrificed by decapitation at 24 h after the last vitamin A administration. The cerebral cortex was dissected out in ice immediately after the rat was sacrificed and stored at −80°C for posterior oxidative stress analyzes. Samples were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4) using a Potter-Elvehjem-type glass homogenizer. The homogenates were centrifuged (700*g*, 5 min) to remove cellular debris. Supernatants were used to all biochemical assays described herein. All the results were normalized by the protein content using bovine albumin as standard [29]. The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH) as previously described [30]. Briefly, tissue homogenates were divided into two aliquots of 200 µl. Proteins were precipitated by the addition of 100 µl 20% TCA for 5 min on ice, and centrifuged at 4000×*g* for 5 min. The pellet was redissolved in 100 µl 0.2 M NaOH, and 100 µl of 2 M HCl or 10 mM 2,4-DNPH in 2 M HCl added to duplicate aliquots for blanks or the derivatizing of carbonyl groups, respectively. Samples were left for 30 min at room

temperature. Proteins were reprecipitated with 20% TCA, and washed three times with 500 µl 1:1 ethanol:ethyl acetate with 15 min standing periods to remove excess DNPH. Samples were redissolved in 1 ml 8 M urea, pH 2.3, and the absorbance was read at 370 nm. The carbonyl content in nmol/mg protein was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} at 370 nm after subtraction of the blank absorbance according to the following equation: {[(Final Absorbance/22,000) × V]/1,000}/Q, where "V" is the volume (in ml) of the sample utilized in the assay, and "Q" is the amount of protein (in mg) in the volume utilized to perform the carbonyl assay. The results are expressed as nmol carbonyl/mg protein and the experiments were performed in triplicate.

Indirect Enzyme-Linked Immunosorbent Assay to 3-Nitrotyrosine

To realize indirect enzyme-linked immunosorbent assay (ELISA), rat cerebral cortex was rapidly homogenized ($T < 1$ min) in lysis buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 2 mM sodium orthovanadate, and Complete™ protease inhibitor cocktail (Roche). Indirect ELISA assay was performed to analyze changes in the content of nitrotyrosine by utilizing a polyclonal antibody to nitrotyrosine (Calbiochem) diluted 1:2000 in phosphate-buffered saline (PBS) pH 7.4 with 5% albumin. Briefly, microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in PBS with 5% albumin. Plates were then washed four times with wash buffer (PBS with 0.05% Tween-20), and the specific antibodies were added to the plates for 2 h at room temperature. After washing (four times), a second incubation with anti-rabbit antibody peroxidase conjugated (diluted 1:1000) for 1 h at room temperature was carried out. After addition of substrates (hydrogen peroxide and 3,3′,5,5′-tetramethylbenzidine, 1:1–v:v), the samples were read at 450 nm in a plate spectrophotometer. Results are expressed as changes in percentage among the groups and the experiments were performed in triplicate.

Superoxide Anion Radical (O_2^-) Production

Briefly, to obtain submitochondrial particles (SMP), cerebral cortex was dissected and homogenized in 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl and 1 mM EDTA (pH 7.4). The samples were centrifuged for 10 min at 600×*g* (4°C). The supernatants were then centrifuged (8000×*g* for 10 min at 4°C) two times to isolate mitochondria. Then, freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free SMP. The SMP solution was also washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) to ensure Mn-SOD release from mitochondria (centrifugation to wash at 5400×*g* for 10 min at 4°C). To quantify superoxide (O_2^-) production, SMP was incubated in reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 10 mM HEPES–KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 µM catalase, and 1 mM epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32°C, as previously described [11, 31]. The experiments were performed in triplicate.

TNF-α Quantification Through Sandwich ELISA

We have measured TNF-α through commercial kit for enzyme-linked immunosorbent assay (ELISA) according manufacturer's instructions (BD Biosciences). Briefly, tissue samples were collected and suspended in lysis buffer as described above. Following tissue homogenization, the samples were centrifuged, and a portion of the supernatant was reserved for protein concentration measurement, and the remaining was stored at −80°C for posterior TNF-α levels quantification. Capture antibody to TNF-α (1:250) was incubated overnight at 4°C in microtiter plates. After washing (three times with PBS pH 7.4 with 0.05% Tween-20) and blocking the plate for 1 h with 5% BSA, diluted samples (1:2) were added to the plates and were left to incubate for 2 h at room temperature. Then, detection antibody (1:250) was added to the plates and an incubation of 2 h was performed at room temperature. Enzyme reagent (streptavidin-horseradish peroxidase conjugate, 1:250) was added to the plate and was left to react for 30 min at room temperature before reading in a microplate spectrophotometer at 450 nm. A standard curve with purified TNF-α was utilized to obtain a calibration factor. The experiments were performed in triplicate.

Caspase-8 Activity

Caspase-8 activity was determined through a colorimetric commercial kit according manufacturer's instructions (Biotium). The samples were prepared in lysis buffer as mentioned above. Caspase-8 activity was monitored in a microplate spectrophotometer at 495 nm for 180 min at 25°C. Results are expressed as nmol R110 produced/min mg^{-1} protein and were performed in triplicate.

Immunoblot Analysis

Western blot assays were performed to determine RAGE immunocontent in rat cerebral cortex. Then, rat cerebral cortex were rapidly homogenized ($T < 1$ min) in lysis buffer containing 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol,

2 mM sodium orthovanadate, and Complete™ protease inhibitor cocktail (Roche). Laemmli-sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% DTT, 2 mM EDTA) was added to samples (30 μg), and proteins were fractioned by SDS–PAGE and electroblotted onto nitrocellulose membranes. Protein loading and electroblotting efficiency were verified by Ponceau S staining, and membranes were washed and blocked using Tris-buffered saline with Tween (100 mM Tris–HCl, 0.9% NaCl, 0.1% Tween-20, pH 7.5) containing 5% albumin. Membranes were incubated overnight at 4°C with the primary polyclonal antibody to RAGE (1:500). Then, membranes were washed with Tris-buffered saline with Tween and incubated with horseradish peroxidase-linked anti-IgG secondary antibody (1:5000), washed again and the immunoreactivity was detected by enhanced chemiluminescence using ECL Plus kit. Densitometric analysis of the films were performed with ImageQuant software. As an internal control, we utilized β-actin antibody (1:2000).

Statistical Analyzes

Results are expressed as means ± standard error of the mean (SEM); P values were considered significant when $P < 0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test whenever necessary.

Results

Oxidative Stress Parameters

As depicted in Fig. 1a, vitamin A supplementation at any dose tested for 28 days increased the cortical levels of protein carbonylation in rats. Vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg day^{-1} increased 3-nitrotyrosine content in rat cerebral cortex (Fig. 1b). Superoxide anion radical production was observed increased in the cerebral cortex of the rats that received vitamin A supplementation at any dose tested (Fig. 1c).

TNF-α Levels and Caspase-8 Enzyme Activity

Vitamin A supplementation did alter neither TNF-α levels nor caspase-8 enzyme activity, respectively, in this experimental model (data not shown).

RAGE Immunocontent

According to Fig. 2, we observe that vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg day^{-1} increased cortical RAGE immunocontent in rats ($P < 0.05$).

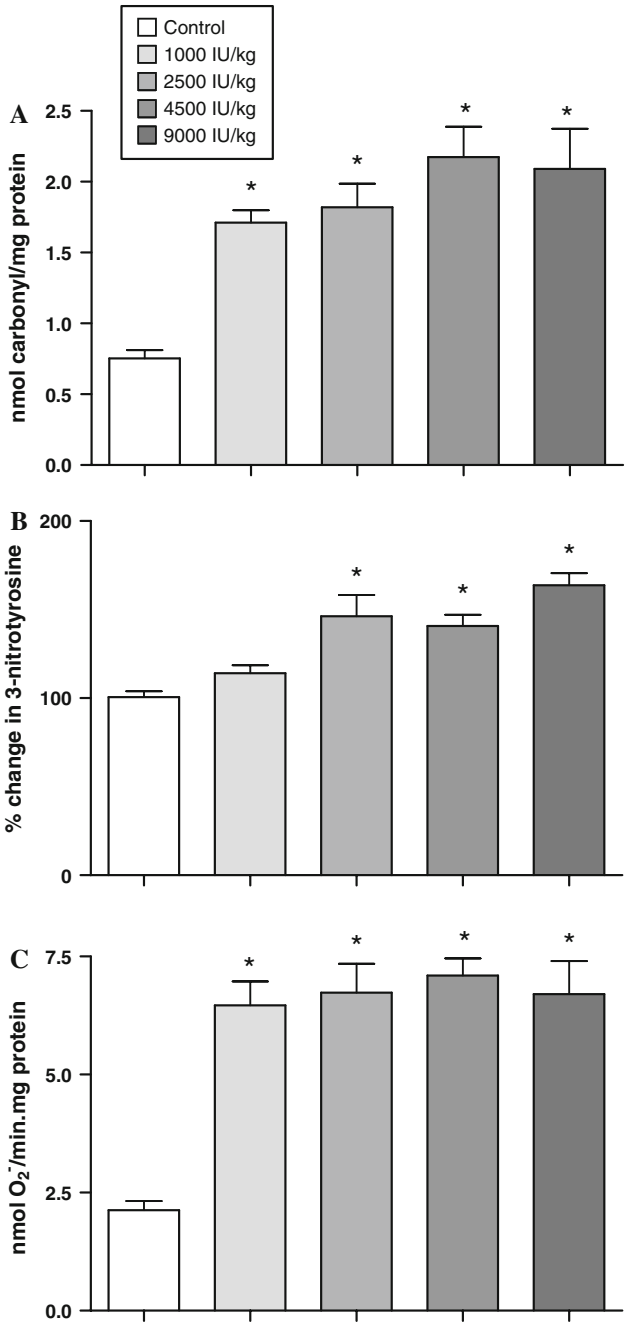

Fig. 1 Effects of vitamin A supplementation on protein carbonylation levels (**a**), 3-nitrotyrosine content (**b**), and superoxide anion radical production (**c**) in the rat cerebral cortex. Data are mean ± SEM ($n = 10$ rats per group) and the experiments were performed in triplicate. *$P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test)

Discussion

Vitamin A therapy, either acute or chronic, has attracted attention due to the negative consequences seen in patients suffering from diseases in the fields of dermatology to oncology. It has been described hepatotoxicity, irritability,

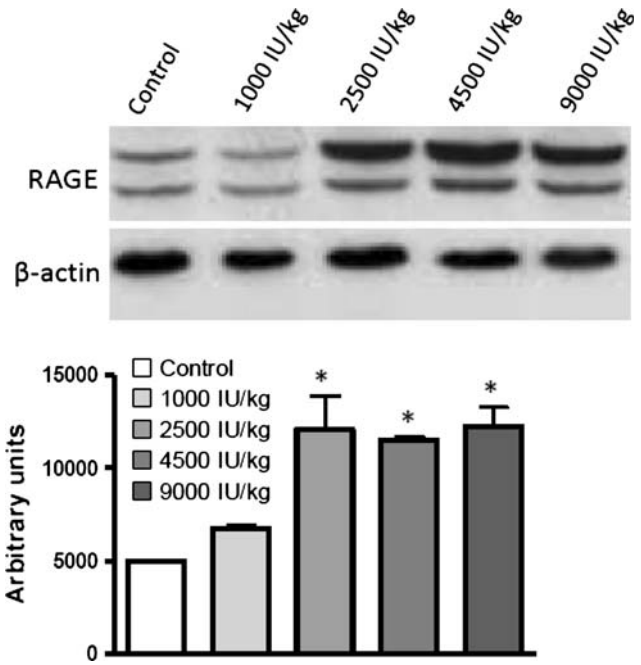

Fig. 2 Effects of vitamin A supplementation on receptor for advanced glycation endproducts (RAGE) immunocontent in rat cerebral cortex. Data are mean ± SEM ($n = 10$ rats per group). A representative gel out of three experiments is shown. *$P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test)

anxiety, and more concerning, depression among subjects receiving vitamin A at doses ranging from 30,000 to 300,000 IU/day [4, 10]. In spite of this, the mechanism by which vitamin A or retinoids induce such cognitive disturbances is not clear. Then, we performed here an experimental model to investigate the effects of vitamin A supplementation at clinical doses (1,000–9,000 IU/kg day^{-1}) on rat cerebral cortex RAGE immunocontent and oxidative stress parameters, as well as TNF-α levels and caspase-8 enzyme activity. We observed a pro-oxidant effect on proteins as consequence of a 28-day vitamin A supplementation, as assessed through protein carbonyl levels and 3-nitrotyrosine content (Fig. 1a, b, respectively). Additionally, we observed increased O_2^- production in cortical SMP (Fig. 1c). Neither TNF-α levels nor caspase-8 enzyme activity did change in this experimental model (data not shown). Interestingly, the immnucontent of RAGE was observed increased in the cerebral cortex of vitamin A-treated rats (Fig. 2). Also, rat food intake and weight gain did not change among the groups (data not shown), suggesting that the effects seen here are not a consequence of changes in rat metabolism, but a direct effect of vitamin A supplementation.

RAGE is a multiligand receptor, which has a wide-range of biological effects, from normal development of mammalian tissues to pro-inflammatory events [25, 26]. In spite of this, RAGE is thought to sustain pro-oxidative effects when up-regulated [32]. Furthermore, it was described increased RAGE levels in subjects suffering from diabetes mellitus, favoring the appearance of symptoms similar to those observed in AD patients, for instance decreased capabilities to learning and memory, as reviewed by Sato et al. [27], thus showing a possible role to RAGE in neurodegenerative diseases. Indeed, it was shown that RAGE is able to transport β-amyloid peptides across the blood–brain barrier, facilitating its accumulation in the extracellular medium [28]. However, we did not find any change in β-amyloid$_{1–40}$ peptide levels in rat cerebral cortex (unpublished data).

Here, we did not observe any change regarding TNF-α levels and caspase-8 enzyme activity (data not shown), suggesting that inflammation is not occurring in the cerebral cortex of vitamin A-treated rats. Moreover, increased O_2^- production (Fig. 1c) is not a consequence of alterations in TNF-α levels, as previously reported [33].

The herein presented study demonstrates, for the first time, that vitamin A supplementation increased RAGE immunocontent and induced oxidative insult in rat cerebral cortex. In addition to the previously reported works from our group and others, we recommend caution when vitamin A at moderate to high doses is the choice in treating human pathologies, since the metabolism of vitamin A did not change significantly among mammalians, mainly rats and humans, as widely demonstrated [3, 5, 34]. Then, it is very likely that some of the molecular alterations seen here may be triggered by long-term vitamin A intake at doses from 30,000 to 300,000 IU/day, as it occurs during leukemia treatment and weight gain therapy utilizing retinol palmitate [4, 10, 35, 36]. Oral vitamin A, as herein applied to the rats, gives rise to a myriad of retinoids, with different reactivity and stability [37]. Hence, it is very speculative to suggest a retinoid responsible for the effects demonstrated here. Additionally, it was reported that plasma retinol levels (and others) did not change even during hypervitaminosis A, indicating that plasmatic or tissue retinol quantification is not a good measurement to indicate vitamin A toxicity [38, 39].

Overall, this work collaborates with the literature in showing the molecular effects of vitamin A supplementation at clinical doses on rat cerebral cortex, a brain region responsible for the maintenance of several cognitive functions implicated in neurodegenerative disturbances. Additionally, when deciding to use vitamin A in an antioxidant therapy, it may be taken into account the possible pro-oxidative effects elicited by such treatment, mainly in SNC tissues due to its low capacity to lead with reactive species.

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Capítulo V

“Pharmacological doses of vitamin A increase caspase-3 activity selectively in cerebral cortex”

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ORIGINAL ARTICLE

Pharmacological doses of vitamin A increase caspase-3 activity selectively in cerebral cortex

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ABSTRACT

Vitamin A exerts a wide range of physiological roles from embryonic to adulthood stages of the mammalian life. However, there is a great concern regarding the deleterious effects of vitamin A use even therapeutically. It was shown that vitamin A induces behavioral impairments, for instance, anxiety-like behavior and depression, in experimental animals and humans. Caspases are enzymes associated with cell death; however, there is a role for such enzymes also in synaptic plasticity. Then, based on previously published data, we have investigated the effects of vitamin A supplementation at clinical doses (1000–9000 IU/kg/day) for 28 days on caspase-3 and caspase-8 activities in adult rat cerebral cortex, cerebellum, striatum, and hippocampus. Furthermore, we have quantified TNF-α levels, a pro-inflammatory cytokine that, besides other biological roles, trigger the extrinsic apoptotic pathway in several cellular types, in those rat brain regions. Interestingly, we found increased caspase-3 activity only in rat cerebral cortex. In all the other regions caspase-3 and caspase-8 activities did not change, as well as the levels of TNF-α. The presented results, herein, indicate that more caution is needed regarding vitamin A clinical use and, also importantly, the consumption of vitamin A-fortified foods, which are not exclusively distributed among vitamin A-deficient subjects.

INTRODUCTION

Vitamin A exerts an important role in modulating cell differentiation and apoptosis in a wide range of mammalian tissues, including the central nervous system (CNS) [1,2]. In addition, there is an important therapeutic application for vitamin A at high doses on the treatment of patients suffering from cancer or dermatological disturbances [3–7]. In spite of this, it was experimentally demonstrated that some retinoids suppressed neurogenesis in hippocampal neurons, consequently decreasing the capacity to learn and inducing depression in adult mice when administrated at doses closely related to those prescribed to patients suffering from acne [8,9]. Recently, we have shown that vitamin A at clinical doses induced anxiety-like behavior in adult rats chronically subjected to retinol palmitate (vitamin A) supplementation [10,11].

It has long been postulated that vitamin A and retinoids have anti-oxidant properties in several experimental models (see below). However, it was demonstrated that retinol (vitamin A) impairs the redox environment in in vitro and in vivo experiments. We found increased levels of oxidative stress markers in cultured Sertoli cells, which were attributed to increased free radical production in mitochondria [12,13]. Recently, we have found that retinol induces mitochondrial swelling in vitro, with concomitant cytochrome *c* release and increased superoxide anion radical ($O_2^{-\bullet}$) production [14]. Furthermore, retinol induces oxidant-associated cell death (apoptosis) in cultured Sertoli cells by a mechanism involving mitochondrial impairment

[13]. Indeed, we have also demonstrated recently that vitamin A supplementation increased the levels of oxidative damage markers in mitochondrial membranes isolated from adult rat cerebral cortex and cerebellum, showing that vitamin A not only in vitro is able to induce mitochondrial dysfunction [10].

Apoptosis is a physiologically necessary event to maintain tissue homeostasis, for instance, number of viable cells and, consequently, morphology. Apoptosis is triggered through at least two different, but not exclusive, cascades of signaling pathways. The first is dependent on cytochrome *c* release from mitochondria and is called intrinsic apoptotic pathway. Inversely, the extrinsic pathway is dependent on external signals to the cells, for instance, tumor necrosis factor-α (TNF-α) that will induce several cytosolic and nuclear events culminating in organized cell death through apoptotic bodies formation. In both intrinsic and extrinsic apoptotic pathways, the cysteine–aspartate proteases (caspases) are needed as either initiators (e.g., caspase-9) or effectors (e.g., caspase-3, caspase-8) to the process to develop successfully. Oxidative stress plays an important role in triggering apoptosis, and an example of this is the oxidatively regulated release of cytochrome *c* from cardiolipin and, consequently, from mitochondria. In the cytosol, cytochrome *c* interacts with other apoptotic proteins originating the apoptosome, the initiator of the intrinsic apoptotic pathway [15]. In addition to cell death, caspase-3 is associated to a process known as synaptic apoptosis, in which there is loss of dendrites in neurons [16]. In addition, it has been described a role to caspase-3 in long-term potentiation of synaptic transmission, indicating a role to caspases in synaptic plasticity [17,18]. Then, caspase-3 impairment or altered activity may affect neuronal homeostasis and, more importantly, cognitive function.

Increased rates of cell death, either through apoptosis or necrosis, were found in several pathologies, including Alzheimer's disease and Parkinson's disease [19]. Indeed, there is great interest in investigate any factor that is able to induce cellular death because of its importance in the whole tissue functioning. Then, in the herein presented experiments, we have analyzed whether vitamin A supplementation at clinical doses (1000–9000 IU/kg/day) for 28 days alters caspase-3 (effector of the intrinsic apoptotic pathway) and caspase-8 (effector of the extrinsic apoptotic pathway) activities in adult rat cerebral cortex, cerebellum, striatum, and hippocampus. In addition, we performed a measurement of TNF-α levels on the same rat brain regions. We aimed to investigate the in vivo effect of vitamin A supplementation, in both intrinsic and extrinsic apoptotic pathways in an attempt to elucidate the mechanism by which this micronutrient, when used at clinical doses, impairs cognition in adult rats, as mentioned above. The retinol palmitate (vitamin A) doses tested here are commonly applied in cancer treatment in both children and adult humans [5–7]. For example, vitamin A (as retinol palmitate) is administrated at doses exceeding 150 000 IU/day to infants and children suffering from some types of cancer [6]. Additionally, very-low-weight-preterm infants receive vitamin A at a maximum daily dose of 8500 IU/kg during weight gain treatment, which occurs during an undetermined period, as reviewed in [20].

EXPERIMENTAL PROCEDURES

Animals

Adult male Wistar rats (290–320 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light-dark cycle (7:00–19:00 h), at a temperature-controlled colony room (23 ± 1 °C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. Our research protocol was approved by the Ethical Committee for Animal Experimentation of the Federal University of Rio Grande do Sul.

Drugs and reagents

Arovit® (retinol palmitate, a water-soluble form of vitamin A) was purchased from Roche, Sao Paulo, SP, Brazil. Caspase-8 activity assay kit was purchased from Biotium, Inc., Hayward, CA, USA. TNF-α assay kit was obtained from BD Biosciences, San Diego, CA, USA. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A treatment was prepared daily and it occurred by protecting from light.

Treatment

The animals were treated once a day for 28 days. All treatments were carried out at night (i.e. when the animals are more active and take a greater amount of food) to ensure maximum vitamin A absorption, as this

vitamin is better absorbed during or after a meal. The animals were treated with vehicle (0.15 M saline), 1000, 2500, 4500, or 9000 IU/kg of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0.6 mL during each period of interest. Adequate measures were taken to minimize pain or discomfort. The animals were killed by decapitation at 24 h after the last vitamin A administration. The cerebral cortex, cerebellum, striatum, and hippocampus were dissected out in ice immediately after the rat was killed and were homogenized in lysis buffer, as mentioned below.

Caspase-3 activity

Caspase-3 activity was determined through a fluorometric commercial kit according manufacturer's instructions (Sigma). Briefly, the samples were homogenized in lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT, 1% inhibitory protease cocktail), and centrifuged at 10 000 ***g*** for 15 min at 4 °C. The supernatants were used to determine caspase-3 assay in a microplate fluorometer at 360 nm excitation and 460 nm emission for 180 min at 25 °C. Results are expressed as nanomol AMC produced/minute per milligram protein.

Caspase-8 activity

Caspase-8 activity was determined through a colorimetric commercial kit according manufacturer's instructions (Biotium, Inc.). The samples were homogenized as described to investigate caspase-3 activity. However, caspase-8 activity was monitored in a microplate spectrophotometer at 495 nm for 180 min at 25 °C. Results are expressed as nanomol R110 produced/minute per milligram protein.

TNF-α quantification

We have measured TNF-α through commercial kit for ELISA according manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Briefly, tissue samples were collected and suspended in lysis buffer containing protease inhibitors. Following cell lysis, the homogenate was centrifuged, and a portion of the supernatant was reserved for protein concentration measurement, and the remaining was stored at −80 °C for posterior TNF-α levels quantification. The samples were read in a microplate spectrophotometer at 450 nm after following the manufacturer's protocol.

Statistical analyses

The results are expressed as means ± SEM; P values were considered significant when $P < 0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test whenever necessary.

RESULTS

Caspase-3 activity

Vitamin A supplementation at any dose tested induced an increase in caspase-3 activity in rat cerebral cortex ($P < 0.05$; *Figure 1a*), but did not alter caspase-3 activity in other rat brain regions (*Figure 1b–d*).

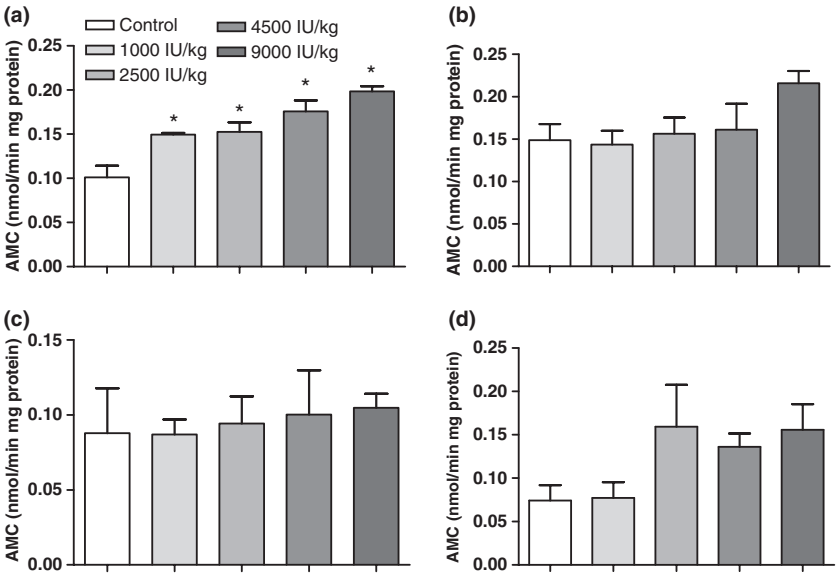

Figure 1 Effect of vitamin A supplementation on caspase-3 activity in rat cerebral cortex (a), cerebellum (b), striatum (c), and hippocampus (d). Data are mean ± SEM (n = 8–10 per group). *$P < 0.05$ vs. control group (one-way ANOVA followed by the post hoc Tukey's test).

Caspase-8 activity

Vitamin A supplementation did not change caspase-8 activity in the rat brain regions herein investigated (*Figure 2a–d*).

TNF-α levels

Similar to the results obtained regarding caspase-8 activity, the levels of TNF-α were not altered in vitamin A-treated rats (*Figure 3a–d*).

DISCUSSION

Retinol palmitate is prescribed at high doses, i.e. 150 000 IU/day retinol palmitate, for example, to patients suffering from pathologies in the field of oncology [6,7]. In spite of this, there is a lack of information in the scientific literature regarding the consequences of the use of this vitamin A compound at high doses even clinically. In this regard, we decided to investigate whether retinol

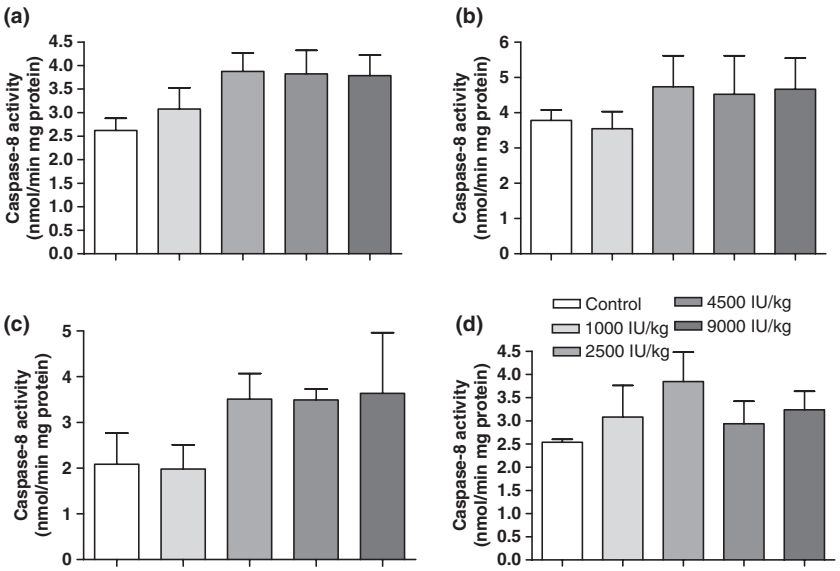

Figure 2 Effect of vitamin A supplementation on caspase-8 activity in rat cerebral cortex (a), cerebellum (b), striatum (c), and hippocampus (d). Data are mean ± SEM (n = 8–10 per group).

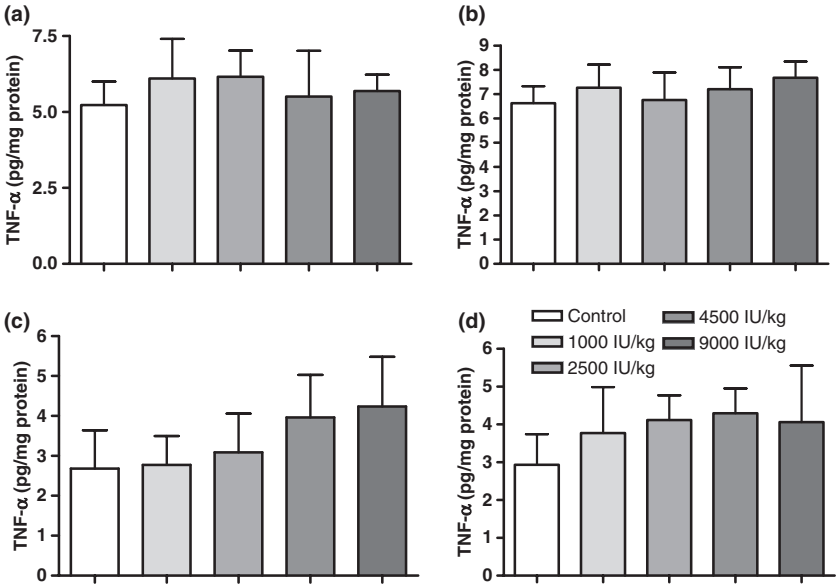

Figure 3 Effect of vitamin A supplementation on TNF-α levels in rat cerebral cortex (a), cerebellum (b), striatum (c), and hippocampus (d). Data are mean ± SEM (n = 8–10 per group).

palmitate (vitamin A) supplementation altered caspases enzyme activities in some rat brain areas involved in the maintenance of cognition. We observed increased caspase-3 enzyme activity in rat cerebral cortex, but not in other brain areas. Importantly, food intake and rat body weight did not change among the experimental groups in this protocol (data not shown), indicating that vitamin A did not induce such central effects as a consequence of impaired rat metabolism.

Vitamin A is a modulatory agent that regulates both cell death and differentiation physiologically at both developmental and adulthood stages of the mammalian life [2,21]. However, clinical application of vitamin A, or its derivatives – the retinoids, is commonly associated with increased rates of cell death, culminating with tissue dysfunction [22]. Recently, we have reported that retinol induces cell death through the apoptotic machinery in cultured Sertoli cells in an oxidative stress-dependent manner [13]. Indeed, other research groups have showed that retinoids are able to induce suppression of neurogenesis and increase rates of neuronal loss in hippocampus, effects that might culminate in behavioral disturbances in mice [8]. Based on these previous findings, we have investigated whether vitamin A induces cell death in some rat brain regions, precisely cerebral cortex, cerebellum, striatum, and hippocampus. Interestingly, we found increased caspase-3 activity only in rat cerebral cortex in vitamin A chronically treated rats.

It was recently reported that an anti-acne retinoid induces a decrease in metabolic rates in the cortex of patients under treatment of severe acne [23]. However, such treatment did not alter patient behavior. On the contrary, we have found anxiety-like behavior in adult rats under vitamin A supplementation at clinical doses commonly applied in the treatment of patients suffering from diseases in the field of oncology and dermatology [11]. Then, we hypothesized that increased rates of cell death could be part of the mechanism sustaining that behavioral impairment. However, in the present study, we have found increased caspase-3 activity, which is very likely to culminate in increased cell death rates, only in rat cerebral cortex, and not other brain regions that have been implicated in the anxiety-like behavior pathologically, as for example hippocampus [24,25]. Interestingly, neither TNF-α nor caspase-8 enzyme activity did alter in this experimental model, suggesting that, at least in part, inflammation is not a consequence of vitamin A supplementation at moderate to high doses. Further studies are necessary to clarify the exact mechanism by which vitamin A supplementation selectively increased caspase-3 enzyme activity in rat cerebral cortex.

Overall, the presented results, herein, indicate at least part of the mechanism by which vitamin A supplementation induces behavioral impairments in adult rats. It remains to be elucidated the exact mechanism of induction of anxiety-like behavior as previously reported. Some attention is needed among public health researchers regarding vitamin A distribution among children and elderly population, as these subjects are more sensitive to the deleterious effects of hypervitaminosis A. In addition, vitamin A-fortified foods, which are not exclusively distributed to vitamin A-deficient people, need to be re-analyzed to avoid increased and unnecessary vitamin A intake [26], which may result from cumulative doses of vitamin A. Further studies are necessary to elucidate whether there is a consequence resulting from increased caspase-3 enzyme activity in synaptic transmission in rat cerebral cortex.

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Capítulo VI

“Impaired redox state and respiratory chain enzyme activities in the cerebellum of vitamin A-treated rats”

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Impaired redox state and respiratory chain enzyme activities in the cerebellum of vitamin A-treated rats

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ABSTRACT

Vitamin A is a micronutrient that participates in the maintenance of the mammalian cells homeostasis. However, excess of vitamin A, which may be achieved through increased intake of the vitamin either therapeutically or inadvertently, induces several deleterious effects in a wide range of mammalian cells, including neuronal cells. Vitamin A is a redox-active molecule, and it was previously demonstrated that it induces oxidative stress in several cell types. Therefore, in the present work, we investigated the effects of vitamin A supplementation at clinical doses (1000–9000 IU/(kg day)) on redox environment and respiratory chain activity in the adult rat cerebellum. Glutathione-*S*-transferase (GST) enzyme activity was also measured here. The animals were treated for 3, 7, or 28 days with vitamin A as retinol palmitate. We found increased levels of molecular markers of oxidative damage in the rat cerebellum in any period analyzed. Additionally, vitamin A supplementation impaired cerebellar mitochondrial electron transfer chain (METC) activity. GST enzyme activity was increased in the cerebellum of rats chronically treated with vitamin A. Based on our results and data previously published, we recommend more caution in prescribing vitamin A at high doses even clinically, since there is a growing concern regarding toxic effects associated to vitamin A intake.

1. Introduction

The importance of vitamin A to the normal brain function is undeniable and has been well studied (see Lane and Bailey, 2005 for a review). However, there is a growing concern about the possible toxic effects induced by vitamin A supplementation at high doses even therapeutically. It has long been demonstrated that vitamin A is teratogenic (Adams, 1993) and induces neuronal malformations (Cohlan, 1953). Moreover, the use of vitamin A derivatives has been associated with the induction of depression in mice (O'Reilly et al., 2006) and humans (Myhre et al., 2003). Recently, a vitamin A derivative was found to suppress the hippocampal cellular proliferation, consequently inducing disturbances in the hippocampal-dependent learning in mice (Crandall et al., 2004). Additionally, excessive intake of vitamin A as a dietary supplement has been demonstrated to induce some parameters of toxicity in humans (Lam et al., 2006).

Vitamin A is a redox-active molecule, and has been demonstrated to induce oxidative stress *in vitro* (see below). Vitamin A treatment induced lipid peroxidation, protein carbonylation, and DNA damage and increased the activity of antioxidant enzymes in cultured Sertoli cells (Dal-Pizzol et al., 2000, 2001). Vitamin A also induced genotoxicity and cellular preneoplasic transformation in some experimental models (Klamt et al., 2003). Moreover, it was found that vitamin A increases the superoxide anion ($O_2^{-\bullet}$) production in liver mitochondria, inducing an increase in mitochondrial lipid peroxidation, and increasing the release of cytochrome *c* from the organelle (Klamt et al., 2005). All-*trans* retinoic acid, a bioactive vitamin A derivative, was also demonstrated to induce oxidative stress in cultured Sertoli cells (Conte da Frota et al., 2006). Recently, we have shown that vitamin A supplementation either acutely or chronically is able to induce a pro-oxidant state in some rat brain structures and also to alter rat behavior (De Oliveira and Moreira, 2007; De Oliveira et al., 2007a,b, 2008).

Therefore, the present study was performed to investigate the effects of sub-acute vitamin A (retinol palmitate) supplementation at clinical (1000–9000 IU/(kg day)) doses on the redox environment and mitochondrial electron transfer chain (METC) activity of the adult rat cerebellum. The retinol palmitate doses used in this work are widely applied in the treatment of patients suffering from diseases in the field of dermatology and oncology. For example, it was described that both infants and adults may be treated with retinol palmitate at doses exceeding 300,000 IU/day

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(Allen and Haskell, 2002; Myhre et al., 2003; Mactier and Weaver, 2005).

2. Experimental procedures

2.1. Animals

Adult male Wistar rat (280–320 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (lights on 7 a.m.), at a temperature-controlled colony room (23 ± 1 °C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior recommendations for animal care.

2.2. Drugs and reagents

Arovit® (retinol palmitate), a water-soluble form of vitamin A, was purchased from Roche, Sao Paulo, SP, Brazil. All other chemicals were purchased from Sigma, St. Louis, MO, USA.

2.3. Treatment

The animals were treated once a day during three different periods: 3, 7, or 28 days. All treatments were carried out during night period (i.e. the period in which the animals are more active, consequently, eating more) in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. The animals were gavaged once a day with vehicle (0.15 M NaCl), 1000, 2500, 4500, or 9000 IU/kg retinol palmitate in a maximum volume of 0.8 mL during each period of interest. Adequate measures were taken to minimize pain or discomfort.

2.4. Oxidative stress analyses

The animals were sacrificed by decapitation at 24 h after the last vitamin A administration. Cerebellum was dissected out immediately (>1 min) after the rats were sacrificed and was homogenized in phosphate-buffered saline (PBS) pH 7.4 and centrifuged at 700 × *g* for 5 min at 4 °C in order to precipitate cellular debris. Then, it was stored at −80 °C and oxidative stress analyses were performed at the day following the rat sacrifice. Protein concentration was determined in rat cerebellum using bovine serum albumin as a standard (Lowry et al., 1951).

2.4.1. Thiobarbituric acid reactive species (TBARS)

As an index of lipid peroxidation, we used the formation of TBARS during an acid-heating reaction, which is widely adopted as a method for the measurement of lipid redox state, as previously described (Draper and Hadley, 1990). Briefly, an aliquot of each sample (0.3 mL) was mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and was centrifuged at 10,000 × *g* for 10 min at 4 °C. Supernatants were collected (0.5 mL) and were mixed with 0.5 mL of 0.67% thiobarbituric acid (TBA), and then heated in a boiling water bath for 25 min. TBARS were determined by the absorbance at 532 nm. We have obtained TBARS concentration in the samples from a calibration curve that was performed using 1,1,3,3-tetramethoxypropane as standard, which was subjected to the same treatment as that applied to the supernatants of the samples. Results are expressed as nmol TBARS/mg protein.

2.4.2. Measurement of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine (DNPH) as previously described (Levine et al., 1990). Briefly, tissue homogenates were divided into two aliquots of 200 μl. Proteins were precipitated by the addition of 100 μl 20% TCA for 5 min on ice, and centrifuged at 4000 × *g* for 5 min. The pellet was redissolved in 100 μl 0.2 M NaOH, and 100 μl of 2 M HCl or 10 mM 2,4-DNPH in 2 M HCl added to duplicate aliquots for blanks or the derivatizing of carbonyl groups, respectively. Samples were left for 30 min at room temperature. Proteins were reprecipitated with 20% TCA, and washed three times with 500 μl 1:1 ethanol:ethyl acetate with 15 min standing periods to remove excess DNPH. Samples were redissolved in 1 mL 8 M urea, pH 2.3, and the absorbance was read at 370 nm. The carbonyl content in nmol/mg protein was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} at 370 nm after subtraction of the blank absorbance according to the following equation: {[(final absorbance/22,000)*V*]/1000}/*Q*, where "*V*" is the volume (in mL) of the sample utilized in the assay, and "*Q*" is the amount of protein (in mg) in the volume utilized to perform the carbonyl assay.

2.4.3. Measurement of non-protein thiol content

Briefly, a supernatant aliquot of 20% TCA precipitated homogenates was diluted in SDS 0.1% and 0.01 M 5,5′-dithionitrobis-2-nitrobenzoic acid (DTNB) in ethanol was added and the intense yellow color was developed and read at 412 nm after 20 min (Ellman, 1959). A blank sample was run simultaneously, except for the absence of DTNB. Non-protein thiol content was calculated after subtraction of the blank absorbance through the following equation utilizing the molar extinction coefficient of 13,600 M^{-1} cm^{-1}: {[(final absorbance/13,600)*V*]/1000}/*Q*, where "*V*" is the volume (in mL) of the sample utilized in the assay, and "*Q*" is the amount of protein (in mg) in the volume utilized to perform the carbonyl assay.

2.4.4. Superoxide anion radical ($O_2^{-\bullet}$) production

Briefly, to obtain submitochondrial particles (SMP), cerebellum was dissected and homogenized in 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl and 1 mM EDTA (pH 7.4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free SMP. The SMP solution was also washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) to ensure Mn–SOD release from mitochondria. To quantify superoxide ($O_2^{-\bullet}$) production, SMP was incubated in reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 10 mM HEPES–KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 μM catalase, and 1 mM epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32 °C and a molar extinction coefficient of 4 mM^{-1} cm^{-1} was applied to calculate the rate of $O_2^{-\bullet}$ production as previously described (Poderoso et al., 1996; De Oliveira and Moreira, 2007).

2.4.5. Antioxidant enzyme activity estimations

Superoxide dismutase activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation, as previously described (Misra and Fridovich, 1972). Catalase (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm (Aebi, 1984). Glutathione peroxidase (GPx) activity was determined by measuring the rate of NAD(P)H oxidation at 340 nm as described (Flohé and Günzler, 1984). A ratio between SOD and CAT activities (SOD/CAT) was applied to better understand the effect of vitamin A supplementation upon these two oxidant-detoxifying enzymes that work in sequence converting superoxide anion to water (Halliwell and Gutteridge, 1999).

2.5. Mitochondrial electron transfer chain (METC) activity

To obtain SMP from cerebellum in order to assess METC activity, we have homogenized the tissue in 250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4, and 50 IU/mL heparin buffer. The samples were centrifuged 1000 × *g* and the supernatants were colleted. Then, the samples were frozen and thawed three times, and METC activity detection was performed as described below.

2.5.1. Complex I activity

Complex I was determined by the rate of NADH-dependent ferricyanide reduction at 420 nm in a spectrophotometer (ε = 1 mM^{-1} cm^{-1}) (Cassina and Radi, 1996).

2.5.2. Complex I–CoQ-III activity

Complex I–CoQ-III activity was determined by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength (ε = 19.1 mM^{-1} cm^{-1}). The reaction buffer contained 20 mM potassium phosphate, pH 8.0, 2.0 mM KCN, 10 μM EDTA, 50 μM cytochrome *c*, and 20–45 μg supernatant protein. The reaction started by the addition of 25 μM NADH and was monitored at 30 °C for 3 min before the addition of 10 μM rotenone, after which the activity was monitored for an additional 3 min. Complex I–III activity was the rotenone-sensitive NADH:cytochrome *c* oxidoreductase activity (Shapira et al., 1990).

2.5.3. Complex II and succinate dehydrogenase (SDH) activities

Complex II (succinate-DCPIP-oxidoreductase) activity was measured by following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol (DCPIP) at 600 nm with 700 nm as reference wavelength (ε = 19.1 mM^{-1} cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8.0 μM DCPIP was preincubated with 48–80 μg supernatant protein at 30 °C for 20 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction was started by the addition of 40 μM DCPIP and was monitored for 5 min at 30 °C. Succinate dehydrogenase (SDH) activity was assessed by adding 1 mM phenazine methasulphate to the reaction mixture. Then, SDH activity was monitored for 5 min at 30 °C at 600 nm with 700 nm as reference wavelength (Fischer et al., 1985).

2.5.4. Complex II–CoQ-III activity

Complex II–CoQ-III activity was measured by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength (ε = 21 mM^{-1} cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate was preincubated with 50–100 μg supernatant protein at 30 °C for 30 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction started by the addition of 0.6 μg/mL cytochrome *c* and monitored for 5 min at 30 °C (Fisher et al., 1985).

2.5.5. Complex IV activity

Complex IV activity was measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength (ε = 19.15 mM^{-1} cm^{-1}). The reaction buffer contained 10 mM

potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg supernatant protein and the reaction was started with the addition of 0.7 μg reduced cytochrome *c*. The activity of complex IV was measured at 25 °C for 10 min (Rustin et al., 1994).

2.6. Glutathione-S-transferase (GST) activity

Glutathione-*S*-transferase (GST) activity was determined spectrophotometrically according to the method of Habig et al. (1974). GST activity was quantified in cerebellum homogenates in a reaction mixture containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 1 mM glutathione as substrates in 0.1 M sodium phosphate buffer, pH 6.5, at 37 °C. Enzyme activity was calculated by the change in the absorbance value from the slope of the initial linear portion of the absorbance time curve at 340 nm for 5 min. Enzyme activity was expressed as nmol of CDNB conjugated with glutathione/min mg^{-1} protein.

2.7. Statistical analyses

Biochemical data is expressed as means ± S.E.M. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 12.0) software. *P*-values were considered significant when $p < 0.05$. Differences in experimental groups were determined by the one-way ANOVA followed by the post hoc Tukey's test.

3. Results

3.1. Oxidative stress markers

Lipid peroxidation was not found increased in the cerebellum of the rats that received vitamin A for 3 days. However, vitamin A supplementation at 2500, 4500, or 9000 IU/(kg day) induced a significant increase in lipid peroxidation in the cerebellum of the rats treated with vitamin A for 7 days ($p < 0.05$). Vitamin A supplementation at any dose tested induced a 1.5–2.0-fold increase of lipid peroxidation in the cerebellum of the rats that received vitamin A for 28 days ($p < 0.05$) (Fig. 1A). We did not observe any alteration in the levels of protein carbonylation in the cerebellum of the rats treated with vitamin A for 3 days. However, protein carbonylation increased in the cerebellum of the rats that received vitamin A at 2500, 4500, or 9000 IU/(kg day) for 7 or 28 days ($p < 0.05$) (Fig. 1B). Non-protein thiol content in the cerebellum of rats treated with vitamin A did not change acutely. However, we observed a decrease in this parameter in the cerebellum of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/(kg day) for 28 days ($p < 0.05$) (Fig. 1C). Vitamin A supplementation induced a 1.7–2.1-fold increase of superoxide anion radical ($O_2^{-\bullet}$) production in cerebellar SMP in any period studied ($p < 0.05$) (Fig. 1D).

3.2. Antioxidant enzymes activities

Superoxide dismutase (SOD) activity increased about 1.5–1.7-fold in the cerebellum of the rats that received vitamin A supplementation at any dose tested for 3 days ($p < 0.05$). However, SOD activity did not change in the cerebellum of the rats that received vitamin A for 7 days. Chronically, SOD activity was found increased in the cerebellum of the rats that received vitamin A at any dose tested ($p < 0.05$) (Fig. 2A). CAT activity decreased in the cerebellum of the rats treated with vitamin A at 2500, 4500, or 9000 IU/(kg day) for 3 days ($p < 0.05$). Vitamin A supplementation at any dose tested induced a 1.5–2.0-fold decrease of CAT activity in the cerebellum of the rats treated for either 7 or 28 days ($p < 0.05$) (Fig. 2B). GPx activity did not change in the cerebellum of the rats in this experimental model (Fig. 2C). We observed a 2.5–3.2-fold increase of SOD/CAT ratio in the cerebellum of the rats treated with vitamin A at 2500, 4500, or 9000 IU/(kg day) for 3 days ($p < 0.05$). Vitamin A supplementation at any dose tested increased the SOD/CAT ratio in the cerebellum of the rats treated for either 7 or 28 days ($p < 0.05$) (Fig. 2D).

3.3. GST activity

GST activity did not change in the cerebellum of the rats that received vitamin A supplementation for 3 or 7 days. Vitamin A supplementation at 4500 or 9000 IU/(kg day) for 28 days induced an increase (1.4–1.6-fold) in cerebellar GST activity ($p < 0.05$) (Fig. 2E).

3.4. Mitochondrial electron transfer chain (METC) activity

Complex I activity did not change in the cerebellum of the rats that received vitamin A supplementation for 3 days. However, vitamin A supplementation at 4500 or 9000 IU/(kg day) induced an increase (1.3–1.6-fold) in cerebellar complex I activity ($p < 0.05$) (Fig. 3A). Chronically, vitamin A supplementation at 2500, 4500, or 9000 IU/(kg day) increased cerebellar complex I activity (1.4–2.1-

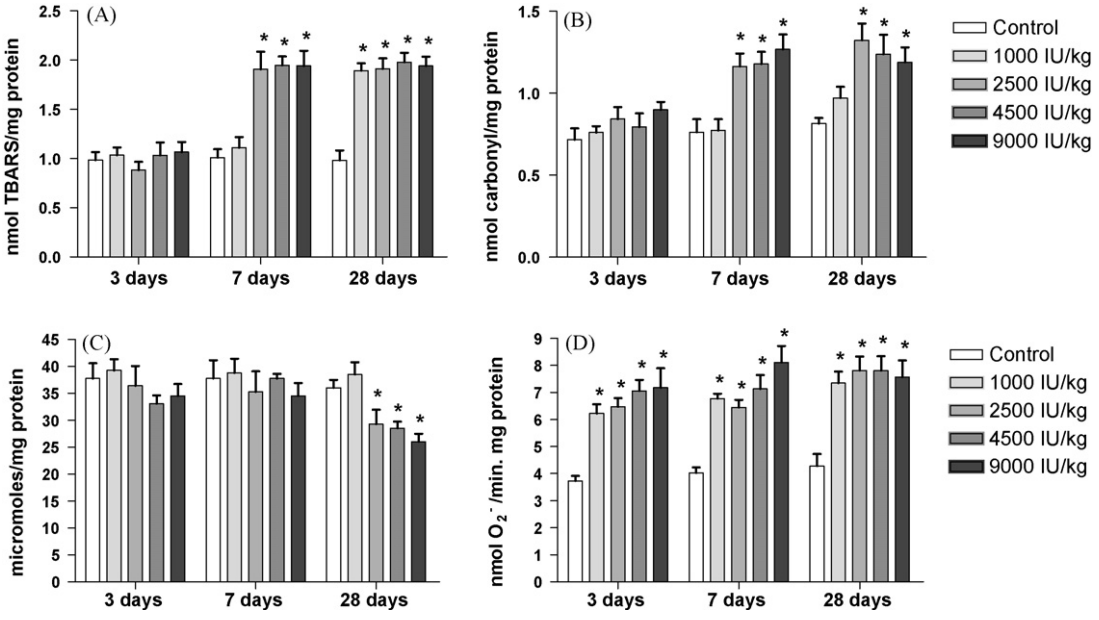

Fig. 1. Effects of acute and chronic vitamin A supplementation on lipid peroxidation (A), protein carbonylation (B), non-protein thiol content (C) and superoxide anion radical production (D) in the rat cerebellum. Data are mean ± S.E.M. of 9–12 animals per group performed in duplicate. *$p < 0.05$ (one-way ANOVA followed by Tukey's test).

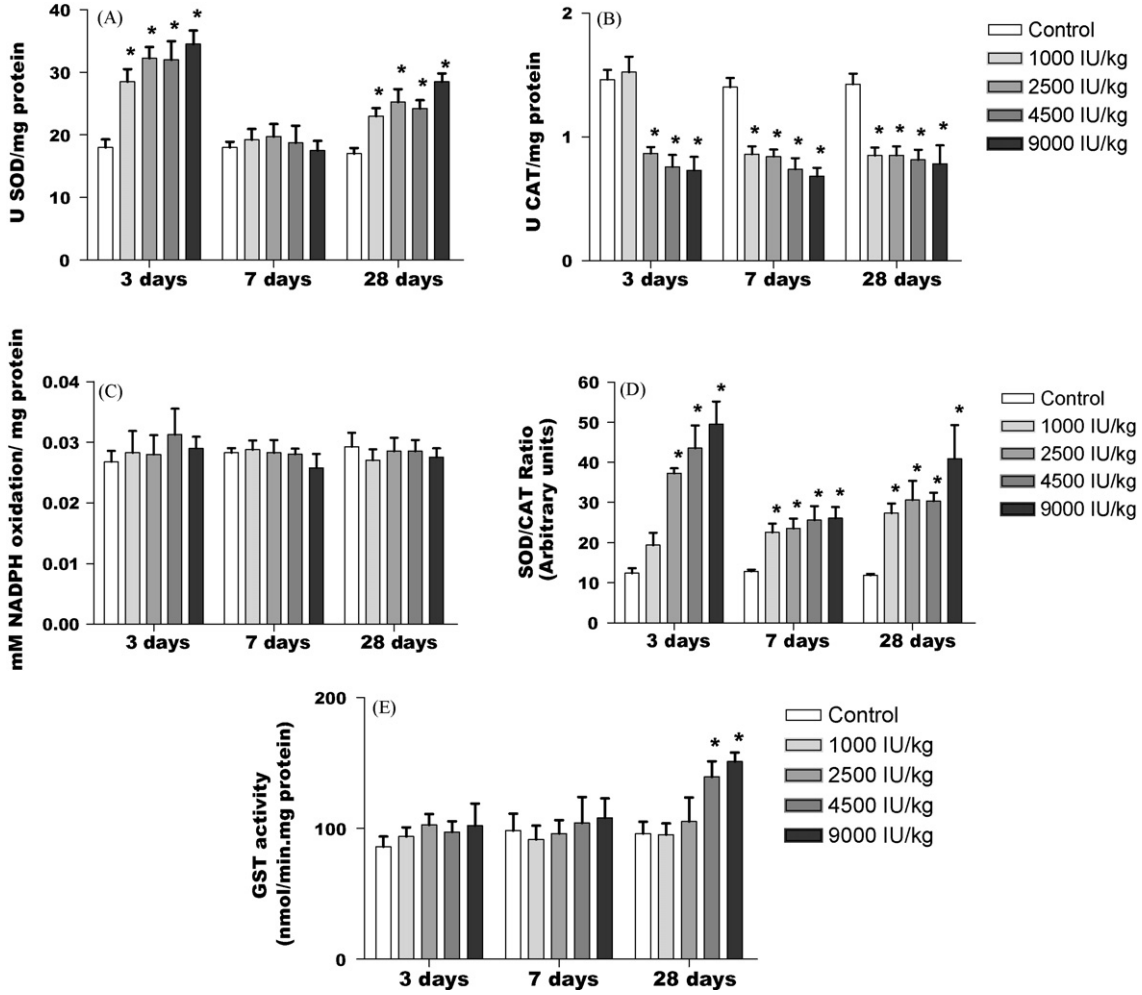

Fig. 2. Effects of acute and chronic vitamin A supplementation on SOD (A), CAT (B), GPx (C) activities in the rat cerebellum. Cerebellar SOD/CAT ratio is shown in (D). The effects of vitamin A supplementation on GST enzyme activity is shown in (E). Data are mean ± S.E.M. of 9–12 animals per group performed in triplicate. *$p < 0.05$ (one-way ANOVA followed by Tukey's test).

fold) ($p < 0.05$) (Fig. 3A). Similarly, complex I–III activity did not change in the cerebellum of the rats that were treated for 3 days with vitamin A (Fig. 3B). Complex I–III activity was found increased (1.2–1.4-fold) in the cerebellum of the rats that received vitamin A at 4500 or 9000 IU/(kg day) for 7 days ($p < 0.05$) (Fig. 3B). Vitamin A supplementation at 2500, 4500, or 9000 IU/(kg day) for 28 days increased cerebellar complex I–III activity ($p < 0.05$) (Fig. 3B). Complex II, SDH, and complex II–III activities did not change in this experimental model (Fig. 3C, D, and E, respectively). Interestingly, complex IV activity did not change in this study (Fig. 3F). However, vitamin A supplementation at 4500 or 9000 IU/(kg day) for 28 days did decrease complex IV activity in the rat cerebellum ($p < 0.05$) (Fig. 3F).

4. Discussion

In this work, we have found that vitamin A supplementation induced a pro-oxidant state in the rat cerebellum. We have observed increased levels of oxidative stress markers in the cerebellum of vitamin A-treated rats (Fig. 1A–C). Furthermore, vitamin A supplementation modulated antioxidant enzyme activity, indicating that either reactive oxygen species or free radicals may be produced in excess during vitamin A supplementation (Fig. 2A and B). Importantly, METC activity was modulated negatively, since an increase was found in the first complexes of the chain, and a decrease was observed in complex IV activity, suggesting uncoupling of the METC activity (Fig. 3).

In addition to increased lipid peroxidation and protein carbonylation levels, we quantified non-protein thiol levels in the cerebellum of vitamin A-treated rats (Fig. 1C). Non-protein thiol levels, which are mainly represented by reduced glutathione (GSH), were decreased in the cerebellum of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/(kg day) for 28 days, suggesting that non-enzymatic antioxidant defenses may be modulated by vitamin A supplementation at high doses *in vivo*, reinforcing the data showing the pro-oxidant effect of such treatment on the rat CNS.

Interestingly, GPx enzyme activity did not change in the cerebellum of the rats under vitamin A supplementation (Fig. 2C). However, SOD and CAT enzymes activities were modulated (Fig. 2A and B). SOD and CAT enzymes are mainly regulated allosterically by $O_2^{-\bullet}$ and H_2O_2. SOD enzyme has its activity increased by $O_2^{-\bullet}$ but decreased by excessive H_2O_2 concentration (Halliwell and Gutteridge, 1999). On the other hand, CAT enzyme is allosterically inhibited by $O_2^{-\bullet}$ (Kono and Fridovich, 1982; Shimizu et al., 1984). Indeed, vitamin A supplementation induced excessive $O_2^{-\bullet}$ production in SMP isolated from adult rat cerebellum (De Oliveira and Moreira, 2007). Then, we suggest that this free radical may be acting on both SOD and CAT enzymes activity, activating the former

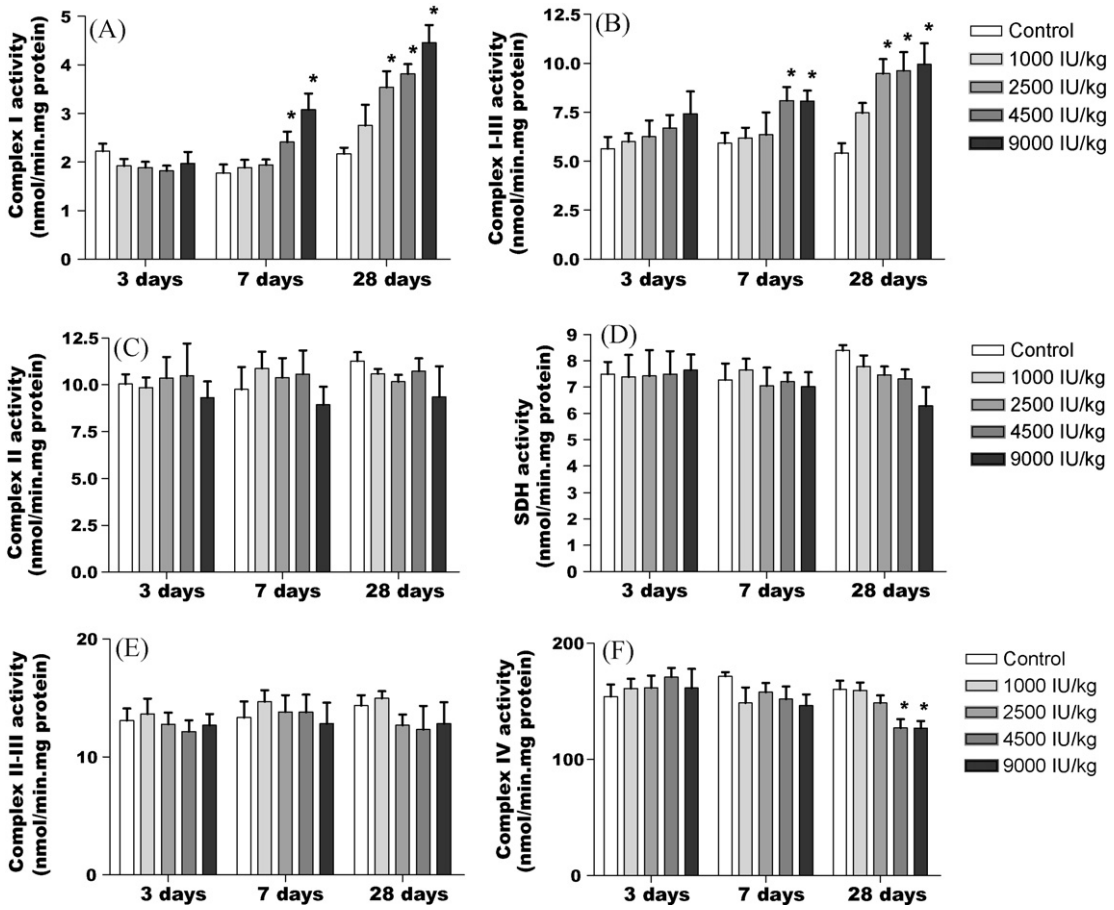

Fig. 3. Effects of acute and chronic vitamin A supplementation on complex I (A), complex I–III (B), complex II (C), SDH (D), complex II–III (E), and Complex IV (F) activities in the rat cerebellum. Data are mean ± S.E.M. of 9–12 animals per group performed in triplicate. $^{*}p < 0.05$ (one-way ANOVA followed by Tukey's test).

and inhibiting the later. Consequently, an imbalance in the SOD/CAT ratio was observed (Fig. 2D) in the cerebellum. Increased SOD/CAT ratio favors the maintenance of high levels of H_2O_2, since SOD converts $O_2^{-\bullet}$ to H_2O_2, but CAT is not able to metabolize H_2O_2 to water, as previously described (Halliwell, 2006).

METC activity was differently modulated by vitamin A supplementation, as shown in Fig. 3. First, we observed an increase in complex I and complex I–III activities (Fig. 3A and B). Second, we did find a decreased complex IV activity in the cerebellum of vitamin A-treated rats (Fig. 3F). Together, these results suggest that vitamin A supplementation induced an impairment in the cerebellar METC, which may give rise to increased $O_2^{-\bullet}$ production due to electron leakage from the METC (Halliwell, 2006). Then, it is plausible to find increased $O_2^{-\bullet}$ production in this experimental model (Fig. 1D).

We have recently reported that vitamin A at the same doses investigated here induced behavioral disturbances in adult rats, as mentioned above. In those analyses, we observed decreased exploration and locomotion in both open field and light–dark box. Those results might indicate, at least in part, some cerebellar dysfunction, or impairment in cerebellum-associated behavior, since the mammalian cerebellum is involved in both planning and execution of movement, as previously described (Gordon, 2007). However, more investigations are necessary to elucidate the role of the cerebellum in the behavioral impairments induced by vitamin A supplementation. In addition, oxidative stress is not the only causative factor in behavioral deficits, and other molecular mechanisms could be responsible for those changes, for instance nuclear signaling promoted by vitamin A or its metabolites. Importantly, recent evidences from our laboratory indicate that cell death is not occurring in the cerebellum of vitamin A-treated rats (unpublished data), indicating that this parameter is not altered in this experimental model.

This work, together with previously published data, indicates a toxic effect of vitamin A supplementation on the adult rat brain. Vitamin A is therapeutically applied at high doses as an alternative treatment in patients suffering from several diseases, for instance leukemia (Tsunati et al., 1990; Norum, 1993; Fenaux et al., 2001). Furthermore, vitamin A is given to very-low-weight preterm infants at very high doses (about 8500 IU/(kg day)) to improve their growing (Mactier and Weaver, 2005). Unfortunately, we are not able to indicate a vitamin A derivative responsible for the effects herein demonstrated, since there are several retinoids that are able to act in different ways within the cells, including genomic and non-genomic actions. Indeed, some excellent works have found that vitamin A and/or its derivatives – the retinoids – may induce cognitive declines in both mice and humans (Myhre et al., 2003; Hull and D'Arcy, 2003; O'Reilly et al., 2006). Then, we suggest more attention on both therapeutic and inadvertently vitamin A use by humans, since oxidative stress is able to disturb several biological phenomena, including neuronal signaling and neurotransmission, which may induce several behavioral deficits.

Conflict of interest

Authors state that they have no conflicts of interest.

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Parte III

4. DISCUSSÃO

Uma das características químicas mais evidentes da vitamina A – e de seus derivados, os retinóides – é sua atividade redox, sendo a vitamina A classificada como uma molécula redox ativa. Assim, dependendo das circunstâncias (*i.e.*, sua concentração no meio, qualidade redox celular, momento metabólico, entre outras), poderá exercer seu poder antioxidante ou, ao contrário, induzir pulsos pró-oxidantes. Esse aparente antagonismo pode ter papel fisiológico, pois se tem evidenciado cada vez mais que sinais pró-oxidantes modulam, fisiologicamente, o destino celular (Halliwell, 2006). Ainda, a vitamina A, tanto direta quanto indiretamente (via retinóides), atua na fisiologia celular via ligação a receptores nucleares (efeito genômico), induzindo a expressão de proteínas alvo, as quais participarão de diferentes processos celulares. Fora este papel de manutenção da homeostasia celular, o uso de vitamina A como agente terapêutico tem sido observado. A aplicação de vitamina A ao longo de tratamentos de patologias variadas, tais como distúrbios dermatológicos, ganho de peso em bebês prematuros e leucemias, mostra que a utilidade de vitamina A não se limita àquilo observado fisiologicamente (Tsunati *et al*., 1990; Tsunati *et al*., 1991; Fenaux *et al*., 2001; Myhre *et al*., 2003; Mactier e Weaver, 2005). No entanto, a toxicologia associada ao uso de vitamina A em doses nem sempre consideradas elevadas tem trazido mais cautela quanto à dosagem administrada aos pacientes nos mais diversos casos onde sua prescrição se torna importante. Em fase aguda de intoxicação, a vitamina A pode induzir enjôo, dor de cabeça, tontura, distúrbios gastrointestinais (vômito, diarreia) e inchaço cerebral. Cronicamente, os efeitos colaterais resultantes da utilização de vitamina A são mais severos, acarretando, inclusive, diminuição na qualidade de vida, com queda no rendimento do paciente, levando a um impacto também

econômico em sua vida (Allen e Haskell, 2002; Myhre *et al*., 2003). Dentre estes efeitos crônicos, a irritabilidade, a ansiedade, a diminuição nas capacidades de aprendizado e de memória, e a depressão são os mais citados.

No que diz respeito ao efeito deletério da vitamina A sobre a cognição, tem sido descrito que pacientes mantidos cronicamente sob tratamento com ácido retinóico 13-*cis* (aplicado contra acne nodular) podem apresentar déficits de aprendizado e de memória e, mais drasticamente, fora observado depressão e aumento nas taxas de suicídio entre tais pacientes (Hull e D'Arcy, 2003). Sugere-se que o ácido retinóico 13-*cis* (que também pode ser originado a partir da vitamina A ingerida como palmitato de retinol) desempenhe este papel negativo sobre o comportamento, mas ainda não há uma compreensão acerca dos mecanismos envolvidos nesta alteração. Ainda, palmitato de retinol, uma forma esterificada de vitamina A facilmente adquirida em farmácias populares, é capaz de induzir efeitos semelhantes em humanos perante exposição crônica a este fármaco, incluindo ansiedade e irritabilidade, além de distúrbios de peso e de sono (Myhre *et al*., 2003). No entanto, há poucos dados na literatura que possam ajudara delinear a maneira pela qual a vitamina A induz efeitos tão fortes sobre a cognição humana.

Em trabalhos anteriores, observamos que a suplementação com vitamina A na forma de palmitato de retinol é capaz de induzir ansiedade e diminuição nas atividades locomotoras e exploratórias de ratos Wistar adultos mantidos aguda e cronicamente neste protocolo. Também encontramos aumento nos níveis de marcadores de estresse oxidativo nas regiões do hipocampo, estriado, *substantia nigra*, córtex cerebral e cerebelo (De Oliveira e Moreira, 2007; DE Oliveira *et al*., 2007a,b; De Oliveira *et al*., 2008). No entanto, ainda não é sabido se alteração induzida por vitamina A no ambiente redox tem alguma participação nas alterações cognitivas resultantes deste modelo. Também não fora

analisado previamente se o tratamento com vitamina A poderia induzir agregação de proteínas e/ou morte neuronal, processos estes que contribuem substancialmente para o desenvolvimento de processos neurodegenerativos.

Assim, foi investigado, no presente trabalho, se haveria algum efeito das suplementações aguda e crônica com palmitato de retinol (Arovit®) sobre o ambiente redox total e mitocondrial de diferentes regiões cerebrais de ratos machos Wistar adultos (90 dias), já que retinóides podem induzir disfunção mitocondrial, a qual participa, de diferentes maneiras, de processos de neurotoxicidade e neurodegeneração. Além disso, o conteúdo celular de α- e β-sinucleínas foi avaliado, bem como os níveis do receptor de dopamina D2 e do receptor para produtos terminais de glicação avançada (RAGE). Quanto ao destino celular, a atividade das enzimas caspase-3 e caspase-8 e os níveis de fator tumoral de necrose-α (TNF-α) foram quantificados, já que pulsos pró-oxidantes podem induzir morte celular por vias dependentes da liberação mitocondrial de citocromo c e da atividade de caspases. Também avaliamos se a suplementação com vitamina A seria capaz de induzir depressão neste modelo experimental, visto que este é um dos efeitos mais alarmantes resultantes do uso crônico e, por vezes, excessivo desta vitamina em humanos.

Nas regiões cerebrais investigadas neste trabalho (*substantia nigra*, estriado, hipocampo, hipotálamo, córtex frontal e córtex cerebral total), fora evidenciado que a suplementação com vitamina A, nas doses tidas atualmente como seguras para aplicação no tratamento de diferentes patologias (1000 – 9000 UI/kg.dia^{-1}), induziu disfunção mitocondrial, tanto no que diz respeito à qualidade redox de biomoléculas mitocondriais quanto na função bioenergética desta organela. Na *substantia nigra*, a suplementação com vitamina A em qualquer dose testada (1000, 2500, 4500 ou 9000 UI/kg.dia^{-1}) pelo período

de 28 dias, induziu aumento na atividade dos complexos I-III, II, II-III e da enzima succinato desidrogenase (SDH), mas nenhuma alteração foi observada na atividade do complexo IV (Cap. I; Fig. 1). Já no estriado dos animais, somente as doses de 4500 e de 9000 UI/kg.dia^{-1} alteraram a função mitocondrial, induzindo aumento na atividade do complexo I-III e diminuição na do complexo IV (Cap. I; Fig. 2). Tanto no caso da *substantia nigra* quanto do estriado, esse aumento em parte da atividade dos complexos (aqui representando o fluxo de elétrons entre um complexo e outro ou a atividade do complexo isolado), mas a ausência de efeito no complexo IV (no caso da *substantia nigra*) ou a diminuição (no caso do estriado) na atividade deste favorece a redução parcial de O_2, levando a um aumento na produção de radical ânion superóxido ($^{\bullet}O_2^-$), já que a taxa de transferência de elétrons para o O_2 (aceptor final de elétrons na cadeia transportadora mitocondrial) no complexo IV não acompanha o recebimento elevado de elétrons vindos dos outros complexos cuja atividade se encontra aumentada pelo tratamento com vitamina A. Realmente, a taxa de produção mitocondrial de $^{\bullet}O_2^-$ foi encontrada aumentada nestas duas regiões cerebrais (Cap. I; Fig. 3A e C). Além disso, um aumento significativo na peroxidação de lipídios componentes de membranas mitocondriais do eixo nigro-estriatal foi induzido pela suplementação diária de vitamina A (Cap. I; Fig. 3B e D). Este aumento na concentração de moléculas intermediárias no processo de peroxidação lipídica pode ter sido causado pelo radical hidroperoxil ($HO_2^{-\bullet}$), o qual é oriundo do $^{\bullet}O_2^-$ em ambientes ácidos (Halliwell, 2006). Como a função mitocondrial conta com oscilações vigorosas na concentração de prótons entre o espaço intermembranas e a matriz mitocondrial, a formação de $HO_2^{-\bullet}$ acaba sendo favorecida, e contribui no ataque aos lipídios de membrana mitocondrial. A capacidade antioxidante não-enzimática também foi quantificada nas

regiões da *substantia nigra* e do estriado neste modelo experimental, e observamos que o tratamento com vitamina A favorece o consumo de moléculas sem atividade enzimática, mas que colaboram na manutenção do estado redox neuronal (Cap. I; Fig. 4).

Em conjunto com a quantificação da taxa de produção de $^{\bullet}O_2^-$, averiguamos a quantidade de 3-nitrotirosina no eixo nigro-estriatal de ratos suplementados com vitamina A. Encontramos aumento na formação de 3-nitrotirosina tanto em amostras nigrais quanto naquelas estriatais dos animais aos quais fora administrada vitamina A nas doses de 4500 e 9000 UI/kg.dia^{-1} (Cap. I; Fig. 6). A quantificação da formação de 3-nitrotirosina é uma maneira de investigar se o tratamento induziu estresse nitrosativo, sendo que a 3-nitrotirosina é um marcador do dano gerado por peroxinitrito ($ONOO^-$), o qual é originado da reação entre $^{\bullet}O_2^-$ e óxido nítrico ($NO^{\bullet}$) (Alvarez e Radi, 2003; Calabrese *et al*., 2007). Indiretamente, este resultado indica que, além de a suplementação com vitamina A estar aumentando a produção de $^{\bullet}O_2^-$, aquela de $NO^{\bullet}$ pode estar sendo aumentada, tendo em vista que a reação entre $^{\bullet}O_2^-$ e $NO^{\bullet}$ é necessária ao surgimento do peroxinitrito, cuja produção pode ser investigada via formação de 3-nitrotirosina. O $NO^{\bullet}$ é capaz de induzir disfunção mitocondrial, com conseqüente diminuição na atividade de complexos da cadeia transportadora de elétrons mitocondrial e, assim, facilitação da produção de $^{\bullet}O_2^-$ (Riobó *et al*., 2001; Calabrese *et al*., 2007). No entanto, mais estudos são necessários para esclarecer o papel do $NO^{\bullet}$ nas alterações disparadas pela suplementação com vitamina A. Assim, a suplementação diária com vitamina A teve como conseqüência, também, estresse nitrosativo no eixo nigro-estriatal de ratos adultos.

A atividade da enzima de fase II de detoxificação glutationa S-transferase (GST) foi encontrada aumentada no eixo nigro-estriatal de animais submetidos à suplementação com

vitamina A nas doses de 4500 e 9000 UI.kg.dia-1 (Cap. I; Fig. 5). Aumento na atividade da GST pode indicar que moléculas apolares potencialmente tóxicas estão sendo conjugadas com glutationa reduzida (GSH) e, posteriormente, exocitadas das células (Sheehan *et al*., 2001). Assim, há aumento no consumo de GSH, a qual é considerada a mais importante agente antioxidante não-enzimática em mamíferos. Desta maneira, a suplementação com vitamina A favorece o estabelecimento de momentos pró-oxidantes que podem se tornar mais duradouros, levando, por exemplo, a uma disfunção bioenergética, conforme visto anteriormente no que diz respeito à função mitocondrial, entre outros parâmetros que podem estar sendo afetados.

Neste trabalho, foram avaliadas as quantidades de α- e β-sinucleínas, duas pequenas proteínas neuronais necessárias à sinalização sináptica (Goedert, 2001; Lotharius e Brundin, 2002). Encontramos que a suplementação com vitamina A aumentou a quantidade de α-sinucleína no eixo nigro-estriatal (Cap. I; Fig. 7). O acúmulo de α-sinucleína tem papel central em distúrbios neurodegenerativos, tais como doença de Parkinson e doença de Lewy, nos quais agregados protéicos de α-sinucleína são encontrados no citosol de neurônios dopaminérgicos dos indivíduos afetados (Kim e Lee, 2008). Agregados de α-sinucleína são capazes de, fora causar perturbação da homeostasia celular por mecanismos físicos, inibir a atividade do proteassomo, o qual é responsável pela degradação de proteínas marcadas devido ao seu tempo de permanência na célula (Lindersson *et al*., 2004). Então, o acúmulo de α-sinucleína, através da inibição do proteassomo, pode favorecer a perpetuação de um ciclo vicioso onde proteínas agregadas levam a um aumento na quantidade de mais proteínas agregadas, as quais podem participar da disfunção redox já em andamento. Já a quantidade de β-sinucleína não apresentou qualquer variação neste

modelo experimental (Cap. I; Fig. 8). Mesmo a ausência de alteração, neste caso, é importante, pois a β-sinucleína é um agente modulador da expressão e da qualidade de α-sinucleína, cuja concentração citosólica não deve alcançar valores críticos que possam levar à disfunção neuronal (Goedert, 2001). Então, há um aparente desequilíbrio em relação ao par α-sinucleína e β-sinucleína, favorecendo um acúmulo da primeira sem um acompanhamento da segunda.

Também analisamos a quantidade do receptor de dopamina do tipo D2 do eixo nigro-estriatal dos animais mantidos neste protocolo experimental. Encontramos diminuição na quantidade de D2 no estriado, mas não na *substantia nigra* (Cap. I; Fig. 9). Uma diminuição na quantidade deste receptor pode levar a um aumento nas taxas de liberação de dopamina a partir da *substantia nigra* em direção ao estriado, já que este receptor age por retroação negativa quanto à liberação daquele neurotransmissor. Assim, quanto menor a quantidade de receptor D2, maior a liberação de dopamina (Bozzi e Borrelli, 2006). No entanto, a dopamina é uma molécula que pode sofrer auto-oxidação, reação com o O_2 pela qual $^{\bullet}O_2^-$ é produzido (Halliwell, 2006). Este aumento na liberação de dopamina pode favorecer, então, aumento na produção citosólica de $^{\bullet}O_2^-$, em adição àquela mitocondrial previamente citada neste texto. Além disso, a dopamina oxidada gera semi-quinonas, as quais são ainda mais reativas que a dopamina e, ao se ligarem a grupamentos tióis de proteínas, induzem perda de conformação e, potencial agregação destas, levando a um acúmulo de proteínas no citosol de um tipo celular que já apresenta um aumento na quantidade de α-sinucleína, um inibidor do proteassomo. A degradação de dopamina é um processo pró-oxidante de grande importância ao ambiente redox neuronal, já que, via a enzima monoamina oxidase, a dopamina é degradada e há produção de peróxido de

hidrogênio (H_2O_2) citosólico, mas muito próximo da mitocôndria, pois a monoamina oxidase está ancorada à membrana mitocondrial externa (Cohen e Kesler, 1999). Assim, origina-se uma fonte de H_2O_2 citosólica, mas que também pode atingir a mitocôndria e seu interior, a matriz mitocondrial, tendo em vista que o H_2O_2 é uma molécula difusível, capaz de cruzar membranas biológicas facilmente, levando um pulso pró-oxidante àquela organela, a qual contém grandes concentrações de íons ferro (Fe^{2+}) e cobre (Cu^{+}), facilitando, então, a ocorrência da reação de Fenton, originando o radical hidroxil ($^{\bullet}OH$), o qual é o mais instável e, portanto, o mais potente de todos os radicais livres conhecidos (Halliwell, 2006). Na verdade, encontramos aumento na atividade da enzima monoamina oxidase no eixo dopaminérgico neste desenho experimental (dados não mostrados), mas serão realizados outros ensaios para averiguação da importância desta enzima no desequilíbrio redox e bioenergético observados neste estudo. Além disso, o excesso de dopamina citosólica pode favorecer a entrada desta na mitocôndria, induzindo aumento na atividade da enzima óxido nítrico sintase mitocondrial, a qual pode passar a produzir mais $NO^{\bullet}$ perante esta situação (Czerniczyniec *et al.*, 2007). Embora o aumento na quantidade de 3-nitrotirosina evidenciado neste trabalho possa contar com este mecanismo (é importante lembrar que o $NO^{\bullet}$ é difusível, podendo sair da mitocôndria de acordo com sua concentração), é necessário um número maior de análises para assegurar as vias pelas quais a vitamina A induz estresse nitrosativo no eixo nigro-estriatal.

Embora a suplementação com vitamina A tenha afetado diretamente a função mitocondrial, nenhuma alteração na atividade da enzima pró-apoptótica caspase-3 foi observado neste protocolo (Cap. I; Fig. 10A e C). Como as mitocôndrias são alvos da toxicidade induzida por vitamina A e, em estudos anteriores de nosso grupo de pesquisa, foi

encontrado aumento na liberação de citocromo c mitocondrial com conseqüente aumento nas taxas de apoptose de células de Sertoli tratadas com retinol, esperávamos encontrar um aumento na atividade desta enzima, a qual é um efetor da morte celular por apoptose em diferentes tipos celulares de mamíferos (Hengartner, 2001). Também não encontramos qualquer variação na concentração de TNF-α neste protocolo (Cap. I; Fig. 10B e D). Estes resultados indicam, pelo menos em parte, que o eixo nigro-estriatal não sofre de perdas neuronais induzidas por vitamina A. Além disso, não há um indício de neuro-inflamação corrente no eixo dopaminérgico.

No córtex frontal, região cerebral envolvida com processos cognitivos como aprendizado e memória e tomada de decisão, entre outras, e afetada em casos de distúrbios neurodegenerativos de diferentes graus de agressividade (ansiedade, doença bipolar, doença de Alzheimer), a suplementação com vitamina A induziu efeitos modestos no ambiente redox. As doses de 4500 e 9000 UI/kg.da^{-1} induziram, após 28 dias de suplementação com a vitamina, um aumento no nível de marcadores de dano oxidativo em lipídios e proteínas (Cap.II; Fig. 1A e B). Já a oxidação de grupamentos tiol foi observada somente nos animais que receberam a maior dose testada (Cap. II; Fig. 1C). A atividade da enzima antioxidante Cu/Zn-SOD cortical foi aumentada nos animais que receberam suplementação com vitamina A na dose de 9000 UI/kg.dia^{-1}, enquanto a atividade de CAT não foi alterada, levando a um desequilíbrio na relação entre as atividades de Cu/Zn-SOD e CAT (SOD/CAT) nesta mesma dose (Cap. II; Fig. 2A, B e C). Este desequilíbrio na SOD/CAT pode indicar uma sobra, pelo menos momentânea, em H_2O_2, agente com características pró-oxidantes e de difusibilidade que favorece a mobilidade do desequilíbrio redox.

Em partículas submitocondriais do córtex frontal dos animais que receberam suplementação com vitamina A, encontramos aumento na produção de $^{\bullet}O_2^-$, na peroxidação lipídica e na carbonilação de proteínas, sendo que nenhuma alteração fora observada na concentração de grupamentos tiol (Cap. II; Fig. 3A-D). O aumento na produção de $^{\bullet}O_2^-$ pode favorecer peroxidação lipídica, conforme citado anteriormente (via $HO_2^{-\bullet}$) e esta, por sua vez, pode induzir carbonilação em proteínas, levando a um ciclo de oxidação a biomoléculas mitocondriais (Berg *et al*., 2004). Por outro lado, encontramos uma sutil alteração na atividade de complexos da cadeia transportadora de elétrons, sendo que a única alteração foi observada na forma de um aumento na atividade do complexo I-III, sem um acompanhamento nos outros complexos da cadeia (Cap. II; Fig. 4A-E). Mais uma vez, como observado no eixo dopaminérgico, esse desequilíbrio entre o fluxo de elétrons entre os complexos I-III e o complexo IV pode levar a uma sobra de elétrons, representada por saturação dos complexos, em porções que podem favorecer a produção de $^{\bullet}O_2^-$ via redução parcial de O_2. Assim, sugerimos que esta disfunção na atividade da cadeia transportadora de elétrons pode levar a um aumento na taxa de produção de $^{\bullet}O_2^-$.

No que diz respeito ao destino celular no córtex frontal, não encontramos qualquer alteração na atividade das enzimas pró-apoptóticas caspase-3 e caspase-8, bem como na concentração de TNF-α, o qual dispararia morte celular via caspase-8 (Cap. II; Fig. 5 e 6). Esse resultado se assemelha àquele obtido nas regiões cerebrais do eixo dopaminérgico, onde nem a atividade de caspase-3 nem a concentração de TNF-α variaram (Cap. I; Fig. 10).

Em trabalhos anteriores, observamos que a suplementação com vitamina A induz comportamento tipo ansiedade em animais mantidos sob este tratamento por 28 dias (De

Oliveira *et al*, 2007a). Além disso, encontramos que, neste mesmo modelo experimental, os animais apresentavam tanto a atividade locomotora quanto aquela exploratória diminuídas (De Oliveira *et al*., 2007a,b). No entanto, pelos testes realizados até o momento, ainda não era possível afirmar se aquele comportamento tipo ansiedade observado contava com comportamento relacionado à depressão simultaneamente. Então, averiguamos se a suplementação com vitamina A seria capaz de induzir comportamento relacionado à depressão via utilização de três testes específicos diferentes. No entanto, não observamos qualquer alteração neste parâmetro neste desenho experimental (Cap. II; Fig. 7). Assim, sugerimos que a vitamina A é capaz de induzir comportamento tipo ansiedade nestes animais, mas não comportamento relacionado à depressão. Para reforçar nossos dados prévios mostrando que a suplementação por 28 dias com vitamina A induzia comportamento tipo ansiedade, realizamos mais um teste que fornece dados que podem indicar a presença de comportamento tipo ansiedade. Em campo aberto, registramos que os animais tratados com vitamina A, em qualquer dose testada, em relação aos animais tratados com salina (controle): 1) demoravam mais tempo para se locomover ao centro do campo aberto pela primeira vez ("latency time"); 2) entravam menos vezes no centro do campo aberto; 3) permaneciam mais tempo sob comportamento do tipo congelamento ("freezing"); 4) se locomoviam menos pelo campo ("crossings"); e 5) excretavam um maior número de bolos fecais (Cap. II; Tabela 1). Em conjunto, estes dados reforçam a teoria de que a suplementação com vitamina A é ansiogênica. No entanto, este efeito ansiogênico não conta com morte neuronal nem com neuro-inflamação.

Em alguns casos, o uso exagerado de vitamina A (que ocorre mais facilmente via ingestão aumentada de palmitato de retinol) pode fazer surgir distúrbios alimentares graves em seres humanos, o que pode favorecer o estabelecimento do processo patológico da

ansiedade (Myhre *et al*., 2003). Então, decidimos analisar os efeitos da suplementação com vitamina A nas mesmas doses citadas anteriormente (1000 – 9000 UI/kg.dia^{-1}) sobre o hipotálamo de ratos Wistar machos, já que esta área cerebral faz parte do controle refinado sobre ingestão alimentar e metabolismo energético em mamíferos (Gao e Horvath, 2007). Embora os efeitos colaterais da vitamina A contem com surgimento de distúrbios alimentares, encontramos apenas alterações sutis no hipocampo dos animais submetidos a este modelo experimental.Quanto ao ambiente redox, apenas as doses de 4500 ou 9000 UI/kg.dia^{-1} foram capazes de induzir peroxidação lipídica e carbonilação de proteínas, sendo que a dose mais alta testada fora a única que induziu aumento na quantidade de 3-nitrotirosina (marcador de estresse nitrosativo induzido por $ONOO^-$). Esta mesma dose induziu aumento na atividade da enzima Cu/Zn-SOD, mas nenhuma alteração na atividade de CAT foi observada, não levando, portanto, a um desequilíbrio na SOD/CAT (Cap. III; Fig. Fig. 1A-F). Assim, aquela sobra de H_2O_2 que pode ter sido mais significativa no córtex frontal não aparece aqui, já que a atividade conjunta destas enzimas antioxidantes parece estar alinhada.

Por outro lado, a suplementação com vitamina A induziu um aumento nas taxas de peroxidação lipídica e de produção de $^{\bullet}O_2^-$ mitocondriais (Cap. III; Fig. 2A e B). Ainda, encontramos que as doses de 4500 e de 9000 UI/kg.dia^{-1} induziram um aumento na atividade do complexo I-III, mas uma diminuição na atividade do complexo IV em mitocôndrias hipotalâmicas, sem qualquer alteração nas outras porções da cadeia transportadora de elétrons (Cap. III; Fig. 3). Mais uma vez, mas de maneira mais forte neste caso, a suplementação com vitamina A pode estar levando a um ambiente pró-oxidante onde a produção mitocondrial de $^{\bullet}O_2^-$ é favorecida e, talvez, mesmo amplificada, por uma

disfunção mitocondrial. Em somatório a este efeito, pode estar havendo uma diminuição na produção de ATP, já que a redução de O_2 a H_2O está mais lenta no complexo IV, o que leva a um retardamento nas taxas oxidação e redução de cada complexo da cadeia. Assim, todo o metabolismo energético de neurônios hipotalâmicos, fora aquele impacto redox, pode estar comprometido, levando a uma facilitação da perda da homeostasia da região como um todo. Em estudos futuros, deveremos investigar a atividade da ATP sintase (complexo V) para melhor entender as conseqüências desta suplementação.

No hipotálamo de ratos mantidos sob suplementação com vitamina A, nenhuma alteração nos níveis de α-sinucleína nem de receptor D2 ou de RAGE fora observada (Cap. III; Fig. 4). Ainda, a atividade de caspase-3 e a concentração de TNF-α não variaram neste modelo experimental (Cap. III; Fig. 5). No entanto, a não alteração na atividade de caspase-3 deve ser vista com mais cuidado, tendo em vista que a ativação de caspase-3 é dependente de ATP, que pode estar tendo sua produção afetada pelo presente tratamento, tendo em vista que a atividade do complexo IV foi reduzida pela suplementação (Cap. III; Fig. 3E). No entanto, mais dados são necessários para confirmar esta hipótese.

Em relação ao córtex cerebral total, a suplementação com vitamina em praticamente todas as doses utilizadas neste trabalho induziu dano oxidativo via aumento na carbonilação de proteínas, na formação de 3-nitrotirosina e na produção de $^{\bullet}O_2^-$ (Cap. IV; Fig. 1), além de aumento na quantidade de RAGE (Cap. IV; Fig. 2). O dano oxidativo por meio de carbonilação a proteínas pode diminuir a função destas através de perda de conformação, mas também é possível, através de um mecanismo mais refinado, favorecer a perpetuação do desequilíbrio redox via aumento na quantidade de RAGE, que pode ser ativado por intermediários da carbonilação de proteínas. Já se sabe que a ativação de RAGE pode se dar

via disparo pró-oxidante e, por sua vez, a ativação deste receptor pode manter o dano oxidativo, em um ciclo vicioso que facilita o deslocamento de um desequilíbrio redox de uma célula para a outra (Bierhaus *et al*., 2005). A ativação de RAGE aparece de maneira importante durante o diabetes mellitus, e tem papel central na manutenção do desbalanço redox envolvida nesta patologia (Ramasamy *et al*., 2005). Mais impressionante é o papel de RAGE no surgimento de doença de Alzheimer em indivíduos sofrendo de diabetes mellitus, conforme vem sendo demonstrado. Neste aspecto, RAGE parece participar do transporte de peptídeos β-amilóides da circulação para o sistema nervoso central via BHE (Takuma *et al*., 2009). Se há um papel para RAGE cortical no desenvolvimento de ansiedade e outros transtornos cognitivos nos animais tratados com suplementação de vitamina A, ainda há de ser investigado, mas indícios apontam para um envolvimento de RAGE com distúrbios neurodegenerativos.

Ainda no córtex cerebral total, investigamos a conseqüência da suplementação com vitamina A sobre o destino celular via análise da atividade das enzimas pró-apoptóticas caspase-3 e caspase-8 e de TNF-α. Em um trabalho comparativo, observamos que apenas no córtex cerebral total a atividade de caspase-3 foi aumentada perante este modelo experimental (Cap. V; Fig. 1A). É interessante o fato de que mesmo a dose mais baixa testada foi capaz de induzir aumento na atividade desta enzima no córtex cerebral. No entanto, nem a atividade de caspase-8 nem a quantidade de TNF-α variou em nenhuma das regiões aqui estudadas (Cap. V; Fig. 2 e 3). A enzima caspase-3 é chamada de caspase efetora, pois participa, com papel central, na efetivação da morte celular por apoptose através da via intrínseca, ou seja, dependente da liberação de citocromo c (carreador de elétrons da cadeia transportadora mitocondrial) a partir da mitocôndria. Já a enzima

caspase-8, medeia o processo apoptótico dependente da sinalização que vem de fora da célula e pode ser iniciada por meio do sinal disparado por TNF-α durante processos de inflamação, por exemplo (via extrínseca) (Hengartner, 2001). Então, essa seletividade na ativação de caspase-3 via suplementação com vitamina A pode ter um papel importante no mecanismo de indução de ansiedade nos animais que receberam esta vitamina em qualquer dose testada neste desenho experimental. O córtex cerebral é tem sua homeostasia afetada durante distúrbios cognitivos, incluindo ansiedade e depressão. Além disso, já foi mostrado que a utilização prolongada (4-6 meses ou mais de tratamento) de ácido retinóico 13-*cis* (fármaco anti-acne) induz diminuição na capacidade bioenergética no córtex cerebral de pacientes mantidos sob este tratamento, mas nenhuma relação com destino celular fora estabelecida (Bremner *et al*., 2005).

Quanto ao cerebelo, encontramos disfunção redox já em um tratamento agudo com vitamina A (3 e 7 dias de duração) (Cap. VI; Fig. 1 e 2). Não o dano oxidativo, mas a produção de $^{\bullet}O_2^-$ já aparece após 3 dias de tratamento em qualquer dose testada. A partir de 7 dias de tratamento, o nível de marcadores de estresse oxidativo já é aparente e significativamente diferente do controle. A atividade das enzimas antioxidantes SOD e CAT, mas não a de GPx, estão alteradas, sendo que há um aumento na atividade enzimática da SOD e diminuição na de CAT (Cap. VI; Fig. 2). Uma provável explicação para esta discrepância quanto à atividade das enzimas antioxidantes pode estar na elevada taxa de produção de $^{\bullet}O_2^-$, o qual ativa alostericamente a SOD, mas pode inibir a CAT (Shimizu *et al*., 1984), levando a um desequilíbrio na SOD/CAT, conforme verificado (Cap. VI; Fig. 2D). Este desequilíbrio na SOD/CAT pode favorecer, conforme mencionado acima, um acúmulo de H_2O_2, ainda mais pela não alteração na atividade da enzima GPx (Cap. VI; Fig.

2C). Além dos efeitos mencionados, verificamos disfunção mitocondrial cerebelar após a suplementação com vitamina A em diferentes períodos de tratamento, contando com aumento na atividade do complexo I e do fluxo de elétrons entre os complexos I-III (Cap. VI; Fig. 3A e B) e diminuição na do complexo IV (Cap. VI; Fig. 3F). Conforme discutido anteriormente, esta disfunção entre o fluxo de elétrons em cada complexo da cadeia transportadora pode levar a um aumento na taxa de produção de $^{\bullet}O_2^-$ mitocondrial (Halliwell, 2006).

Neste trabalho, investigamos as conseqüências da suplementação com vitamina A em doses utilizadas com freqüência no tratamento de diversas patologias indo desde distúrbios dermatológicos até alguns tipos de câncer, passando por terapia de ganho de peso em bebês prematuros em animais experimentais. Embora a utilização desta vitamina como fármaco seja mundial, pouco se sabe acerca das conseqüências moleculares de sua utilização. Devido ao acesso inviável de amostras de SNC de humanos que estejam passando por este tratamento, a investigação dos efeitos disparados pela vitamina A neste tecido fica limitada a modelos experimentais, os quais apresentam uma similaridade proveitosa quanto ao metabolismo da vitamina A quando comparados a humanos (Kerr *et al*., 1982; Nulman *et al*., 1998; Hendrix *et al*., 2004). Algumas similaridades entre este modelo experimental e aquilo observado em humanos cuja ingestão diária de vitamina A seja elevada nos indicam um caminho seguro a seguir nesta investigação. Um exemplo é o de que suplementação com vitamina A na forma de palmitato de retinol por períodos superiores a 20 dias é um indutor mais comum de irritabilidade e ansiedade, mas não de depressão, conforme reportado (Allen e Haskell, 2002; Myhre *et al*., 2003). Isto é exatamente o que foi observado ao longo desta pesquisa, embora alguns efeitos moleculares

(desbalanço redox e disfunção mitocondrial, diminuição na quantidade de receptor D2, e aumento na quantidade de RAGE e na atividade de caspase-3) mostrem que, se a utilização de vitamina A for mantida, poderá se estabelecer um comportamento relacionado à depressão, de acordo com aquilo observado em humanos (Myhre *et al*., 2003).

Distúrbios cognitivos são causa freqüente de diminuição no rendimento do indivíduo afetado, podendo levar a conseqüências mais drásticas economicamente em um âmbito mais geral (vida pessoal do paciente, sua família, seu trabalho/empresa). Além disso, os gastos com o tratamento de patologias neurodegenerativas, no sistema público de saúde brasileiro, são considerados elevados, comprometendo, por vezes, o próprio tratamento do paciente, tendo em vista o alto custo dos medicamentos. Alguns trabalhos mostram uma possível relação entre consumo elevado de vitamina A e surgimento de esquizofrenia entre humanos, mas não há ainda um mecanismo elucidado que explique como se dá essa interação (Goodman, 1998).

Ainda mais alarmante é o que vem sendo mostrado em trabalhos que utilizam dados populacionais para estudar os efeitos da suplementação com vitaminas sobre a qualidade de vida dos utilizadores deste hábito alimentar. Recentemente, Bjelakovic e colaboradores (Bjelakovic *et al*., 2007) mostraram que o aumento no consumo de vitamina A aumenta o risco de vida dos indivíduos que a utilizam diariamente ou alternando os dias ao longo da semana. Não houve uma investigação quanto à função cognitiva destes pacientes, mas não se descartou a hipótese de haver comprometimento central em conjunto com outras alterações deletérias.

5. CONCLUSÃO

5.1. CONCLUSÃO GERAL

Então, a suplementação com vitamina A em doses terapêuticas pode induzir distúrbio neurodegenerativo através de um mecanismo complexo que conta com disfunção mitocondrial, dano oxidativo e aumento na atividade de caspase-3, uma efetora da morte celular por apoptose. Certamente, outras investigações deverão ser estabelecidas a fim de encontrar se há relação entre estes parâmetros alterados e o distúrbio cognitivo evidenciado.

5.2. CONCLUSÕES ESPECÍFICAS

Baseando-se nos resultados obtidos no presente trabalho, podemos concluir que:

1) A suplementação com vitamina A em doses clinicamente tidas como seguras teve como conseqüências desequilíbrio redox e disfunção na atividade de complexos mitocondriais, contando com aumento na produção de $O_2^{-\bullet}$, dano oxidativo a biomoléculas mitocondriais (aumento na peroxidação lipídica e carbonilação de proteínas), aumento na atividade do complexo I-III e diminuição na do complexo IV (em alguns casos, só o primeiro efeito fora observado);
2) O tratamento induziu aumento na quantidade de α-sinucleína no eixo dopaminérgico, mas não em outras regiões onde este parâmetro foi avaliado. A ausência de efeito sobre a quantidade de β-sinucleína pode favorecer o aumento em α-sinucleína, tendo em vista o papel de chaperona e de regulador da expressão desta sobre a primeira;

3) A diminuição na quantidade de receptor D2 estriatal encontrada como efeito adverso do tratamento pode ter papel importante no desbalanço redox observado em trabalhos anteriores no grupo considerando a relação entre vitamina A e sistema nervoso central, já que este receptor regula a liberação de dopamina no eixo nigro-estriatal, conforme explicado na Discussão;
4) Ainda, o aumento no imunoconteúdo de RAGE pode atuar como um mantenedor do desequilíbrio redox, tendo em vista sua participação na facilitação de processos pró-oxidantes.
5) Também pode ser importante a participação da enzima caspase-3 no estabelecimento de declínio cognitivo (ansiedade, principalmente, que já foi observado anteriormente em nossos trabalhos), já que esta enzima tanto participa do processo de morte celular apoptótico quanto pode diminuir o número de botões sinápticos, o que afeta, conseqüentemente, a qualidade neuronal. Então, não é necessário haver perda neuronal, mas a simples diminuição na quantidade de botões sinápticos afetaria em muito a qualidade de sinal entre neurônios.

6. PERSPECTIVAS

A partir dos resultados obtidos na presente Tese, pretendemos continuar investigando as conseqüências moleculares da suplementação com vitamina em modelo experimental animal e em cultivos celulares. Análises de translocação de peptídeos β-amilóides e da proteína α-sinucleína serão importantes na tentativa de analisar a comunicação citosol-mitocôndria, que pode tomar papel central no processo neurodegenerativo induzido por vitamina A. Ainda, uma análise mais detalhada abordando a localização da enzima caspase-3 ativada nos auxiliará se a mesma atua diminuindo a área de contato interneuronal ou se está, realmente, levando os neurônios corticais a morrer por apoptose. Já realizamos tratamentos mais longos, de forma crônica, com vitamina A em animais adultos, utilizando doses mais baixas, que se aproximam muito daquilo ingerido por usuários de suplementação vitamínica, e encontramos efeitos deletérios importantes, principalmente estresse nitrosativo em mitocôndrias e diminuição nos níveis de fator neurotrófico derivado do cérebro (BDNF), além de aumento na concentração de peptídeos β-amilóide$_{1-40}$ e $_{1-42}$.

Portanto, mais estudos serão úteis ao esclarecimento destes pontos ainda sem resposta completa.

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ANEXO

Fora os trabalhos discutidos acima, os quais fazem parte do corpo principal da tese, análises bioquímicas relevantes ao ambiente redox do fígado dos animais tratados com vitamina A também foram realizadas e constarão neste documento como artigos em anexo. Os trabalhos em questão são os seguintes:

1) "Short-term vitamin A supplementation at therapeutic doses induces a pro-oxidative state in the hepatic environment and facilitates calcium-ion-induced oxidative stress in rat liver mitochondria independently from permeability transition pore formation". Autores: Marcos Roberto de Oliveira, Max William Soares Oliveira, Rodrigo Lorenzi, Ricardo Fagundes da Rocha, José Cláudio Fonseca Moreira, publicado no periódico Cell Biology and Toxicology (DOI 10.1007/s10565-008-9111-9).
2) "Evaluation of redox and bioenergetics states in the liver of vitamin A-treated rats". Autores: Marcos Roberto de Oliveira, Max W. Soares Oliveira, Mariana Leivas Muller Hoff, Guilherme Antônio Behr, Ricardo Fagundes da Rocha, José Cláudio Fonseca Moreira, publicado no periódico European Journal of Pharmacology, v. 610, pp. 99-105 (2009).

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Short-term vitamin A supplementation at therapeutic doses induces a pro-oxidative state in the hepatic environment and facilitates calcium-ion-induced oxidative stress in rat liver mitochondria independently from permeability transition pore formation

Detrimental effects of vitamin A supplementation on rat liver redox and bioenergetic states homeostasis

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Abstract There is a growing body of evidence showing that vitamin A induces toxic effects in several experimental models and in human beings. In the present work, we have investigated the effects of short-term vitamin A supplementation on the adult rat liver redox status. We have found that vitamin A at therapeutic doses induces a hepatic oxidative insult. Furthermore, we have observed increased antioxidant enzyme activity in the liver of vitamin-A-treated rats. Additionally, some mitochondrial dysfunction was found since superoxide anion production was increased in vitamin-A-treated rat liver submitochondrial particles, which may be the result of impaired mitochondrial electron transfer chain activity, as assessed here. We have also isolated rat liver mitochondria and challenged it with 75 μM $CaCl_2$, a non-oxidant agent that is able to induce mitochondrial oxidative stress indirectly. We have found that mitochondria isolated from vitamin-A-treated rat liver are more sensitive to $CaCl_2$ than control mitochondria regarding the redox status. Importantly, vitamin A seems to alter mitochondrial redox status independently of the participation of the mitochondrial permeability transition pore, which is activated by Ca^{2+} ions since cyclosporin A did not prevent the oxidative insult elicited by Ca^{2+} addition. Overall, we show here that mitochondria are a target of vitamin-A-associated toxicity also in vivo.

Keywords Vitamin A · Oxidative stress · Rat liver · Mitochondria · Calcium ions

Abbreviations

CsA	Cyclosporin A
METC	Mitochondrial electron transfer chain
MPTP	Mitochondrial permeability transition pore
SMP	Submitochondrial particles

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Introduction

Vitamin A is a micronutrient that is necessary in all stages of mammalian development. During adulthood, either vitamin A (retinol) or its derivatives (retinoids) also participate in the maintenance of mammalian organism homeostasis (Napoli 1999). The liver is the site where vitamin A is stored in an esterified form, namely retinyl palmitate (Olson and Gunning 1983; Napoli 1996). Vitamin A esters are more stable than free retinol and will be metabolized when retinol serum levels fall (Napoli 1996). In spite of this, there is a growing body of evidence suggesting that vitamin A may induce or facilitate liver toxicity. Low vitamin A doses might protect liver damage induced through CCl_4 administration (Rosengren et al. 1995). However, chronic treatment with vitamin A alone induces liver damage (Russell et al. 1974) and fails in protecting liver against CCl_4-induced toxicity (Elsisi et al. 1993a, b; Badger et al. 1996).

Therapeutically, vitamin A and retinoids are administrated in some pathological conditions at high doses, for instance leukemia and dermatological diseases (Norum 1993; Fenaux et al. 2001; Myhre et al. 2003). Short-term vitamin A exposition (i.e., days to weeks) at high doses is known to induce a myriad of side effects, including diarrhea and cognitive impairments (Geelen 1979; Myhre et al. 2003; O'Reilly et al. 2006). Additionally, vitamin A exposition as a food supplement or fortification may lead to intoxication, as recently published (Lam et al. 2006). However, the exact mechanism of such toxicity remains to be established. It is well established that retinoids are potent inducers of mitochondrial dysfunction, for instance mitochondrial swelling and leakage of cytochrome *c* (Rigobello et al. 1999; Klamt et al. 2005), with such an event culminating in increased reactive oxygen species (ROS) production (Klamt et al. 2005). Cytochrome *c* release to cytosol is a pro-apoptotic event, and ROS production is thought to participate positively in such event (Hengartner 2000; Ott et al. 2002; Kagan et al. 2004). Indeed, it has been shown that vitamin A and retinoids induce apoptosis in different cell types (Pfahl and Piedrafita 2003). However, excessive ROS or reactive nitrogen species production inhibit apoptosis progression since the caspases are dependent on a reduced thiol group in their active site to cleave their substrates (Hengartner 2000).

It has been demonstrated that vitamin A is a redox-active molecule whose behavior is pro- or antioxidant depending on its concentration. Recently, we have found that both short- and long-term vitamin A supplementation increased superoxide anion ($O_2^{-\bullet}$) production in submitochondrial particles (SMP) isolated from rat cerebral cortex and cerebellum (De Oliveira and Moreira 2007). Interestingly, we did not observe any adaptation to the insult promoted by vitamin A supplementation in the long-term exposition, showing that it is an important toxic effect of this vitamin A in those brain regions. Previously reported data also confirm the capacity of vitamin A (retinol) and its derivatives in inducing alterations in the cellular redox environment, including protein carbonylation, lipid peroxidation, and DNA damage induced by free radicals (Moreira et al. 1997; Dal-Pizzol et al. 2000; Klamt et al. 2000; Dal-Pizzol et al. 2001; Frota et al. 2004; De Oliveira et al. 2007a, b).

Therefore, we decided to investigate the effects of short-term vitamin A supplementation on rat liver regarding the redox effects of such treatment. Moreover, we have assessed mitochondrial electron transfer chain (METC) activity in the liver of vitamin-A-treated rats. We have also isolated liver mitochondria to analyze similar parameters. In addition, we have challenged isolated mitochondria with calcium, a non-oxidant agent, that is able to induce mitochondrial swelling and, indirectly, increases free radical production in mitochondria. The main objective of this work was to investigate whether a non-oxidant agent such as calcium ion was able to induce mitochondrial dysfunction in mitochondria isolated from vitamin-A-treated rats regarding redox environment parameters.

Materials and methods

Animals

We used adult male Wistar rats (90 days old; 250–320 g) obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (7:00–19:00 h) at 23±1°C. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals recommendations for animal care. The Ethical Committee for animal experimentation of

the Federal University of Rio Grande do Sul approved our research protocol.

Drugs and reagents

Arovit® (retinyl palmitate, a water soluble form of vitamin A) was purchased from Bayer, Sao Paulo, SP, Brazil. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A was prepared daily, and it occurred by protecting from light and by utilizing amber vials.

Drug administration

The animals were treated once a day for 3 or 7 days. All treatments were carried out at night (i.e., when the animals are more active and take a greater amount of food) in order to ensure maximum vitamin A absorption since this vitamin is better absorbed during or after a meal. The animals were randomly separated between groups and were treated with vehicle (0.15 M saline; control group), 1,000, 2,500, 4,500, or 9,000 IU/kg of retinyl palmitate (vitamin A) administered orally via a metallic gastric tube (gavage) in a maximum volume of 0.8 mL during each period of interest. We utilized six to seven animals in each experimental group. Vitamin A content in the diet of the animals was 12,500 IU/kg food. The daily vitamin A ingestion present only in the chow was calculated within the range of 350–400 UI/day. In the vitamin A supplementation groups, the rats were treated with vitamin A at doses ranging from 300 to 320 IU/day (1,000 IU/kg/day group), from 750 to 800 IU/day (2,500 IU/kg/day group), from 1,350 to 1,440 IU/day (4,500 IU/kg/day group), and from 2,700 to 2,880 IU/day (9,000 IU/kg/day group). Adequate measures were taken to minimize pain or discomfort.

Preparations of the samples

The animals were sacrificed by decapitation at 24 h after the last vitamin A administration. The liver was dissected out in ice immediately after the sacrifice and stored at −80°C for posterior biochemical analyses. The liver was homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged (700×*g*, 5 min) to remove cellular debris. Supernatants were used for all biochemical assays described herein. SMP and intact mitochondria were isolated as described below. The results regarding liver redox state were normalized by the protein content using bovine albumin as standard (Lowry et al. 1951).

Isolation of submitochondrial particles (SMP)

Briefly, to obtain SMP, liver was dissected and homogenized in 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl, and 1 mM EDTA (pH 7.4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free SMP. The SMP solution was also washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) to ensure Mn–superoxide dismutase (SOD) release from mitochondria (Poderoso et al. 1996; De Oliveira and Moreira 2007).

Isolation of intact mitochondria

Mitochondria from fresh rat liver were isolated as described elsewhere (Klamt et al. 2005). Briefly, liver of Wistar rats suspended in ice-cold isolation buffer A (220 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, and 0.5 mg/mL fatty-acid free bovine serum albumin) was gently homogenized with a glass-homogenizer and centrifuged at 2000×*g* for 10 min at 4°C. Approximately three quarters of the supernatant was further centrifuged at 10,000×*g* for 10 min at 4°C in a new tube. The fluffy layer of the pellet was removed by gently shaking with buffer A, and the firmly packed sediment was resuspended in the same buffer without EGTA and centrifuged at 10,000×*g* for 10 min at 4°C. The mitochondrial pellet was resuspended in buffer B (210 mM mannitol, 70 mM sucrose, 10 mM HEPES–KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, and 4 μg/mL rotenone). This procedure, which was designed to isolate intact mitochondria rather than to recover all of that present in the liver, yielded about 20 mg of mitochondrial protein per gram of liver.

Incubation of intact rat liver mitochondria

After the end of the in vivo treatments, the animals were killed as mentioned above. Liver mitochondria were isolated as described, and 1 mg/mL mitochondria were incubated (in the presence of O_2) for 10 min at 25°C with or without $CaCl_2$ and/or co-treatments. Co-treatments consisted of 1 mM dithiothreitol (DTT),

1 mM reduced glutathione (GSH), 200 U/mL native purified superoxide dismutase (SOD), 50 U/mL catalase (CAT), 850 nM cyclosporine A (CsA), 50 μM bongkrekic acid (BKA), or 10 μM Trolox™ (an analogue of vitamin E). Co-treatments, when used, were added to the incubation buffer with or without $CaCl_2$ in the beginning of the assay. After the ending of the incubation period, the samples were centrifuged at 10,000×*g* for 10 min at 4°C, and the mitochondrial pellet was resuspended in phosphate-buffered saline (pH 7.4) or in 140 mM KCl, 20 mM Tris–HCl (pH 7.4) buffer to assess oxidative stress markers or superoxide anion ($O_2^{-\bullet}$) production, respectively. To assess $O_2^{-\bullet}$ production in intact mitochondria, we froze and thawed mitochondria three times to obtain mitochondrial membranes without mitochondrial matrix so that mitochondrial SOD did not interfere with the assay as mentioned above.

Antioxidant enzyme activity estimations

SOD activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described (Misra and Fridovich 1972). Catalase (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm (Aebi 1984). A ratio between SOD activity and CAT activity (SOD/CAT ratio) was applied to better understand the effect of vitamin A supplementation upon these two oxidant-detoxifying enzymes that work sequentially converting the superoxide anion to water (Halliwell 2006). An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

Thiobarbituric acid reactive species (TBARS)

As an index of lipid peroxidation, we detected hepatic and mitochondrial thiobarbituric acid reactive species (TBARS) formation through a hot and acidic reaction. This is widely adopted as a method for measurement of lipid redox state, as previously described (Draper and Hadley 1990). Briefly, the samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 25 min. TBARS were determined by absorbance in a spectrophotometer at 532 nm. We have obtained TBARS concentration in the samples from a calibration curve that was performed using 1,1,3,3-tetramethoxypropane as standard, which was subjected to the same treatment as that applied to the supernatants of the samples. Results are expressed as nanomoles TBARS per milligram protein.

Measurement of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) as previously described (Levine et al. 1990). Briefly, tissue homogenates or mitochondria were divided into two aliquots of 200 μl. Proteins were precipitated by the addition of 100 μl 20% TCA for 5 min on ice and centrifuged at 4,000×*g* for 5 min. The pellet was redissolved in 100 μl 0.2 M NaOH, and 100 μl of 2 M HCl or 10 mM 2,4-DNPH in 2 M HCl was added to duplicate aliquots for blanks or the derivatizing of carbonyl groups, respectively. Samples were left for 30 min at room temperature. Proteins were reprecipitated with 20% TCA and washed three times with 500 μl 1:1 ethanol/ethyl acetate with 15-min standing periods to remove excess DNPH. Samples were redissolved in 1 mL 8 M urea, pH 2.3, and the absorbance was read at 370 nm. The carbonyl content in nanomoles per milligram protein was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1} at 370 nm after subtraction of the blank absorbance according to the following equation: $\{[(Final\ Absorbance/22,000.22,000) \times V]/1,000\}/Q$, where V is the volume (in milliliters) of the sample utilized in the assay, and Q is the amount of protein (in milligrams) in the volume utilized to perform the carbonyl assay.

Measurement of protein and non-protein thiol content

Briefly, a supernatant aliquot of 20% TCA precipitated homogenates was diluted in SDS 0.1%, and 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB) in ethanol was added, and the intense yellow color was developed and read at 412 nm after 20 min (Ellman 1959). A blank sample was run simultaneously, except for the absence of DTNB. Protein thiol content was calculated after subtraction of the blank absorbance through the following equation utilizing the molar extinction coefficient of 13,600 M^{-1} cm^{-1} {[(*Final*

$Absorbance/13,600) \times V]/1,000\}/Q$, where V is the volume (in milliliters) of the sample utilized in the assay, and Q is the amount of protein (in milligrams) in the volume utilized to perform the carbonyl assay. The free sulfhydryl (–SH) content (non-protein thiol content) was estimated in supernatants of 20% TCA precipitated homogenates by the same method and was calculated according to the same equation described above. Results are expressed as nano- or micromoles SH per milligram protein, depending on the sample analyzed (rat tissue or mitochondria).

Quantification of $O_2^{-\bullet}$ production

To quantify superoxide ($O_2^{-\bullet}$) production, SMP was incubated in reaction medium consisting of 230 mM mannitol, 70 mM sucrose, 10 mM HEPES–KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 μM catalase, and 1 mM epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32°C, as previously described (Poderoso et al. 1996; De Oliveira and Moreira 2007).

Mitochondrial electron transfer chain (METC) enzyme activities

To obtain SMP from rat liver in order to assess METC activity, we homogenized the tissue in 250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4, and 50 IU/mL heparin buffer. The samples were centrifuged 1,000×*g*, and the supernatants were colleted. Then, the samples were frozen and thawed three times, and METC activity detection was performed as described below.

Complex I–CoQ-III activity

Complex I–CoQ-III activity was determined by following the increase in absorbance due to reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength (ϵ=19.1 mM^{-1} cm^{-1}). The reaction buffer contained 20 mM potassium phosphate, pH 8.0, 2.0 mM KCN, 10 μM EDTA, 50 μM cytochrome *c*, and 20–45 μg supernatant protein. The reaction started by addition of 25 μM NADH and was monitored at 30°C for 3 min before the addition of 10 μM rotenone, after which the activity was monitored for an additional 3 min. Complex I–III activity was the rotenone-sensitive NADH/cytochrome *c* oxidoreductase activity (Shapira et al. 1990).

Complex II–CoQ-III activity

Complex II–CoQ-III activity was measured by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength (ϵ=21 mM^{-1} cm^{-1}). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, and 16 mM succinate was preincubated with 50–100 μg supernatant protein at 30°C for 30 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added, and the reaction was started by the addition of 0.6 μg/mL cytochrome *c* and was monitored for 5 min at 30°C (Fisher et al. 1985).

Complex IV activity

Complex IV activity was measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength (ϵ=19.15 mM^{-1} cm^{-1}). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg supernatant protein, and the reaction was started with addition of 0.7 μg reduced cytochrome *c*. The activity of complex IV was measured at 25°C for 10 min (Rustin et al. 1994).

Statistical analyses

Biochemical data are expressed as means ± SEM. All analyses were performed using the Statistical Package for the Social Sciences (SPSS 12.0) software. p values were considered significant when $p<0.05$. Differences in experimental groups were determined by the Student's t test or the one-way ANOVA followed by the post hoc Duncan's test.

Results

Hepatic oxidative damage parameters

Lipid peroxidation

As depicted in Fig. 1a, we found increased lipid peroxidation in the liver of the rats that received vitamin

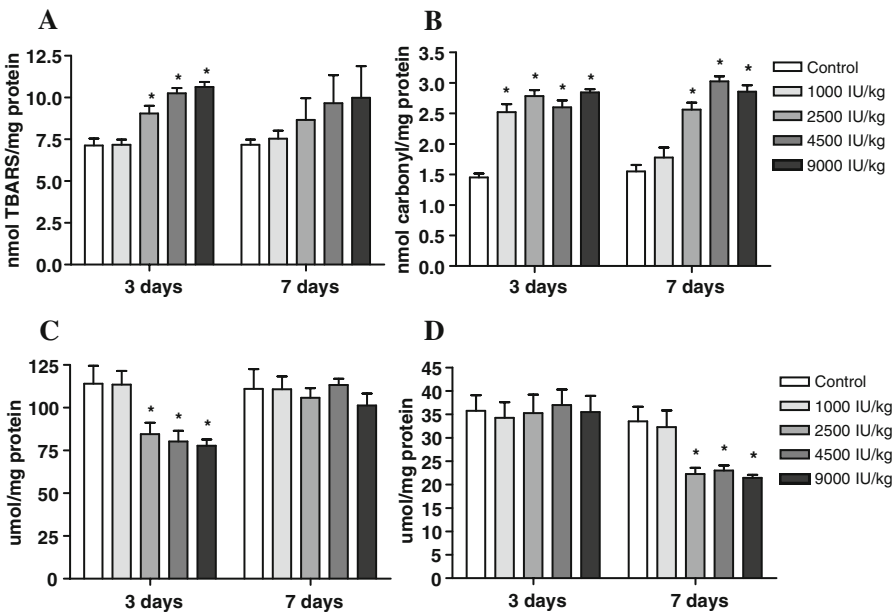

Fig. 1 Effects of short-term vitamin A supplementation on lipid peroxidation (**a**), protein carbonylation (**b**), protein (**c**), and non-protein (**d**) thiol content in the rat liver. Data are mean±SEM (n=7 per group). *p<0.01 (one-way ANOVA followed by the post hoc Duncan's test)

A supplementation at 2,500, 4,500, or 9,000 IU/kg/day for 3 days (1.4- to 1.7-fold; p<0.01). However, hepatic lipid peroxidation levels did not change after vitamin A supplementation for 7 days (Fig. 1a).

Protein carbonylation

We found a 1.3- to 1.7-fold increase of protein carbonylation levels in the liver of the rats that received vitamin A at any dose tested for 3 days (p<0.01; Fig. 1b). Vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day induced a 1.3- to 1.4-fold increase of hepatic protein carbonylation levels in the rats that were treated for 7 days (p<0.01; Fig. 1b).

Protein thiol content

As shown in Fig. 1c, we detected decreased protein thiol content (1.2- to 1.3-fold) in the liver of the rats that were treated with vitamin A at 2,500, 4,500, or 9,000 IU/kg/day for 3 days (p<0.01). However, protein thiol content did not change in the liver of the rats that were treated with vitamin A for 7 days (Fig. 1c).

Non-protein thiol content

Hepatic non-protein thiol content did not change in the rats that received vitamin A supplementation for 3 days (Fig. 1d). We found that vitamin A at 2,500, 4,500, or 9,000 IU/kg/day induced a 1.4- to 1.5-fold decrease of hepatic non-protein thiol content (p<0.01; Fig. 1d).

Antioxidant enzyme activities

SOD activity

Vitamin A supplementation at 9,000 IU/kg/day induced a 1.3-fold increase of SOD activity in the liver of the rats that were treated for 3 days (p<0.05; Fig. 2a). Sustained vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day for 7 days induced a 1.2- to 1.4-fold increase in hepatic SOD activity (p<0.05; Fig. 2a).

CAT activity

We found increased (1.4-fold) CAT activity in the liver of the rats that received vitamin A supplementation at 9,000 IU/kg/day for 3 days (p<0.05; Fig. 2b). CAT enzyme activity did not change in the liver of the rats that received vitamin A supplementation for 7 days (p<0.05; Fig. 2b).

SOD/CAT

Hepatic SOD/CAT increased in the animals that received vitamin A supplementation at 4,500 or 9,000 IU/kg/day for 7 days (p<0.05; Fig. 2c).

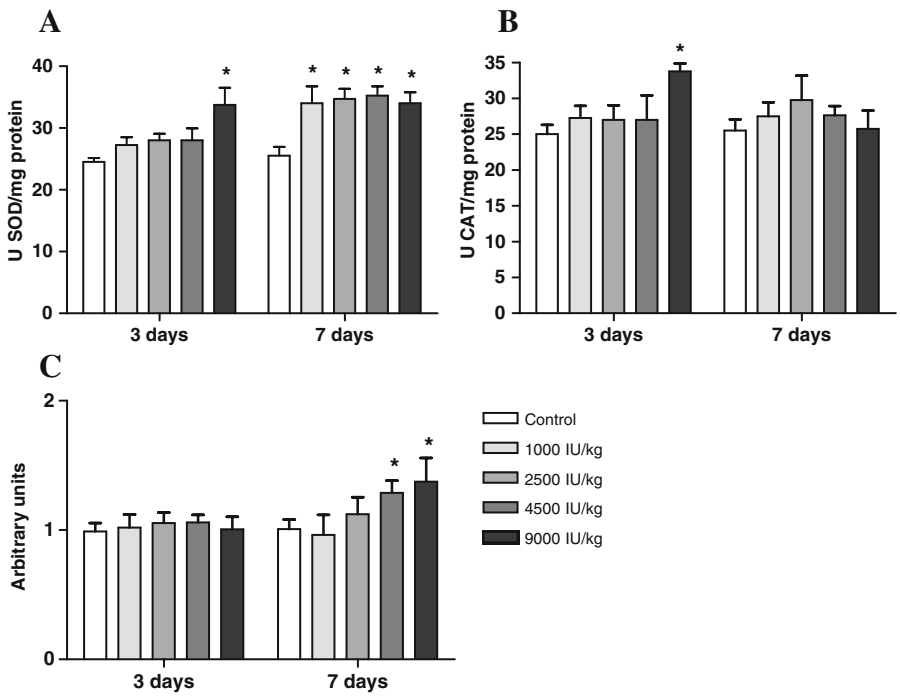

Fig. 2 Effects of short-term vitamin A supplementation on SOD (**a**), CAT (**b**), and SOD/CAT ratio (**c**) in the rat liver. Data are mean±SEM (n=7 per group). *p<0.05 (one-way ANOVA followed by the post hoc Duncan's test)

Hepatic submitochondrial particles (SMP) oxidative parameters

Superoxide anion ($O_2^{-\bullet}$) production

We found increased (1.3- to 1.6-fold; p<0.01) $O_2^{-\bullet}$ production in hepatic SMP of the rats that were treated with vitamin A at 2,500, 4,500, or 9,000 IU/kg/day for 3 days (Fig. 3a). Vitamin A supplementation at any dose tested induced a 1.3- to 1.7-fold increase of $O_2^{-\bullet}$ production in hepatic SMP isolated from the rats that were treated for 7 days (p<0.01; Fig. 3a).

SMP lipid peroxidation

As depicted in Fig. 4b, we found increased (1.7- to 2.7-fold) lipid peroxidation levels in hepatic SMP of the rats that received vitamin A at 2,500, 4,500, or 9,000 IU/kg/day for 3 days (p<0.01). Vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day increased (1.6- to 2.7-fold) hepatic SMP lipid peroxidation levels in the rats that were treated for 7 days (p<0.01; Fig. 3b).

SMP protein carbonylation

Vitamin A supplementation at 9,000 IU/kg/day induced a 1.5-fold increase of SMP protein carbonylation levels in the rats that were treated for 3 days (p<0.01; Fig. 3c). Sustained vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day for 7 days induced a 1.5- to 1.8-fold increase of SMP protein carbonylation levels (Fig. 3c).

SMP protein thiol content

Hepatic SMP protein thiol content did not change in this experimental model (Fig. 3d).

Hepatic mitochondrial electron transfer chain (METC) enzyme activities

As depicted in Fig. 4a, vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg/day for 3 days induced an increase (1.3 to 1.5-fold) in hepatic complex I–III enzyme activity. We observed that vitamin A supplementation at any dose tested for 7 days induced an increase (1.3- to 1.6-fold) in hepatic complex I–III

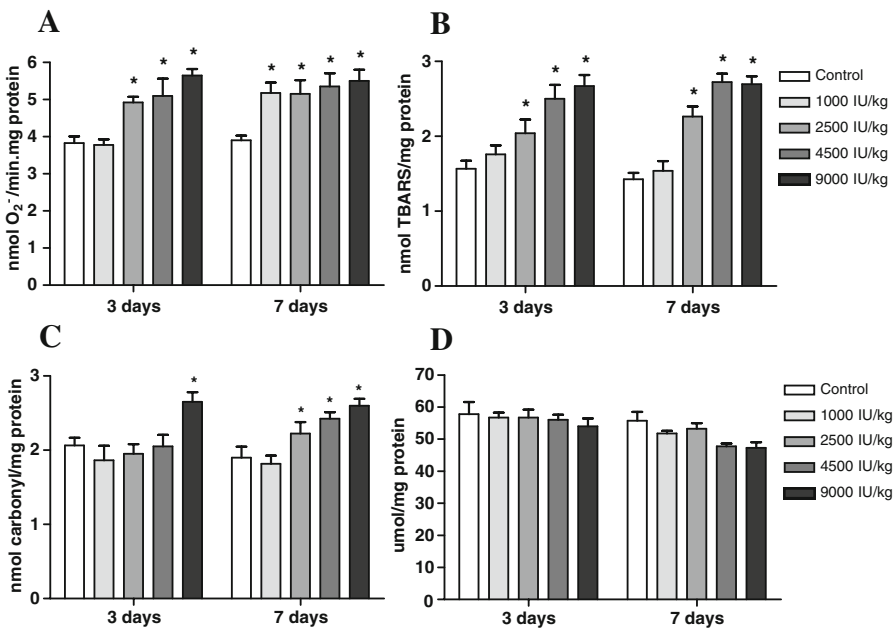

Fig. 3 Effects of short-term vitamin A supplementation on superoxide anion production (**a**), lipid peroxidation (**b**), protein carbonylation (**c**), and protein thiol content (**d**) in SMP isolated form rat liver. Data are mean±SEM (n=7 per group). *p<0.01 (one-way ANOVA followed by the post hoc Duncan's test)

enzyme activity (Fig. 4a). In spite of this, we did not find any change in complex II–III and IV enzyme activities in the liver of vitamin-A-treated rats (Fig. 4b and c).

Incubated hepatic mitochondria

Lipid peroxidation

As shown in Table 1, mitochondria that were isolated from the liver of the rats that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 3 days present higher lipid peroxidation levels when incubated for 10 min with buffer C (p<0.05). Interestingly, 75 μM $CaCl_2$ induced a 2.5- to 2.9-fold increase of lipid peroxidation in liver mitochondria from the animals that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 3 days when compared with mitochondria isolated from the liver of the animals that received saline (vitamin A vehicle) for 3 days (p<0.01). A co-treatment with GSH, SOD, CAT, or Trolox™ decreased lipid peroxidation levels induced by $CaCl_2$ (p<0.05; Table 1).

A very similar effect was observed when mitochondria were isolated from the liver of the rats that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 7 days (Table 1). Co-treatment with mitochondrial permeability transition pore (MPTP) inhibitors did not prevent the oxidative damage induced by 75 μM $CaCl_2$ in mitochondria isolated from vitamin-A-treated rats (Table 2). The co-treatments alone did not alter mitochondrial lipid peroxidation levels (data not shown).

Protein carbonylation

We found increased protein carbonylation levels in incubated mitochondria (in the absence of $CaCl_2$) that were isolated from the liver of the rats that were treated with vitamin A at 9,000 IU/kg for 3 days (p<0.05; Table 3). $CaCl_2$ at 75 μM induced a 1.7- to 2.0-fold increase of protein carbonylation in the mitochondria that were isolated from the liver of the rats that received vitamin A supplementation at 4,500 or 9,000 IU/kg for 3 days (p<0.01; Table 2). $CaCl_2$-induced protein carbonylation was decreased by a co-treatment with GSH, SOD, or Trolox™ (p<0.05; Table 3).

Mitochondria that were isolated from the liver of the rats that were treated with vitamin A at 2,500, 4,500, or 9,000 IU/kg for 7 days showed increased (1.4- to 1.5-fold) protein carbonylation levels even when incubated in the absence of $CaCl_2$ (p<0.05; Table 2). However, the addition of $CaCl_2$ increased (1.7- to 2.0-fold) protein carbonylation levels in mitochondria that were isolated from the animals that received vitamin A at 2,500, 4,500, or 9,000 IU/kg

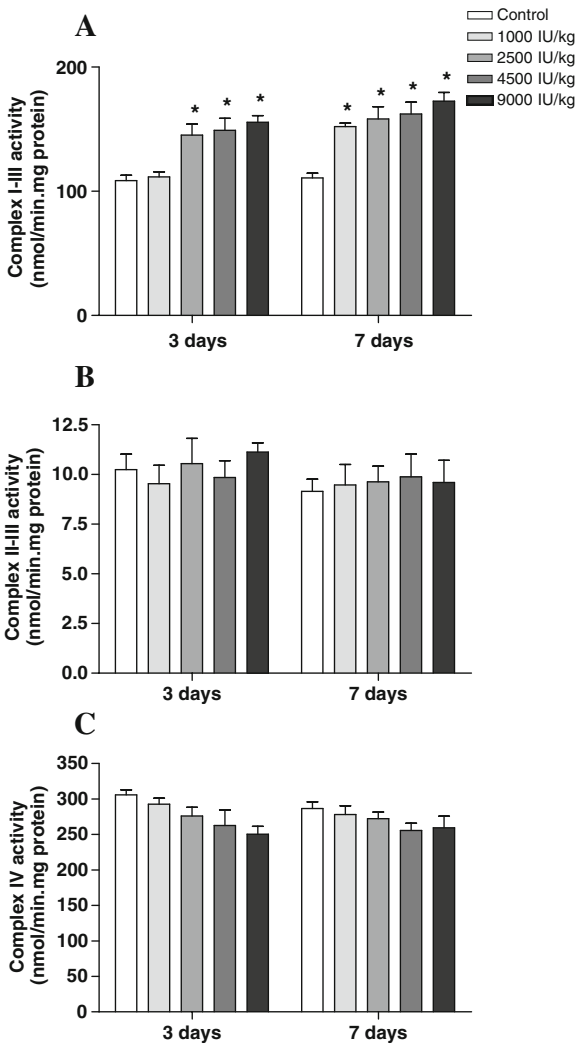

Fig. 4 Effects of short-term vitamin A supplementation on complex I–III (**a**), complex II–III (**b**), and complex IV (**c**) enzyme activities in rat liver mitochondria. Data are mean ± SEM (n=7 per group). *p<0.05 (one-way ANOVA followed by the post hoc Duncan's test)

when compared with mitochondria isolated from the animals that received saline (p<0.01; Table 2). Only the co-treatment with GSH, SOD, or Trolox™ prevented the increase in protein carbonylation induced by $CaCl_2$ (p<0.05; Table 3). Co-treatment with MPTP inhibitors did not prevent the oxidative damage induced by 75 μM $CaCl_2$ in mitochondria isolated from vitamin-A-treated rats (Table 4). The co-treatments alone did not interfere in the mitochondrial protein carbonylation levels (data not shown).

Protein thiol content

As depicted in Table 5, protein thiol content of mitochondria isolated from control or vitamin-A-treated rats did not change when incubated in the absence of $CaCl_2$. However, $CaCl_2$ induced a 1.3- to 1.4-fold decrease of protein thiol content in the mitochondria isolated from the animals that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 3 days when compared to mitochondria isolated from control animals (p<0.05). A co-treatment with GSH, SOD, or Trolox™ prevented the decrease in protein thiol content induced by $CaCl_2$ (p<0.05; Table 5).

We did not detect any change in liver mitochondria isolated from 7-day vitamin-A-treated rats when incubated without $CaCl_2$ regarding the redox state of protein thiol content (Table 5). However, $CaCl_2$ at 75 μM induced a decrease (1.5- to 1.6-fold) in the protein thiol content of the mitochondria that were isolated from the animals that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 7 days when compared to control animals (p<0.05; Table 5). A co-treatment with GSH, SOD, or Trolox™ prevented the decrease in protein thiol content induced by $CaCl_2$ (p<0.05; Table 5). Co-treatment with MPTP inhibitors did not prevent the oxidative damage induced by 75 μM $CaCl_2$ in mitochondria isolated from vitamin-A-treated rats (Table 6). The co-treatments alone did not alter mitochondrial protein thiol content (data not shown).

Superoxide anion ($O_2^{-\bullet}$) production

As shown in Table 7, mitochondria that were isolated from the liver of the rats that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg for 3 days produced more $O_2^{-\bullet}$ after incubation without $CaCl_2$ when compared to mitochondria isolated from the liver of control animals (p<0.05). The addition of $CaCl_2$ increased (2.0- to 2.45-fold) $O_2^{-\bullet}$ production in mitochondria isolated from vitamin-A-treated rats (2,500, 4,500, or 9,000 IU/kg vitamin A, 3 days; p<0.01). A co-treatment with GSH, SOD, or Trolox™ prevented the increase in $O_2^{-\bullet}$ production induced by $CaCl_2$ (p<0.05; Table 7).

We detected increased $O_2^{-\bullet}$ production in mitochondria isolated from the animals that received vitamin A supplementation at any dose tested for

Table 1 Effects of vitamin A supplementation and different antioxidant co-treatments on TBARS levels of isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + DTT	$CaCl_2$ + GSH	$CaCl_2$ + SOD	$CaCl_2$ + CAT	$CaCl_2$ + Trolox
3	Saline	1.02±0.14	1.03±0.04	1.08±0.08	1.06±0.06	1.03±0.09	1.06±0.09	1.03±0.06
	1,000 IU/kg	1.12±0.05	1.21±0.11	1.23±0.07	1.24±0.04	1.12±0.09	1.15±0.1	1.14±0.04
	2,500 IU/kg	1.75±0.07*	2.62±0.2**,***	2.53±0.14	1.63±0.13****	1.64±0.12****	1.7±0.12****	1.77±0.2****
	4,500 IU/kg	1.85±0.08*	2.35±0.06**,***	2.23±0.11	1.79±0.16****	1.86±0.07****	1.73±0.09****	1.75±0.19****
	9,000 IU/kg	2.16±0.16*	2.94±0.2**,***	3.02±0.2	1.96±0.04****	1.91±0.12****	1.95±0.24****	1.96±0.07****
7	Saline	1.03±0.05	1±0.06	1.19±0.08	1.22±0.08	1.2±0.05	1.19±0.01	1.13±0.06
	1,000 IU/kg	1.05±0.05	1.22±0.13	1.16±0.13	1.23±0.11	1.11±0.04	1.1±0.06	1.06±0.04
	2,500 IU/kg	1.91±0.07*	2.79±0.09**,***	2.77±0.07	1.98±0.11****	1.93±0.03****	1.94±0.14****	1.82±0.1****
	4,500 IU/kg	1.9±0.08*	2.72±0.11**,***	2.75±0.22	1.84±0.14****	1.91±0.15****	1.96±0.04****	1.83±0.05****
	9,000 IU/kg	2.1±0.02*	2.84±0.12**,***	2.84±0.13	1.96±0.19****	1.97±0.13****	1.96±0.1****	1.84±0.19****

Values are mean ± SEM. N=6 per group, duplicate

*p<0.05;**p<0.05 different from respective control (i.e., without calcium); ***p<0.01 vitamin A vs. saline; ****p<0.05 different from the respective $CaCl_2$-treated group (one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

7 days after incubation without $CaCl_2$ when compared to mitochondria isolated from the liver of control animals treated during the same period (p<0.05; Table 7). The addition of $CaCl_2$ increased $O_2^{-\bullet}$ production (2.0- to 2.7-fold) in mitochondria isolated from the rats that received vitamin A supplementation at any dose tested when compared to saline-treated animals (control animals; p<0.01; Table 7). A co-treatment with GSH, SOD, or Trolox™ prevented the increase in $O_2^{-\bullet}$ production induced by $CaCl_2$ (p<0.05; Table 7). Co-treatment with MPTP inhibitors did not prevent the oxidative damage induced by 75 μM $CaCl_2$ in mitochondria isolated from vitamin-A-treated rats (Table 8). The co-treatments alone did not alter mitochondrial $O_2^{-\bullet}$ production levels (data not shown).

Discussion

In this work, we investigated the short-term effects of vitamin A supplementation on rat liver redox parameters. In addition, we have isolated SMP and intact mitochondria from the liver of the animals that

Table 2 Effects of vitamin A supplementation and MPTP inhibitors on TBARS levels of isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + CsA	$CaCl_2$ + BKA
3	Saline	1.02±0.14	1.03±0.04	1.09±0.1	1.04±0.08
	1,000 IU/kg	1.12±0.05	1.21±0.11	1.2±0.11	1.21±0.05
	2,500 IU/kg	1.75±0.07*	2.62±0.2**,***	2.51±0.14	2.61±0.09
	4,500 IU/kg	1.85±0.08*	2.35±0.06**,***	2.39±0.18	2.37±0.16
	9,000 IU/kg	2.16±0.16*	2.94±0.2**,***	3.09±0.11	3.02±0.13
7	Saline	1.03±0.05	1±0.06	0.97±0.03	1.15±0.11
	1,000 IU/kg	1.05±0.05	1.22±0.13	1.15±0.07	1.17±0.08
	2,500 IU/kg	1.91±0.07*	2.79±0.09**,***	2.75±0.08	2.85±0.09
	4,500 IU/kg	1.9±0.08*	2.72±0.11**,***	2.84±0.08	2.88±0.16
	9,000 IU/kg	2.1±0.02*	2.84±0.12**,***	2.73±0.21	2.75±0.13

Values are mean ± SEM. N=6 per group, duplicate

*p<0.05; **p<0.05 different from respective control (i.e., without calcium; one-way ANOVA followed by the post hoc Tukey's test, Student's t test); ***p<0.01 vitamin A vs. saline

Table 3 Effects of vitamin A supplementation and different antioxidant co-treatments on protein carbonylation levels of isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + DTT	$CaCl_2$ + GSH	$CaCl_2$ + SOD	$CaCl_2$ + CAT	$CaCl_2$ + Trolox
3	Saline	1.82±0.06	1.74±0.13	1.61±0.12	1.54±0.09	1.59±0.09	1.66±0.1	1.71±0.06
	1,000 IU/kg	1.92±0.07	2.12±0.06	2.02±0.1	1.98±0.09	1.91±0.12	1.93±0.08	2.05±0.07
	2,500 IU/kg	1.9±0.04	1.97±0.19	1.96±0.14	2.05±0.14	1.95±0.05	1.95±0.06	1.95±0.07
	4,500 IU/kg	1.92±0.07	3.02±0.17**·***	2.9±0.06	2.01±0.13****	2.10±0.15****	2.99±0.19	1.95±0.13****
	9,000 IU/kg	2.67±0.09*	3.53±0.11**·***	3.36±0.2	2.55±0.12****	2.26±0.14****	3.44±0.18	2.43±0.25****
7	Saline	1.86±0.06	1.9±0.07	1.91±0.08	1.93±0.12	1.93±0.11	2.06±0.05	1.92±0.07
	1,000 IU/kg	1.92±0.08	1.89±0.06	1.8±0.18	1.92±0.09	2.07±0.08	1.89±0.05	1.74±0.23
	2,500 IU/kg	2.52±0.22*	3.2±0.13**·***	3.14±0.07	2.44±0.13****	2.26±0.2****	3.27±0.17	2.35±0.11****
	4,500 IU/kg	2.77±0.06*	3.7±0.16**·***	3.88±0.11	2.61±0.16****	2.44±0.16****	3.56±0.08	2.6±0.16****
	9,000 IU/kg	2.67±0.18*	3.85±0.07**·***	3.75±0.1	2.6±0.15****	2.52±0.16****	3.77±0.14	2.54±0.14****

Values are mean ± SEM. N=6 per group, duplicate

*p<0.05; **p<0.05 different from respective control (i.e., without calcium); ***p<0.01 vitamin A vs. saline; ****p<0.05 different from the respective $CaCl_2$-treated group (one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

received vitamin A at different doses and analyzed the effects of vitamin A supplementation on the mitochondrial redox state. We have found that vitamin A supplementation for 3 or 7 days exerts a pro-oxidant state on rat liver and on isolated hepatic mitochondria, as well as on mitochondria that were incubated with $CaCl_2$, a non-oxidant agent that is able to, indirectly, induce oxidative stress in mitochondria through mitochondrial dysfunction, for instance mitochondrial swelling (Cai and Jones 1998).

Vitamin A is, as previously mentioned, a redox-active molecule, which is able to induce—or facilitate—oxidative stress in several experimental models. We have recently reported increased superoxide anion ($O_2^{\bullet-}$) production in SMP isolated from rat cerebral cortex and cerebellum (De Oliveira and Moreira 2007). Furthermore, we have previously reported that retinol induces mitochondrial swelling in vitro and, consequently, induces cytochrome c release from mitochondria (Klamt et al. 2005). In the present work, we demonstrate, for the first time, that mitochondria isolated from the livers of rats that received vitamin A at therapeutic doses for 3 or 7 days is more sensitive to $CaCl_2$ regarding the redox effects of this challenge.

Table 4 Effects of vitamin A supplementation and MPTP inhibitors on protein carbonylation levels of isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + CsA	$CaCl_2$ + BKA
3	Saline	1.82±0.06	1.74±0.13	1.78±0.13	1.71±0.08
	1,000 IU/kg	1.92±0.07	2.12±0.06	1.94±0.1	1.95±0.17
	2,500 IU/kg	1.9±0.04	1.97±0.19	2.21±0.14	2.24±0.12
	4,500 IU/kg	1.92±0.07	3.02±0.17**·***	3.05±0.13	2.95±0.19
	9,000 IU/kg	2.67±0.09*	3.53±0.11**·***	3.32±0.19	3.54±0.12
7	Saline	1.86±0.06	1.9±0.07	1.96±0.04	1.94±0.11
	1,000 IU/kg	1.92±0.08	1.89±0.06	1.99±0.11	1.87±0.08
	2,500 IU/kg	2.52±0.22*	3.2±0.13**·***	3.19±0.15	3.21±0.08
	4,500 IU/kg	2.77±0.06*	3.7±0.16**·***	3.66±0.17	3.7±0.11
	9,000 IU/kg	2.67±0.18*	3.85±0.07**·***	3.87±0.07	3.85±0.16

Values are mean ± SEM. N=6 per group, duplicate

*p<0.05; **p<0.05 different from respective control (i.e., without calcium; one-way ANOVA followed by the post hoc Tukey's test, Student's t test); ***p<0.01 vitamin A vs. saline

Table 5 Effects of vitamin A supplementation and different antioxidant co-treatments on protein sulfhydryl content of isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + DTT	$CaCl_2$ + GSH	$CaCl_2$ + SOD	$CaCl_2$ + CAT	$CaCl_2$ + Trolox
3	Saline	56.2±2.31	57.4±1.8	58.6±2.2	56.5±1.2	56.3±0.82	57.3±0.9	57.5±1.78
	1,000 IU/kg	56.6±1.37	58.3±1.56	59.6±0.78	59.2±1.2	59.4±2.26	57.9±1.85	58.4±2.24
	2,500 IU/kg	55.7±2.25	41.7±2.7*·**	41±2.02	54.5±1.99***	53.3±1.37***	41.9±1.84	56±1.24***
	4,500 IU/kg	56.7±0.7	39.7±2.28*·**	37.6±2.73	58.4±1.28***	52.4±0.72***	39.7±3.53	58.6±1.54***
	9,000 IU/kg	58.5±0.77	39.9±1.32*·**	40±3.12	59.6±0.82***	52±1.06***	42.9±3.32	58.7±2.1***
7	Saline	58.7±0.7	60±1.13	58.8±1.85	58.7±1.37	59.8±1.31	58.8±1.08	57.6±1.75
	1,000 IU/kg	61±0.9	58.8±0.8	60.9±1.33	58.8±1.72	59.2±1.28	59.8±1.14	59.7±1.33
	2,500 IU/kg	60.2±1.42	40.6±1.07*·**	38.4±1.8	60.6±1.92***	63.2±2.3***	40.4±3.17	61.3±1.71***
	4,500 IU/kg	62.1±0.49	40.76±1.41*·**	41.1±1.57	62.1±0.96***	63.3±1.77***	44.3±3.13	60.2±2.14***
	9,000 IU/kg	60.4±2.88	38.1±0.78*·**	42.3±4.1	60.6±2.73***	61.1±3.3***	44.9±3.91	61.9±1.98***

Values are mean ± SEM. N=6 per group, duplicate

*p<0.05 vitamin A vs. saline; **p<0.05 different from respective control (i.e., without calcium); ***p<0.05 different from the respective $CaCl_2$-treated group (one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

Vitamin A supplementation induced lipid peroxidation, protein carbonylation, and oxidation of protein thiol groups in rat liver in vivo (Fig. 1a,b,c, and d). Interestingly, we did not find any change in lipid peroxidation levels in the liver of the rats that received vitamin A supplementation for 7 days (Fig. 1a). Also, we did not find any change in CAT enzyme activity in the same period (Fig. 2b). At least in part, the non-altered lipid peroxidation levels may be due to decreased rates of hydrogen peroxide production in rat liver in such period since CAT enzyme is not altered. Hydrogen peroxide is a diffusible reactive oxygen species, which is capable of inducing oxidative damage mainly to lipids due to its capacity to cross biological membranes (Halliwell 2006). Non-protein thiol content was decreased only in the animals that receive vitamin A supplementation for 7 days (Fig. 1d), showing that this treatment is able to, at least in part, consume some liver glutathione (GSH), which might facilitate the pro-oxidant effects that were observed in both SMP and intact mitochondria isolated from rat liver (Fig. 3a,b,c, and d; Tables 1, 3, 5, and 7). We found increased SOD and CAT enzyme activities in the liver of the rats that received vitamin A (Fig. 2a, and b). These results indicate an increased $O_2^{\bullet-}$ production since this pro-

Table 6 Effects of vitamin A supplementation and MPTP inhibitors on protein sulfhydryl content of isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + CsA	$CaCl_2$ + BKA
3	Saline	56.2±2.31	57.4±1.8	58.4±1.3	58.2±2.8
	1,000 IU/kg	56.6±1.37	58.3±1.56	58.7±1.45	58.5±1.5
	2,500 IU/kg	55.7±2.25	41.7±2.7*·**	42.7±1.19	39.5±2.27
	4,500 IU/kg	56.7±0.7	39.7±2.28*·**	42.6±1.18	39.4±1.74
	9,000 IU/kg	58.5±0.77	39.9±1.32*·**	39.7±1.9	40.4±2.7
7	Saline	58.7±0.7	60±1.13	59.1±1.13	59.3±2.25
	1,000 IU/kg	61±0.9	58.8±0.8	58.8±2.1	59±1.58
	2,500 IU/kg	60.2±1.42	40.6±1.07*·**	39.8±2.6	41±2.08
	4,500 IU/kg	62.1±0.49	40.76±1.41*·**	39.7±2.33	40.2±1.47
	9,000 IU/kg	60.4±2.88	38.1±0.78*·**	40.1±1.67	39.8±2.65

Values are mean ± SEM. N=6 per group, duplicate

*p<0.05 vitamin A vs. saline; **p<0.05 different from respective control (i.e., without calcium; one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

Table 7 Effects of vitamin A supplementation and different antioxidant co-treatments on superoxide anion production in isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + DTT	$CaCl_2$ + GSH	$CaCl_2$ + SOD	$CaCl_2$ + CAT	$CaCl_2$ + Trolox
3	Saline	1.05±0.1	1.03±0.1	1.07±0.05	1.05±0.07	1±0.02	1.06±0.05	1.09±0.08
	1,000 IU/kg	1.14±0.06	1.12±0.04	1.11±0.07	1.12±0.05	1.19±0.05	1.08±0.05	1.18±0.07
	2,500 IU/kg	1.44±0.06*	2.1±0.13**·***	2.05±0.04	1.33±0.17****	1.32±0.26****	2.19±0.11	1.12±0.05****
	4,500 IU/kg	1.54±0.14*	2.4±0.12**·***	2.12±0.07	1.43±0.07****	1.24±0.27****	1.91±0.13	1.29±0.1****
	9,000 IU/kg	1.66±0.1	2.53±0.11**·***	2.29±0.14	1.3±0.14****	1.23±0.13****	2.15±0.03	1.2±0.06****
7	Saline	1.09±0.04	1.06±0.05	1.03±0.02	1.04±0.04	1.32±0.03	1.1±0.08	1.04±0.09
	1,000 IU/kg	1.59±0.17*	2.16±0.05**·***	2.15±0.06	1.51±0.25****	1.16±0.04****	1.15±0.02	1.43±0.22****
	2,500 IU/kg	1.98±0.09*	2.65±0.09**·***	2.5±0.06	1.7±0.26****	1.67±0.14****	2.17±0.09	1.74±0.19****
	4,500 IU/kg	1.99±0.17*	2.53±0.08**·***	2.7±0.12	1.83±0.11****	1.89±0.05****	2.2±0.09	1.92±0.11****
	9,000 IU/kg	2.09±0.12*	2.84±0.08**·***	2.86±0.06	1.78±0.31****	1.8±0.09****	2.26±0.06	1.76±0.17****

Values are mean ± SEM. N=6 per group, duplicate

*p<0.05; **p<0.05 different from respective control (i.e., without calcium); ***p<0.1 vitamin A vs. saline; ****p<0.05 different from the respective $CaCl_2$-treated group (one-way ANOVA followed by the post hoc Tukey's test, Student's t test)

oxidant agent allosterically activates SOD, which in turn produces H_2O_2 by dismutation of $O_2^{\bullet-}$ (Halliwell 2006). Actually, we found increased $O_2^{\bullet-}$ production in SMP isolated from the livers of the animals that received vitamin A supplementation (Fig. 3a). According to Fig. 4, we observed increased complex I–III enzyme activity, but unaltered complexes II–III and IV enzyme activities, suggesting mitochondrial uncoupling and, consequently, gave rise to increased $O_2^{\bullet-}$ production, as mentioned above.

The effects seen here when mitochondria were challenged with $CaCl_2$ might be a consequence of increased levels of markers of oxidative stress that were found in vivo in SMP, which represent mitochondrial membranes without mitochondrial matrix content (Poderoso et al. 1996). We found increased lipid peroxidation and protein carbonylation and decreased protein thiol content in SMP isolated from rat liver (Fig. 3a–d). This in vivo oxidative insult might facilitate the in vitro observed impairment in the mitochondrial redox state that was triggered by $CaCl_2$ treatment. Indeed, it was previously reported that oxidation of thiol groups induces mitochondrial dysfunction in several experimental models (Kowaltowski

Table 8 Effects of vitamin A supplementation and MPTP inhibitors on superoxide anion production in isolated rat liver mitochondria incubated with 75 μM $CaCl_2$

Period (days)	Groups	Control	$CaCl_2$	$CaCl_2$ + CsA	$CaCl_2$ + BKA
3	Saline	1.05±0.1	1.03±0.1	1.03±0.02	1.23±0.07
	1,000 IU/kg	1.14±0.06	1.12±0.04	1.17±0.06	1.04±0.07
	2,500 IU/kg	1.44±0.06*	2.1±0.13**·***	2.02±0.03	2.19±0.16
	4,500 IU/kg	1.54±0.14*	2.4±0.12**·***	2.25±0.1	2.29±0.1
	9,000 IU/kg	1.66±0.1	2.53±0.11**·***	2.48±0.06	2.37±0.09
7	Saline	1.09±0.04	1.06±0.05	1.05±0.02	1.07±0.05
	1,000 IU/kg	1.59±0.17*	2.16±0.05**·***	1.24±0.06	1.08±0.03
	2,500 IU/kg	1.98±0.09*	2.65±0.09**·***	2.02±0.04	2.07±0.17
	4,500 IU/kg	1.99±0.17*	2.53±0.08**·***	2.3±0.07	2.24±0.11
	9,000 IU/kg	2.09±0.12*	2.84±0.08**·***	2.64±0.12	2.64±0.1

Values are mean ± SEM. N=6 per group, duplicate

*p<0.05; **p<0.05 different from respective control (i.e., without calcium; one-way ANOVA followed by the post hoc Tukey's test, Student's t test); ***p<0.1 vitamin A vs. saline

et al. 1998; Kim et al. 2003; Aon et al. 2007). It is important to mention that the $CaCl_2$ concentration used here is considered to be low to moderate when compared to other reports (Borutaite et al. 1999; Duchen 2000). Higher $CaCl_2$ concentrations, for instance 150–300 μM, could induce effects not associated with the ability of calcium ions to open the MPTP.

In Tables 1 and 2, we observed that a co-treatment with DTT, CsA, or BKA did not protect isolated mitochondria against the pro-oxidant state induced by $CaCl_2$ regarding lipid peroxidation. However, the absence of effect of DTT is expected since this thiol reducer agent does not pass through biological membranes. Interestingly, addition of reduced glutathione (GSH), SOD, CAT, or Trolox™ effectively promote mitochondrial protection against $CaCl_2$-induced redox impairment (Table 1, 3, 5, and 7). It is important to mention, again, that $CaCl_2$ induces a pro-oxidant state in mitochondria through indirect action since this molecule did not present an intrinsic capacity to induce oxidative stress directly. The redox effects induced by $CaCl_2$ is very likely to be consequence of $CaCl_2$-dependent MPTP induction, as previously reported (Zamzami and Kroemer 2001). In spite of this, our data indicate a potential toxic effect of vitamin A upon rat liver mitochondria that is independent of MPTP activation. It is noteworthy that neither CsA nor BKA, two inhibitors of the MPTP, did protect mitochondria against $CaCl_2$ pro-oxidant effects (Tables 2, 4, 6, and 8). Protein carbonylation, oxidation of protein thiol content, and $O_2^{\bullet-}$ production were prevented when GSH, SOD, or Trolox™ were added to the incubation buffer, but CAT was ineffective in preventing those oxidant insults induced by $CaCl_2$ (Tables 3, 5, and 7, respectively), suggesting that H_2O_2 did not participate in altering those redox parameters in this experimental model. Taken together, these data reinforce our hypothesis that vitamin A is able to induce a mitochondrial impairment that is, at least in part, dependent on the redox environment. However, it was also demonstrated that vitamin A is a membranolytic agent (Goodall et al. 1980). This effect might be responsible for all the other effects that were seen in the present work since the doses applied here are considered to be high, even though therapeutically used (Norum 1993; Fenaux et al. 2001). Mitochondrial membrane breakage in vivo could facilitate, for example, $O_2^{\bullet-}$ production since increased mitochondrial cytochrome *c* leakage facilitates electron transfer chain uncoupling (Borutaite et al. 1999; Klamt et al. 2005). Consequently, it could induce increased oxidative stress parameters in mitochondrial membranes.

Other important research groups have demonstrated that short-term vitamin A supplementation attenuated CCl_4-induced liver toxicity through increased CYP4502E1 protein levels (Inder et al. 1999). However, in a previous study, it was demonstrated that vitamin A facilitates acetaminophen-induced hepatotoxicity (Rosengren and Sipes 1995). Indeed, we have recently demonstrated that both short- and long-term vitamin A supplementation have induced a pro-oxidant insult in some rat brain regions and also altered the behavior of the animals, for instance, inducing anxiety-like behavior and decreasing both locomotion in and exploration of an open-field apparatus (De Oliveira and Moreira 2007; De Oliveira et al. 2007b).

Vitamin A supplementation, either therapeutically or inadvertently, is a cause of concern among researchers since this vitamin is promptly delivered to several human tissues through its binding proteins (Napoli 1999). This feature favors the induction of early signs of vitamin A toxicity. However, in some cases, some side effects are very difficult to be diagnosed since it alters, for example, cognition (Myhre et al. 2003). Indeed, some reports have been published showing that vitamin A or some of its derivatives induces depression in humans (Jick et al. 2000; Hull and D'Arcy 2003).

Based on the results presented here and also in previously reported data, we conclude that vitamin A is able to induce a pro-oxidant state in rat liver by, at least in part, inducing an in vivo impairment in the mitochondrial redox state (as assessed through hepatic SMP isolation), which might be responsible for the effects seen when $CaCl_2$ was used as an in vitro challenging agent against mitochondrial integrity. The results shown here indicate that liver mitochondria isolated from vitamin-A-treated rats are more sensitive to a non-oxidant agent when compared to saline-treated rats, i.e., the in vivo vitamin-A-induced oxidative damage favors the in vitro observed mitochondrial redox impairment. Unfortunately, it is almost impossible to indicate the vitamin A metabolite responsible for the observed effects, given the vast number of vitamin A metabolites existing. Addition-

ally, some vitamin A derivatives are very unstable molecules and would exert its effects too fast to be detected due to its high reactivity (Napoli 1999). Also, case reports of vitamin A toxicity have shown serum retinol concentrations within normal limits (Ellis et al. 1986; Croquet et al. 2000; Mills and Tanumihardjo 2006), indicating that serum retinol is not a good measure of vitamin A status during toxicity. Our data could be useful to explain some previously reported studies showing that the liver of rats that received vitamin A supplementation are more sensitive to some chemically stressful conditions. In addition, the herein observed impairment in METC activity may culminate in increased rates of superoxide anion production in the liver of patients treated under high vitamin A doses even acutely. We have observed here increased superoxide anion production levels in the liver of the rats that received vitamin A. Increased rates of superoxide anion production would lead to decreased hepatic function if persistent. Then, the results presented here may be useful to elucidate, at least in part, the mechanism by which vitamin A interferes with tissue homeostasis even during short-term exposure to high levels of this vitamin.

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Evaluation of redox and bioenergetics states in the liver of vitamin A-treated rats

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ABSTRACT

Vitamin A is normally stored in the mammalian liver and is physiologically released depending on the need of the organism for the vitamin. However, there is a compelling evidence showing that even the liver is affected by conditions of high vitamin A intake. Based on these previously reported findings showing negative effects of vitamin A on mammalian tissues, we have investigated the effects of a supplementation with vitamin A at clinical doses (1000–9000 IU/kg day^{-1}) on some rat liver parameters. We have analyzed hepatic redox environment, as well as the activity of the mitochondrial electron transfer chain in vitamin A-treated rats. Additionally, activity of the detoxifying enzyme glutathione S-transferase was checked. Also, caspase-3 and caspase-8 and tumor necrosis factor-α levels were quantified to assess either cell death or inflammation effects of vitamin A on rat liver. We found increased free radical production and, consequently, increased oxidative damage in biomolecules in the liver of vitamin A-treated rats. Interestingly, we found increased mitochondrial electron transfer chain activity, as well as glutathione-S-transferase enzyme activity. Neither caspases activity, nor tumor necrosis factor-α levels change in this experimental model. Our results suggest a pro-oxidant, but not pro-inflammatory effect of vitamin A on rat liver.

1. Introduction

The liver is the main site of vitamin A storage in mammalian organisms (Napoli, 1999; Ross and Zolfaghari, 2004). Its function in regulating vitamin A metabolism is crucial to the physiology of other tissues. On the other hand, vitamin A is equally important to hepatic homeostasis. It was demonstrated, for example, that vitamin A normal levels are required to maintain rat liver mitochondria morphology, which is closely related to the mitochondrial electron transfer chain activity (Seward et al., 1966; Esteronell et al., 2000). Furthermore, it was reported that either vitamin A deficiency or hypervitaminosis A (200,000 IU/kg/day) impairs cell cycle in adult rat liver and lung, the former inducing cell cycle arrest and the later favoring a proliferative state in both liver and lung cells (Borrás et al., 2003). Indeed, in patients submitted to chronic vitamin A treatment, it was observed that hepatotoxicity is sometimes followed by mild cognitive impairments (Myhre et al., 2003). These findings suggest that vitamin A levels modulate a wide range of hepatic parameters that could culminate in toxicity.

The ability to alter the redox environment in *in vitro* and *in vivo* experimental models is also a consequence of increasing vitamin A concentration. It was demonstrated that retinol (vitamin A) induces oxidative DNA damage through increased superoxide anion radical ($O_2^{-\bullet}$) production (Murata and Kawanishi, 2000). We have reported that vitamin A induces lipid peroxidation, protein carbonylation, DNA oxidative damage, and modulates the activity of antioxidants enzymes in cultured Sertoli cells (Dal-Pizzol et al., 2001; Pasquali et al., 2008). Additionally, $O_2^{-\bullet}$ production was found in rat liver mitochondria treated with increasing doses of vitamin A, consequently inducing mitochondrial lipid peroxidation and swelling of the organelle, which resulted in an increase in mitochondrial cytochrome *c* release, a pro-apoptotic factor (Klamt et al., 2005). Recently, it has been shown that retinol induces apoptotic cell death in cultured Sertoli cells through an oxidative stress-related process (Klamt et al., 2008).

Based on previously reported works demonstrating the pro-oxidant effects of vitamin A on rat tissues, we investigated here the effects of a chronic supplementation (28 days) with retinol palmitate, a vitamin A supplement commercially available at drug stores, at pharmacological doses (1000–9000 IU/kg day^{-1}) on the hepatic redox state, mitochondrial electron transfer chain activity, caspases-3 and -8 enzymes activities, and tumor necrosis factor-α levels in adult Wistar rats. The retinol palmitate doses tested here are within a therapeutic range commonly applied in cancer treatment in both children and adult humans (Fenaux et al., 2001; Allen and Haskell, 2002; Myhre et al., 2003; Mactier and Weaver, 2005).

2. Experimental procedures

2.1. Animals

Adult male Wistar rats (290–320 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle

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(7:00–19:00 h), at a temperature-controlled colony room (23 ± 1 °C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80–23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

2.2. Drugs and reagents

Arovit® (retinol palmitate, a water-soluble form of vitamin A) was purchased from Roche, Sao Paulo, SP, Brazil. Caspase-8 activity assay kit was purchased from Biotium, Inc., Hayward, CA, USA. Tumor necrosis factor-α assay kit was obtained from BD Biosciences, San Diego, CA, USA. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A treatment was prepared daily and it occurred by protecting vitamin A from light.

2.3. Treatment

The animals were treated once a day for 28 days. All treatments were carried out at night (*i.e.* when the animals are more active and take a greater amount of food) in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. The animals were treated with vehicle (0.15 M saline; $n = 8$–10 animals), 1000 ($n = 10$), 2500 ($n = 10$), 4500 ($n = 10$), or 9000 IU/kg ($n = 10$) of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0.6 ml. Adequate measures were taken to minimize pain or discomfort.

2.4. Oxidative stress and antioxidant enzyme activities analyses

Before sacrifice, the animals were anesthetized with ketamine plus xylazine (100 mg/kg and 14 mg/kg, respectively). The animals were sacrificed by decapitation at 24 h after the last vitamin A administration. The liver was dissected out in ice immediately after the rat was sacrificed and stored at −80 °C for posterior oxidative stress analyses. The homogenates were centrifuged (700 *g*, 5 min at 4 °C) to remove cellular debris. Supernatants were used to all biochemical assays described herein. All the results were normalized by the protein content using bovine albumin as standard (Lowry et al., 1951).

2.4.1. Thiobarbituric acid reactive species

As an index of lipid peroxidation, we used the formation of thiobarbituric acid reactive species during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described (Draper and Hadley, 1990). The samples were mixed with 0.6 ml of 10% trichloroacetic acid and 0.5 ml of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 min. Thiobarbituric acid reactive species were determined by the absorbance in a spectrophotometer at 532 nm. Results are expressed as thiobarbituric acid reactive species /mg protein.

2.4.2. Measurement of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine as previously described (Levine et al., 1990). Proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in dinitrophenylhidrazine and the absorbance read in a spectrophotometer at 370 nm. Results are expressed as nmol carbonyl/mg protein.

2.4.3. Measurement of protein thiol content

Briefly, an aliquot was diluted in SDS 0.1% and 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid in ethanol was added and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min (Ellman, 1959). Results are expressed as μmol SH/mg protein.

2.4.4. Enzyme-linked immunosorbent assay (ELISA) to 3-nitrotyrosine

Indirect ELISA assay was performed to analyze changes in the content of nitrotyrosine by utilizing a polyclonal antibody to nitrotyrosine (Calbiochem) diluted 1:5000 in phosphate-buffered saline (PBS) pH 7.4 with 5% albumin. Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in PBS with 5% albumin. Plates were then washed four times with wash buffer (PBS with 0.05% Tween-20), and the specific antibodies were added to the plates for 2 h at room temperature. After washing (seven times) with wash buffer, a second incubation with anti-rabbit antibody peroxidase conjugated (diluted 1:10,000) for 1 h at room temperature was carried out. After addition of substrates (hydrogen peroxide and 3, 3′, 5, 5′-tetramethylbenzidine 1:1 v:v), the samples were read at 450 nm in a plate spectrophotometer. Results are expressed as changes in percentage among the groups (compared to control group).

2.4.5. Antioxidant enzyme activities estimations

Superoxide dismutase enzyme activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described (Misra and Fridovich, 1972), and the results are expressed as U/mg protein. Catalase activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm (Aebi, 1984), and the results are expressed as U/mg protein. A ratio between superoxide dismutase and catalase enzyme activities were applied to better understand the effect of vitamin A-supplementation upon these two oxidant-detoxifying enzymes that work in sequence converting superoxide anion to water (Halliwell and Gutteridge, 1999). An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

2.5. Oxidative parameters in submitochondrial particles

To obtain submitochondrial particles, liver was dissected and homogenized in 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl and 1 mM EDTA (pH 7.4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free submitochondrial particles. The submitochondrial particles solution was also washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) to ensure Mn-superoxide dismutase release from mitochondria. To quantify superoxide ($O_2^{-\bullet}$) production, submitochondrial particles was incubated in reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 μM catalase, and 1 mM epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32 °C, as previously described (Poderoso et al., 1996; De Oliveira and Moreira, 2007). As a marker of lipid peroxidation, we measured the formation of thiobarbituric acid reactive species during an acid-heating reaction, as previously described (Draper and Hadley, 1990). The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine as previously described above (Levine et al., 1990). Protein thiol content in liver submitochondrial particles samples was determined as described above. Briefly, an aliquot was diluted in SDS 0.1% and 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid in ethanol was added and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min (Ellman, 1959).

2.6. Total radical-trapping antioxidant parameter

The non-enzymatic antioxidant cellular defenses were estimated by the total radical-trapping antioxidant parameter, which determines

the non-enzymatic antioxidant potential of the sample, as previously described (Wayner et al., 1985). Briefly, the reaction was initiated by injecting luminol and 2,2′-Azobis[2-methylpropionamidine]dihydrochloride–a free radical source that produces peroxyl radical at a constant rate–in glycine buffer (0.1 M, pH 8.6), resulting in a steady luminescent emission. Hepatic samples (30 μg of protein) were mixed in glycine buffer in the reaction vial and the decrease in luminescence monitored in a liquid scintillation counter for 60 min after the addition of the sample homogenates. The area under the curve obtained of the chemiluminescence values were transformed to percentage values and compared against the control values.

2.7. Mitochondrial electron transfer chain activity

To obtain submitochondrial particles from rat liver in order to assess mitochondrial electron transfer chain activity, we have homogenized the tissue in 250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4, and 50 IU/ml heparin buffer. The samples were centrifuged 1000×*g* and the supernatants were colleted. Then, the samples were frozen and thawed three times, and mitochondrial electron transfer chain activity detection was performed as described below.

2.7.1. Complex I-CoQ-III activity

Complex I-CoQ-III activity was determined by following the increase in absorbance due to reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\in = 19.1\ mM^{-1}\ cm^{-1}$). The reaction buffer contained 20 mM potassium phosphate, pH 8.0, 2.0 mM KCN, 10 μM EDTA, 50 μM cytochrome *c*, and 20–45 μg supernatant protein. The reaction started by addition of 25 μM NADH and was monitored at 30 °C for 3 min before the addition of 10 μM rotenone, after the which the activity was monitored for an additional 3 min. Complex I–III activity was the rotenone-sensitive NADH:cytochrome *c* oxidoreductase activity (Shapira et al., 1990).

2.7.2. Complex II and succinate dehydrogenase activities

Complex II (succinate-DCPIP-oxidoreductase) activity was measured by following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol at 600 nm with 700 nm as reference wavelength ($\in = 19.1\ mM^{-1}\ cm^{-1}$). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8.0 μM 2,6-dichloroindophenol was preincubated with 48–80 μg supernatant protein at 30 °C for 20 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction was started by addition of 40 μM 2,6-dichloroindophenol and was monitored for 5 min at 30 °C. Succinate dehydrogenase activity was assessed by adding 1 mM phenazine methasulphate to the reaction mixture. Then, succinate dehydrogenase activity was monitored for 5 min at 30 °C at 600 nm with 700 nm as reference wavelength (Fischer et al., 1985).

2.7.3. Complex II-CoQ-III activity

Complex II-CoQ-III activity was measured by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength ($\in = 21\ mM^{-1}\ cm^{-1}$). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate was preincubated with 50–100 μg supernatant protein at 30 °C for 30 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction started by the addition of 0.6 μg/ml cytochrome *c* and monitored for 5 min at 30 °C (Fischer et al., 1985).

2.7.4. Complex IV activity

Complex IV activity was measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\in = 19.15\ mM^{-1}\ cm^{-1}$). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg supernatant protein and the reaction was started with addition of 0.7 μg reduced cytochrome *c*. The activity of complex IV was measured at 25 °C for 10 min (Rustin et al., 1994).

2.8. Glutathione-S-transferase activity

Glutathione-S-transferase activity was determined spectrophotometrically according to the method of Habig et al., 1974. Glutathione-S-transferase activity was quantified in liver homogenates in a reaction mixture containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 1 mM glutathione as substrates in 0.1 M sodium phosphate buffer, pH 6.5, at 37 °C. Enzyme activity was calculated by the change in the absorbance value from the slope of the initial linear portion of the absorbance time curve at 340 nm for 5 min. Enzyme activity was expressed as nmol of CDNB conjugated with glutathione/min mg^{-1} protein.

2.9. Caspase-3 activity

Caspase-3 activity was determined through a fluorimetric commercial kit according manufacturer's instructions (Sigma). Briefly, the samples were homogenized in lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT), and centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatants were used to determine caspase-3 assay in a microplate fluorimeter at 360 nm excitation and 460 nm emission for 180 min at 25 °C. Results are expressed as nmol AMC produced/min mg^{-1} protein.

2.10. Caspase-8 activity

Caspase-8 activity was determined through a colorimetric commercial kit according manufacturer's instructions (Biotium). The samples were prepared as described to investigate caspase-3 activity. However, caspase-8 activity was monitored in a microplate spectrophotometer at 495 nm for 180 min at 25 °C. Results are expressed as nmol R110 produced/min mg^{-1} protein.

2.11. Tumor necrosis factor-α quantification

We have measured tumor necrosis factor-α through commercial kit for enzyme-linked immunosorbent assay according manufacturer's instructions (BD Biosciences). Briefly, tissue samples were collected and suspended in lysis buffer containing protease inhibitors. Following cell lysis, the homogenate was centrifuged, and a portion of the

Table 1
The effects of vitamin A supplementation on rat liver oxidative stress markers levels.

Groups	Lipid peroxidation (nmol TBARS/mg protein)	Protein carbonylation (nmol/mg protein)	Protein thiol content (μmol sulfhydryl/mg protein)	3-Nitrotyrosine content (% change)
Control	7.33 ± 1.05	1.84 ± 0.18	160.5 ± 26.35	100.7 ± 5.5
1000 IU/kg day^{-1}	7.72 ± 1.83	1.92 ± 0.2	181.3 ± 16.2	114.0 ± 7.81
2500 IU/kg day^{-1}	8.0 ± 1.16	1.79 ± 0.22	166.2 ± 20.8	118.9 ± 20.1
4500 IU/kg day^{-1}	11.0 ± 1.34	1.79 ± 0.18	164.3 ± 22.3	141.0 ± 17.7[a]
9000 IU/kg day^{-1}	12.11 ± 1.79[a]	2.23 ± 0.3	173.7 ± 22.2	145.2 ± 7.8[a]

Values are means ± S.D. of 8–10 animals per group.
[a] $P<0.05$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

Table 2
The effects of vitamin A supplementation on rat liver antioxidant and detoxification enzyme activities.

Groups	Superoxide dismutase (U/mg protein)	Catalase (U/mg protein)	SOD/CAT ration (arbitrary units)	Glutathione-S-transferase (nmol CDNB/min mg protein)
Control	29.9 ± 5.1	39.2 ± 3.11	0.76 ± 0.07	372.15 ± 24.9
1000 IU/kg day^{-1}	36.0 ± 7.51	50.5 ± 6.7	0.72 ± 0.2	440.1 ± 153.9
2500 IU/kg day^{-1}	40.21 ± 9.42	49.0 ± 10.1	0.85 ± 0.3	485.3 ± 189.1
4500 IU/kg day^{-1}	55.8 ± 12.2[a]	54.07 ± 10.1	1.02 ± 0.03	553.2 ± 98.2[a]
9000 IU/kg day^{-1}	58.2 ± 4.9[a]	55.9 ± 2.94	1.04 ± 0.14	575.4 ± 28.4[a]

Values are means ± S.D. of 8–10 animals per group.
[a] $P<0.05$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

supernatant was reserved for protein concentration measurement, and the remaining was stored at −80 °C for posterior tumor necrosis factor-α levels quantification. The samples were read in a microplate spectrophotometer at 450 nm.

2.12. Statistical analyses

Data are expressed as means ± standard deviation (S.D.); p values were considered significant when $p<0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test whenever necessary.

3. Results

3.1. Oxidative stress markers and antioxidant enzyme activities analyses

3.1.1. Oxidative/nitrosative damage markers

In this experimental model, only vitamin A supplementation at 9000 IU/kg day^{-1} induced an increase in lipid peroxidation levels in rat liver ($p<0.05$; Table 1). Protein carbonylation levels, as well as protein thiol content, did not change in the liver of vitamin A-treated rats (Table 1). However, we observed increased 3-nitrotyrosine content in the liver of the rats that received vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Table 1).

3.1.2. Antioxidant and detoxifying enzyme activities

Vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} induced a 1.7–1.9-fold increase of rat liver superoxide dismutase enzyme activity ($p<0.05$; Table 2). Catalase enzyme activity did not change in this experimental model (Table 2). Consequently, the hepatic superoxide dismutase/catalase ratio did not change in vitamin A-treated rats (Table 2). Glutathione-S-transferase activity was found increased in the liver of the rats that received vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Table 2).

3.1.3. Superoxide anion radical ($O_2^{-\bullet}$) production and oxidative damage in submitochondrial particles

As depicted in Fig. 2A, vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} induced a 1.8–2-fold increase of $O_2^{-\bullet}$ production in hepatic submitochondrial particles ($p<0.05$; Table 3). Lipid peroxidation levels were found increased in submitochondrial particles isolated of the rats that received vitamin A supplementation at 9000 IU/kg day^{-1} ($p<0.05$; Table 3). Protein carbonylation levels and protein thiol content did not change in the liver of vitamin A-treated rats (Table 3).

3.1.4. Non-enzymatic antioxidant capacity

Chemiluminescence emitted from samples at 1 min of experiment (total antioxidant reactivity–TAR) did not change in the hepatic homogenates from the rats that received vitamin A supplementation (Fig. 1A). Also, TroloxÔ equivalents, an antioxidant parameter, did not change in this experimental model (Fig. 1B). Consequently, area under the curve, which represents a measure of the pro-oxidant capacity of the sample tested, was found unaltered in the liver of the rats that received vitamin A supplementation (Fig. 1C).

3.2. Mitochondrial electron transfer chain activity

Vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} induced a 1.5–1.7-fold increase of complex I–III activity in rat liver ($p<0.05$; Fig. 2A). Complex II–III activity was found increased only in the liver of the rats that were administrated with vitamin A at 9000 IU/kg day^{-1} ($p<0.05$; Fig. 2B). Similarly, complex II activity, as well as succinate dehydrogenase enzyme activity, was found increased only in the liver of the rats that received vitamin A supplementation at 9000 IU/kg day^{-1} ($p<0.05$; Fig. 2C and D). Complex IV activity was found increased in the liver of the rats that were exposed to vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Fig. 2E).

3.3. Caspase-3 and caspase-8 activities and tumor necrosis factor-α levels

Vitamin A supplementation did change neither caspase-3 activity, nor caspase-8 activity in rat liver. Similarly, tumor necrosis factor-α levels did not vary in this experimental model (Table 4).

4. Discussion

In the herein presented work, we have shown that vitamin A supplementation at clinical doses commonly used in the treatment of life-threatening maladies induces an increase in lipid peroxidation, but not protein carbonylation, in rat liver. In addition, we found increased $O_2^{-\bullet}$ production in hepatic submitochondrial particles of vitamin A-treated animals. Increased $O_2^{-\bullet}$ production may be the result of an uncoupled or increased electron flux during oxidative phosphorylation, as previously reviewed (Halliwell, 2006). Indeed, we have found

Table 3
The effects of vitamin A supplementation on oxidative damage levels and superoxide anion radical ($O_2^{-\bullet}$) production in rat liver submitochondrial membranes.

Groups	Lipid peroxidation (nmol TBARS/mg protein)	Protein carbonylation (nmol/mg protein)	Protein thiol content (nmol sulfhydryl/mg protein)	$O_2^{-\bullet}$ production (nmol/min mg protein^{-1})
Control	4.0 ± 0.33	1.24 ± 0.2	111.0 ± 18.27	4.1 ± 0.4
1000 IU/kg day^{-1}	5.47 ± 0.6	1.46 ± 0.37	112.0 ± 11.85	6.1 ± 1.31
2500 IU/kg day^{-1}	5.66 ± 1.42	1.35 ± 0.22	100.7 ± 9.55	7.52 ± 1.21
4500 IU/kg day^{-1}	6.26 ± 1.3	1.28 ± 0.4	107.7 ± 15.3	8.38 ± 2.1[a]
9000 IU/kg day^{-1}	7.7 ± 1.08[a]	1.31 ± 0.62	109.6 ± 12.4	8.45 ± 1.7[a]

Values are means ± S.D. of 8–10 animals per group.
[a] $P<0.05$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

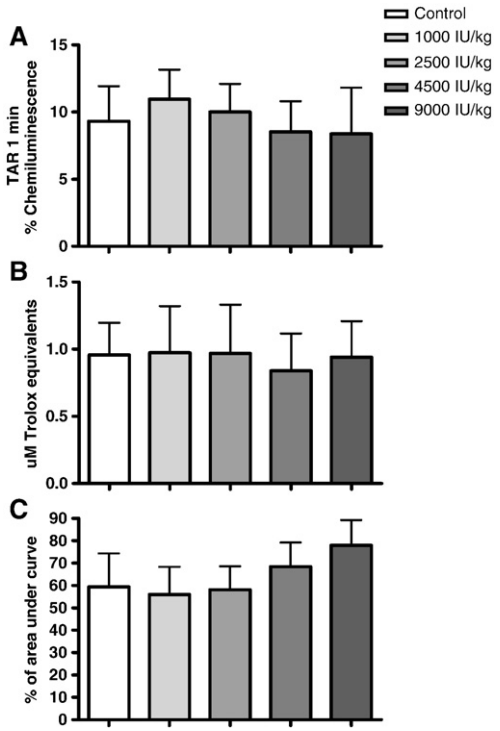

Fig. 1. Effects of vitamin A supplementation on hepatic non-enzymatic antioxidant defenses. The total antioxidant reactivity (TAR) is shown in (A). Trolox™ equivalents and area under the curve are shown in (B) and (C), respectively. Data are mean ± S.D. of 8–10 animals per group performed in triplicate.

increased mitochondrial electron transfer chain activity in the liver of the rats that received vitamin A supplementation for 28 days. Additionally, we have found increased lipid peroxidation levels in mitochondrial membranes. However, even in the presence of an ongoing lipid peroxidation process in hepatic submitochondrial particles, we did not observe any change in caspase-3 or caspase-8 activity. Also, the levels of a pro-inflammatory cytokine, tumor necrosis factor-α, was demonstrated to be unaltered in the present experimental model.

We have recently reported that vitamin A induces a several-fold increase of oxidative stress markers in adult rat hippocampus, striatum, *substantia nigra*, cerebral cortex, and cerebellum and altered rat behavior (De Oliveira and Moreira, 2007; De Oliveira et al., 2007a, b; De Oliveira et al., 2008a, b). Nevertheless, vitamin A did not induce similar effects on rat liver, which may be explained, at least in part, by the fact that liver is the main site of vitamin A storage and metabolism in the mammalian organism, as elegantly reviewed by Napoli (Napoli, 1999). Nevertheless, in some situations, vitamin A aggravates the manifestation of hepatotoxicity in rat, as demonstrated in a CCl_4-induced model of liver disease (Elsisi et al., 1993a, b; Badger et al., 1996). In addition, the differences observed in the studies mentioned above and the present one may be due to increased susceptibility of central nervous system to oxidative insult, since there is a high content of polyunsaturated fatty acids (PUFA) in brain, which are more sensitive to oxidative modifications than other lipid molecules (Halliwell and Chirico, 1993). Furthermore, central nervous system has a decreased activity of antioxidant enzymes activities than liver, which may favor the onset and maintenance of a pro-oxidative state (Halliwell, 2006).

Interestingly, we found increased 3-nitrotyrosine content, but not protein carbonylation in this experimental model regarding oxidative state of proteins (Table 1). Increased 3-nitrotyrosine content indicates increased peroxynitrite ($ONOO^-$) production, reactive specie originated from nitric oxide ($NO^\bullet$) and $O_2^{-\bullet}$ (Halliwell, 2006). Also, we found increased superoxide dismutase enzyme activity, but unaltered catalase enzyme activity in the present experimental model. However, it did not result in an imbalance in the superoxide dismutase/catalase ratio (Table 2). This is in agreement with the results showing unaltered lipid peroxidation (with the exception of the 9000 IU/kg

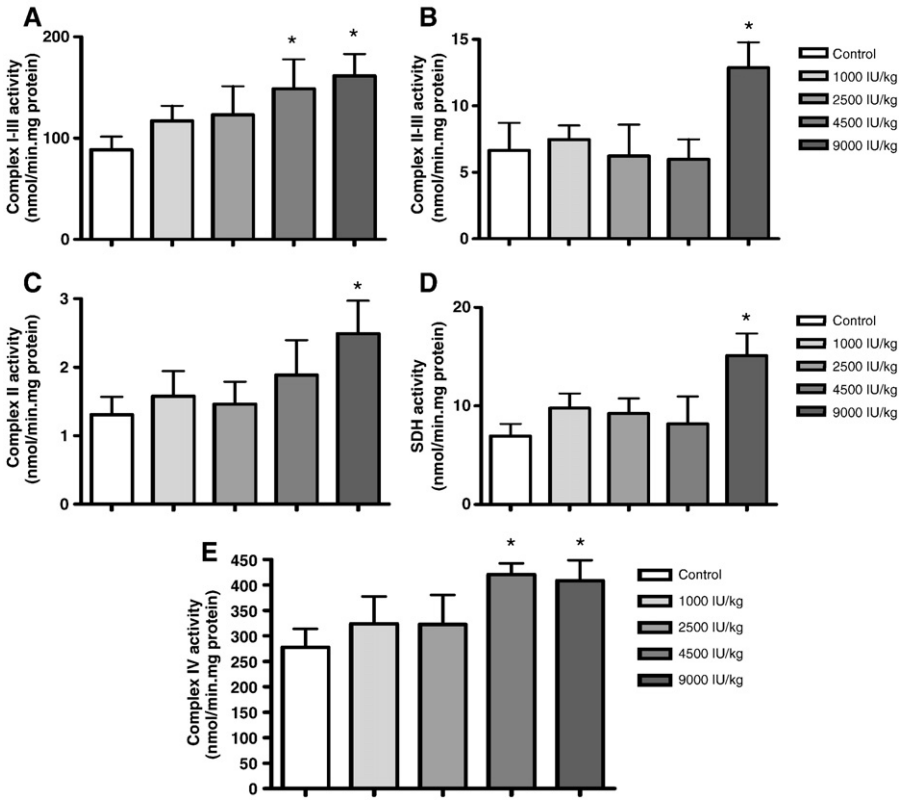

Fig. 2. Effects of vitamin A supplementation on complex I–III (A), complex II–III (B), complex II (C), succinate dehydrogenase (SDH) (D), and Complex IV (E) enzyme activities in the rat liver. Data are mean ± S.D. of 8–10 animals per group performed in triplicate. $^*P<0.05$ (one-way ANOVA followed by Tukey's test).

Table 4
The effects of vitamin A supplementation on caspase-3 and caspase-8 enzyme activities and TNF-α levels.

Groups	Caspase-3 (nmol AMC/min mg protein^{-1})	Caspase-8 (nmol/min mg protein^{-1})	TNF-α levels (pg/mg protein)
Control	0.61 ± 0.13	33.9 ± 9.3	3.3 ± 0.96
1000 IU/kg day^{-1}	0.46 ± 0.08	43.45 ± 20.8	3.93 ± 1.1
2500 IU/kg day^{-1}	1.08 ± 0.64	45.9 ± 1.1	3.8 ± 1.3
4500 IU/kg day^{-1}	0.99 ± 0.33	39.11 ± 7.4	4.2 ± 0.96
9000 IU/kg day^{-1}	0.92 ± 0.3	44.4 ± 7.6	3.9 ± 1.4

Values are means ± S.D. of 8–10 animals per group.

vitamin A dose), protein carbonylation, and protein thiol content, since an imbalance in the superoxide dismutase/catalase ratio is very likely to favor an increase in H_2O_2 production, a reactive oxygen specie that may give rise to more powerful oxidizing agents, as the hydroxyl ($^{\bullet}OH$) radical (Halliwell and Gutteridge, 1999). In regard to non-enzymatic antioxidant defenses, we did not find any change that would suggest that vitamin A modulates such parameter in rat liver (Fig. 1A–C).

The increased mitochondrial electron transfer chain activity observed in this work may represent a response of hepatic cells to the stressful condition imposed by high vitamin A intake, as postulated (Manoli et al., 2007). Indeed, we have seen that the same vitamin A doses that induced oxidative damage in liver samples also increased mitochondrial electron transfer chain activity (Fig. 2A–E). On the other hand, increased mitochondrial electron transfer chain activity may give rise to increased $O_2^{-\bullet}$ production due to electron leakage from the electron transfer chain (Halliwell and Gutteridge, 1999; Halliwell, 2006). Accordingly, we have found increased $O_2^{-\bullet}$ production in submitochondrial particles isolated from vitamin A-treated rats (Table 3).

In a recently published work, our group has demonstrated that retinol (vitamin A) induces apoptosis in cultured Sertoli cells through an oxidative stress-associated process (Klamt et al., 2008). Then, we have investigated here the effect of vitamin A supplementation on caspases activity in the liver of rats. Surprisingly, we did find any change neither in caspase-3 nor caspase-8 activity. Additionally, we did not observe any alteration in the levels of tumor necrosis factor-α cytokine (Table 4), suggesting that, in this experimental model, vitamin A supplementation at therapeutic doses for 28 days did not induce a pro-inflammatory state in rat liver.

Our results indicate a stressful event that is induced through vitamin A supplementation at clinical levels on rat liver. This becomes clearer to be seen when we analyze the activity of both mitochondrial electron transfer chain and glutathione-S-transferase enzyme. As mentioned above, it was previously reviewed that diverse cell types, under stressful conditions, modulate mitochondrial electron transfer chain activity and number of mitochondria in an attempt to produce more ATP as a response to the unfavorable environment (Manoli et al., 2007). Indeed, we have found increased both mitochondrial electron transfer chain and glutathione-S-transferase activities (Fig. 2 and Table 2, respectively).

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