UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Efeitos agudos e crônicos de suplementação com diferentes doses de vitamina A sobre parâmetros de estresse oxidativo e comportamentais em ratos

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Como requisito para obtenção do grau de Mestre em Bioquímica.

Porto Alegre, 2008

Este trabalho foi realizado no Centro de Estudos em Estresse Oxidativo, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, sendo financiado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e pela Pró-Reitoria de Pesquisa desta Universidade (PROPESQ/UFRGS).

Dedicatória

Dedico este trabalho a minha mãe, que sempre me inspirou com sua vontade de trabalhar, e pelo modo honesto e ético pelo qual veio guiando nossas vidas. Te agradeço eternamente pelo esforço dedicado para eu poder estudar. Ninguém nunca tirará de nós o brilho de nossos olhos devido a nossas conquistas. Este trabalho, e outros que virão, serão uma pequena homenagem a ti, Maria Irma de Oliveira.

AGRADECIMENTOS

Este trabalho é fruto de muita perseverança, pois seu começo não foi tão rentável como o aqui demonstrado. Havíamos começado a idéia de tratar animais com vitamina A junto de um co-tratamento com uma droga que induziria hemi-parkinsonismo nos animais. No entanto, muitos destes animais morriam logo após a cirurgia, ou alguns poucos dias depois. No começo, não entendíamos o porquê. Após algumas análises utilizando apenas vitamina A como tratamento, passamos a entender o porquê. Provavelmente, esta vitamina, em doses elevadas, mas mesmo assim utilizadas no tratamento de diversas patologias, sensibilize o sistema nervoso central dos animais, tornando seu óbito uma conseqüência provável. Mudamos de projeto, então, seguindo por uma proposta minha a meu orientador, o qual sou muito grato pela confiança em mim depositada.

Gostaria de agradecer:

A minha mãe, pela força com que demonstra amar a vida, pelo amor por seu trabalho, e pela determinação que demonstra em cada dia de sua vida. Não é à toa que teus olhos brilham tanto. És um ser de força inestimável que, felizmente, esteve sempre ao meu lado, me ensinando a ler a vida, me guiando, se dedicando a mim, exclusivamente, para que eu pudesse estudar e trabalhar, seguindo um sonho meu e teu. Espero continuar dando motivos para teus olhos brilharem por muitos anos. Nosso esforço conjunto já sacudiu muitas montanhas. Obrigado por seres quem és. Obrigado por ser minha amiga. Obrigado por ter-me dado toda a liberdade que deste, para que eu pudesse escolher o melhor para mim, sem influenciar, diretamente, nas minhas escolhas. Isto foi, sem dúvidas, fundamental ao nosso sucesso.

Agradeço a minha madrinha, Clarissa, pela confiança, pelo carinho, pela amizade. São anos de sinceridade um com o outro. Por muitos anos permanecerá assim.

Agradeço ao meu orientador, o Professor José Cláudio, pela confiança em mim depositada. Sempre escutando minhas propostas, as respeitando, e as melhorando. Dando todo o suporte necessário para o desenvolvimento de tais tarefas. Sempre lembrarei de tua confiança, Professor. Certamente, é difícil achar alguém assim neste nosso meio. Confiar pode custar caro, mas tu és, realmente, um profissional da pesquisa, área onde curiosidade e bom-senso, se andarem juntos, resultarão sempre em inúmeros resultados e publicações. Mais uma vez, obrigado pela confiança e, também, pela liberdade de raciocínio que dá aos teus alunos.

Agradeço a minha namorada, Fernanda Jardim, pela compreensão, pela amizade, pelo carinho e cumplicidade. É nosso começo, e deveremos lutar muito para que se mantenha bom como está.

Agradeço a meus amigos, principalmente Chico, Xuri, Thiago, pela parceria cotidiana. Às vezes, se torna impossível um simples conversa, pois as tarefas são muitas e os prazos estão sempre nos mostrando que a pressa é necessária. Agradeço pela compreensão. Estaremos sempre unidos.

Agradeço aos funcionários do nosso Departamento de Bioquímica (segunda casa para muitos, senão a primeira!!): Cléa, Simone, Cláudia, Bebel, Sérgio, seu Waldemar, dona Lia, Marcelo, Glênio, seu Odi, e todos que possam ter me ajudado direta ou indiretamente. Vocês formam uma excelente equipe.

Agradeço aos colegas de laboratório pelas horas agradáveis que passamos juntos no ambiente de trabalho, o que torna muito melhor nosso dia-a-dia: Rodrigo Lorenzi,

Guilherme, Matheus, Marinho, Max, Michael, Mari Hoff, Mari Escobar, Ricardo, Chaca, Juliane, Fernanda Bonatto, Márcio, Mauro, Virgínia, Ricardo Pinho, Fábio!!

Ao professor Tadeu Mello e Souza e suas alunas Roberta e Cris pela ajuda na análise dos comportamentos dos animais, e por tantas cirurgias em estereotáxico: que trabalheira!!!!

Agradeço aos professores do Departamento, em geral, devido ao esforço conjunto para todos melhorarem, ainda mais, esta nossa Casa. Alunos professores, cada um com seu papel, fazendo do Departamento de Bioquímica um lugar cada vez melhor para trabalhar, para descobrir, para crescer. Obrigado por ajudar nossos sonhos, dos alunos, a se tornar realidade.

“In union we stand.”

Overkill

Parte I

RESUMO

A vitamina e seus derivados, os retinóides, participam de processos celulares responsáveis pela manutenção do sistema nervoso central. Estas moléculas induzem, por exemplo, tanto diferenciação quanto morte neuronais, cujas conseqüências são o surgimento de regiões com funções específicas naquele tecido e uma morfologia característica. No entanto, o excesso de vitamina A, ou de retinóides, na dieta, ou devido a uso terapêutico, pode ser teratogênico, por exemplo. Ainda, outros tecidos podem ser afetados pelo excesso de tais moléculas, incluindo o fígado. Não só a teratogênese induzida por vitamina A é importante. Transtornos cognitivos são comuns em usuários de vitamina A/retinóides em altas doses, dentre eles irritabilidade, ansiedade e depressão. Além disso, estudos *in vitro* têm demonstrado um papel redox ativo para a vitamina A, ou seja, dependendo da concentração, ela será antioxidante ou pró-oxidante. Os objetivos deste trabalho foram investigar possíveis alterações no ambiente redox de diferentes regiões cerebrais de ratos adultos, além de investigar modificações comportamentais induzidas pelo tratamento com vitamina A. Neste trabalho, foram utilizados ratos Wistar machos adultos – 90 dias, que foram tratados por 3, 7 ou 28 dias com vitamina A na forma de palmitato de retinol nas doses de 1000, 2500, 4500 ou 9000 UI/kg/dia via intra-gástrica (gavagem). Nas estruturas cerebrais substância negra, estriado e hipocampo, verificamos aumento nos níveis de marcadores de estresse oxidativo (carbonilação de proteínas, peroxidação lipídica e diminuição no conteúdo de tióis reduzidos) e modulação da atividade de enzimas antioxidantes em todos os períodos analisados. Cronicamente, observamos que vitamina A em qualquer dose, mesmo naquelas usualmente consideradas terapêuticas, foi capaz de induzir comportamento tipo-ansiedade nos animais, além de diminuir sua capacidade de locomoção e de exploração. Então, a partir destes resultados e de outros dados já reportados, recomendamos cautela mesmo no uso terapêutico de vitamina A, já que alterações cognitivas são, muitas vezes, silenciosas, tornando seu diagnóstico complicado.

ABSTRACT

Vitamin A and its derivatives, the retinoids, participate in cellular processes that are responsible for the maintenance of the central nervous system. These molecules induce, for example, either neuronal cell differentiation or death, consequently inducing the arisen of cerebral regions with specific function and a characteristic morphology. However, excessive vitamin A, or its retinóides, in the diet, or even due to therapeutic use, may be, for instance, theratogenic. In addition, other tissues may be affected by the excess of such molecules, including the liver. Not only vitamin A-induced theratogenesis is important. Cognitive impairments are common among vitamin A-treated patients, for instance irritability, anxiety, and depression. Furthermore, *in vitro* investigations have demonstrating a redox active role to vitamin A, *i.e.* depending on its concentration, it could be anti- or pro-oxidant. In this work, we aimed to investigate alterations in the redox environment of some rat brain regions, and also to investigate behavioral changes that could be induced by vitamin A supplementation. Here, we have utilized adult male Wistar rats (90-day old) that were treated for 3, 7, or 28 days with vitamin A as retinol palmitate at 1000, 2500, 4500, or 9000 IU/kg/day intra-gastrically (gavage). In the cerebral regions substantia nigra, striatum, and hippocampus, we have verified an increased level of oxidative stress markers (protein carbonylation, lipid peroxidation, and decreased protein and non-protein thiol content) and antioxidant enzymes activities modulation after any period. Chronically, we have observed that vitamin A at any dose, even the therapeutic ones, was able to induce anxiety-like behavior in the animals, in addition to decrease its locomotion in and exploration of the light-dark box and open field. Then, regarding the results obtained in this work and from other reported data, we recommend more caution even in the therapeutic use of vitamin A, since the cognitive alterations may be, frequently, hard to be diagnosed.

LISTA DE ABREVIATURAS

CAT – catalase

Cu^{+}/Zn^{+}-SOD – superóxido dismutase cobre/zinco

DNA – ácido desoxirribonucléico

ERO – espécie reativa do oxigênio

GPx – glutationa peroxidase

GR – glutationa redutase

GSH – glutationa reduzida

H_2O_2 – peróxido de hidrogênio

Mn-SOD – superóxido dismutase manganês

$NO^{\bullet}$ – óxido nítrico

$O_2^{\bullet-}$ – ânion superóxido

$OH^{\bullet}$ – radical hidroxil

PCLAR – proteína celular ligante de ácido retinóico

PCLR – proteína cellular ligante de retinol

PLR – proteína ligante de retinol

RAR – receptor de ácido retinóico

RXR – receptor de retinóides

SNC – sistema nervoso central

SOD – superóxido dismutase

TBARS – espécies reativas ao ácido tiobarbitúrico

TTR – transtiretina

1. INTRODUÇÃO

1.1 Metabolismo da vitamina A

A vitamina A foi uma das primeiras vitaminas a ser descoberta. Em 1909, Stepp observou que a presença de um composto abundante na gema do ovo era essencial à vida. Este composto era parte da fração “A” lipossolúvel de suas extrações, e recebeu o nome de vitamina A (Stepp, 1909).

Junto de seus derivados, os retinóides, a vitamina A é fundamental à manutenção de diversos processos biológicos, tais como desenvolvimento de tecidos durante a embriogênese, visão, diferenciação epitelial, crescimento, metabolismo ósseo, reprodução e regulação do sistema imune, entre outros (Napoli, 1999). As principais fontes de retinóides naturais, na dieta, são gorduras animais, óleo de fígado de peixes (contendo ésteres de retinil) e vegetais amarelos e verdes (que contêm carotenóides, os precursores dos retinóides obtidos a partir de vegetais) (Bellovino, 2003).

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 través 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, inclusive, pode ser excretada da célula.

Devido a sua natureza hidrofóbica, estes compostos são transportados, tanto no plasma sangüíneo, quanto no meio intracelular, ligados a proteínas específicas. Além de tornar possível o tráfego dos retinóides pelos diversos tecidos do organismo, estas proteínas servem como reguladoras dos níveis de retinóides livres, agindo como verdadeiros tampões para aquelas moléculas. As proteínas ligantes de retinol (PLR), as proteínas celulares ligantes de retinol (PCLR), e as proteínas celulares ligantes de ácido retinóico (PCLAR) são as principais, e serão descritas em detalhe no decorrer da dissertação. Além destas, existe a transtiretina (TTR), presente em plasma, que se destina à proteção do complexo PLR-retinol perante degradação, ou excreção renal (Napoli, 1999).

Em 1987, descobriu-se que os retinóides exercem suas funções através da sua ligação a receptores nucleares específicos (receptores de retinóides- RXR- e receptores de ácido retinóico- RAR) que, por sua vez, agem como fatores de transcrição, regulando, portanto, a expressão gênica de seqüências de DNA alvo àquelas moléculas sinalizadoras. Por esta razão, estes compostos têm sido definidos, por muitos autores, como hormônios.

Além da forma tradicionalmente aceita de sinalização mediada por vitamina A, têm se demonstrado que a vitamina pode atuar, em nível celular, de maneira independente do núcleo, por meio de uma ação não genômica. Um exemplo disto é a indução inchamento mitocondrial disparada por retinol, com conseqüente liberação de citocromo c da organela,

um importante agente pró-apoptótico (Klamt et al., 2005). Além disso, nosso grupo demonstrou que a via de sinalização celular ERK ½-CREB pode ser ativada por retinol em concentrações pouco acima do fisiológico no modelo experimental utilizado (Gelain et al., 2006). Recentemente, encontramos que uma suplementação com vitamina A em doses terapêuticas pode induzir aumento de cerca de 100% na produção do ânion superóxido em partículas submitocondriais isoladas de córtex cerebral e de cerebelo, além de induzir aumento no dano em membranas mitocondriais (De Oliveira e Moreira, 2007), o que pode contribuir em diminuir a capacidade bioenergética naquelas estruturas, além de pode influenciar no comportamento animal. Todas estas ações da vitamina A parecem independer de sua ligação a receptores nucleares, mas o exato mecanismo de ação ainda merece ser investigado.

1.2 Radicais livres e estresse oxidativo

Um radical livre é uma espécie química com um, ou mais, elétrons desemparelhados no seu último orbital. As espécies químicas podem ser átomos, como hidrogênio ou cloreto; metais de transição; ou uma molécula onde o elétron desemparelhado esteja localizado no orbital externo. Este elétron desemparelhado confere uma reatividade relativamente alta a esta molécula**,** devido a uma grande tendência de esta adquirir um segundo elétron para este orbital (Halliwell, 2006).

Radicais livres são escritos quimicamente com uma notação para a espécie química seguida de um ponto, o qual indica o elétron desemparelhado, por exemplo, o radical livre ânion superóxido: $O_2^{\bullet -}$.

Quimicamente, radicais livres são caracterizados por sustentarem reações em cadeia, que se autopropaga, onde uma molécula reduzida perde seu elétron para o radical livre, e aquela reduzida se torna, agora, um radical livre, reagindo com outro composto químico, e assim por diante.

Classicamente, as reações de radicais livres são divididas em: a) reações de iniciação; b) reações de propagação; e, c) reações de terminação. Nas reações de iniciação, um radical livre é formado a partir de espécies químicas não-radicais (e portanto estáveis) : AB + C → $A^{\bullet}$+ D + E. Nas reações de propagação, um radical livre, também chamado centro de reação, reage com uma molécula estável, resultando em outro radical livre, ou centro de reação: $A^{\bullet}$+ CD → AC + $D^{\bullet}$. Nas reações de terminação, dois radicais livres cancelam seus elétrons desemparelhados formando um produto estável.

A reatividade química dos radicais livres é determinada pela molécula que carrega este elétron desemparelhado; conseqüentemente, a reatividade varia muito entre um radical e outro. Um modo de expressar e comparar a reatividade química destas moléculas é especificar a meia-vida ($t_{½}$) das mesmas. Uma meia-vida curta indica alta reatividade, e o radical hidroxil é o mais reativo dos radicais livres (sendo, então, o mais instável, ou seja, é aquele que reage mais rapidamente assim que formado).

O radical livre de ocorrência mais comum é o ânion superóxido ($O_2^{\bullet -}$), que é produzido quando uma molécula de oxigênio é reduzida parcialmente, ou seja, quando recebe apenas um elétron, ao invés de receber dois elétrons, sendo, então, formada água desta redução completa. Quantidades excessivas de $O_2^{\bullet -}$ levam a dano tecidual por induzir a produção de hidroxil derivado da reação com peróxido de hidrogênio (H_2O_2) com metais de transição Fe^{+2} ou Cu^{+2}- reação de Fenton. Além disso, ao reagir com óxido nítrico ($NO^{\bullet}$),

$O_2^{\bullet -}$ forma peroxinitrito ($ONOO^-$) que, por sua vez, pode gerar o radical nitrosil (ONOOH) que, ao se decompor, também forma o radical hidroxil.

Os radicais livres podem causar dano oxidativo aos componentes celulares como lipídios, carboidratos, proteínas e DNA devido a sua alta reatividade e natureza oxidante. No entanto, as células contam com defesas contra tais efeitos danosos daquelas moléculas. São as defesas antioxidantes, que podem ser tanto enzimáticas quanto não- enzimáticas. Entre as defesas enzimáticas estão as enzimas superóxido dismutase, catalase e glutationa peroxidase. O tripeptídio glutationa (na forma reduzida- GSH) e as vitaminas (originadas da dieta, como o ácido ascórbico, e a vitamina E, por exemplo) representam defesas antioxidantes não- enzimáticas.

As enzimas citadas acima são tidas como defesas antioxidantes primárias, ou seja, agem diretamente sobre a molécula do radical livre, antes que este possa vir a oxidar uma biomolécula. A enzima superóxido dismutase (SOD) apresenta quatro classes: Mn-SOD (localizada na matriz mitocondrial), Cu, Zn-SOD (citosólica), Ni-SOD e SOD extracelular. Todas estas formas de SOD agem sobre o radical $O_2^{\bullet -}$, transformando-o em peróxido de hidrogênio (H_2O_2) e oxigênio através da seguinte reação:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Já a enzima catalase (CAT), age sobre o peróxido de hidrogênio gerado na reação acima, transformando-o em água por meio da reação:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

A enzima glutationa peroxidase (GPx) também atua sobre o H_2O_2 , no entanto, por meio de um mecanismo diferente. A GPx participa de um ciclo redox junto da enzima glutationa redutase, onde GSH é usada, pela GPx, para transformar H_2O_2 em água; e

NADPH é utilizado pela glutationa redutase para reduzir a glutationa oxidada, na primeira reação, em GSH novamente (Boveris, 1998).

Estresse oxidativo é o termo utilizado em uma situação onde a formação de radicais livres excede a capacidade de transformação destas moléculas em outras não oxidantes pode meio das defesas antioxidantes. Neste caso, podemos dizer que ocorreu um desequilíbrio entre a formação de radicais livres e a atuação da defesa antioxidante. E isto pode ocorrer por diversos motivos, inclusive, inativação de enzimas como SOD e CAT por meio de reações destas com as próprias moléculas oxidantes, onde as enzimas perdem sua característica nativa e, conseqüentemente, sua função. Um exemplo é a inativação de catalase por $O_2^{\bullet -}$ (Shimizu *et al.*, 1984).

Dentro da célula, pode-se encontrar fontes de formação de radicais livres, tais como a cadeia transportadora de elétrons mitocondrial, onde a redução parcial do oxigênio dará origem ao $O_2^{\bullet -}$. Existem dois locais da cadeia transportadora de elétrons de onde elétrons podem vazar, formando o $O_2^{\bullet -}$. O primeiro é a partir da NADH desidrogenase (Complexo I) e o outro a partir do Complexo III.

Embora a mitocôndria seja o local mais importante de produção de radicais livres endógenos, existem outros locais onde estas moléculas oxidantes podem ser formadas. No citosol, por exemplo, a cascata do ácido araquidônico, que produz prostaglandinas e leucotrienos, pode formar ERO quando o lipídio metabolizado é liberado. Ainda, algumas isoenzimas citocromo P-450 também estão descritas como produtoras de ERO (Halliwell, 1984).

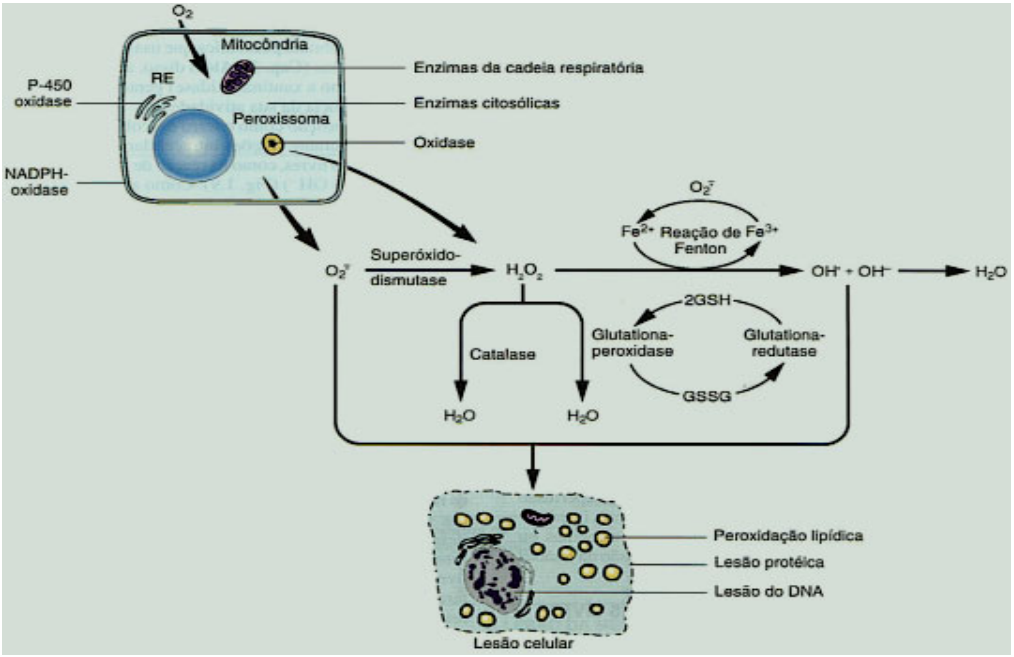

Figura 1. Esquema de formação e neutralização dos radicais livres endógenos (fonte: INTERNET).

1.3 Vitamina A e sistema nervoso central

A vitamina A participa do desenvolvimento de praticamente todos os tecidos dos mamíferos (Napoli, 1999), e durante a vida adulta, continua a exercer importantes papéis na manutenção da homeostasia de diferentes tecidos, incluindo o sistema nervoso central (SNC) (Lane e Bailey, 2005). De fato, o aparato enzimático, incluindo receptores nucleares desta vitamina, é encontrado, em diferentes níveis, em todas as regiões cerebrais de mamíferos adultos (Zetterström *et al.*, 1999; Mey e McCaffery, 2004). No SNC, a vitamina A age induzindo diferenciação celular e apoptose, este último mais importante durante o desenvolvimento.

Mesmo sendo considerada fundamental ao SNC, a vitamina A pode exercer papéis indesejáveis quando atinge certos níveis neste tecido. Recentemente, Myhre e

colaboradores (Myhre *et al.*, 2003) mostraram que a forma hidrossolúvel da vitamina A tem maior potencial tóxico que aquelas lipossolúveis. Dentre os efeitos relacionados ao SNC humano, foram descritos inchaço cerebral (*pseudotumor cerebri*), tontura, irritabilidade, ansiedade e depressão. Estes efeitos são observados em indivíduos que, terapeuticamente ou inadvertidamente, ingerem grandes quantidades de vitamina A por períodos variáveis, desde dias até meses.

O retinóide mais conhecido por apresentar sérios efeitos colaterais em relação à cognição é o ácido retinóico 13-*cis*, o qual é usado para o tratamento crônico (6 meses) anti-acne. Esta droga pode induzir depressão severa, aumentando, inclusive, os índices de suicídio entre os usuários deste tratamento (Hull e D'Arcy, 2003). O tratamento com palmitato de retinol pode induzir aumento nos níveis de ácido retinóico 13-*cis*, no entanto, não é sabido se estes níveis são suficientes para induzir os distúrbios cognitivos observados quando este ácido é aplicado de forma concentrada. O ácido retinóico 13-*cis* é capaz de induzir parada no ciclo celular de uma região hipocampal que mantém a divisão celular mesmo na fase adulta do mamífero, o giro denteado, com conseqüente diminuição na capacidade de aprendizado em camundongos (Crandall *et al.*, 2004). Além disso, esta droga pode induzir depressão após 28 dias de tratamento, conforme mostrado por O'Reilly e colaboradores (O'Reilly *et al.*, 2006).

Embora o ácido retinóico 13-*cis* seja importante em um tipo de tratamento, o palmitato de retinol é mais usado terapeuticamente, incluindo tratamentos de distúrbios de visão e dermatológicos e deficiência de vitamina A. Além disso, no tratamento de leucemias e de bebês prematuros, as doses de vitamina A aplicadas são bastante elevadas (Fenaux *et al.*, 2001; Mactier e Weaver, 2005). No entanto, pouco se sabe sobre os efeitos

da suplementação com palmitato de retinol em relação ao SNC no que diz respeito ao ambiente redox deste e em tarefas comportamentais.

1.4 Comportamento tipo-ansiedade

Este tipo de comportamento, cuja principal característica é a fuga de prováveis riscos ao animal, é considerado evolutivamente favorável à manutenção da vida, já que evitaria exposições arriscadas. No entanto, o excesso de ansiedade causa transtornos tanto a animais quanto a humanos, pois deixar de arriscar-se acarreta diminuição na coleta de alimento para animais, por exemplo, e problemas sociais e financeiros para humanos. Neste trabalho, investigamos se a suplementação com vitamina A em diferentes doses era capaz de induzir tal comportamento em ratos adultos. Em 2003, Myhre e colaboradores (Myhre *et al.*, 2003) mostraram, através de uma meta-análise, que vitamina A na forma hidrossolúvel poderia induzir ansiedade e depressão em humanos adultos. No entanto, apenas este trabalho estudou efeitos de vitamina A hidrossolúvel no comportamento, ou seja, faltam dados, na literatura, a respeito de efeitos desta droga nestes (e em outros) parâmetros.

A caixa claro-escuro é uma ferramenta muito útil na investigação de comportamento tipo-ansiedade em animais, já que é composta de dois compartimentos: um claro e um escuro, sendo o escuro menor que o claro. O compartimento claro, por receber mais iluminação e ser maior, representa um lugar arriscado para o animal, pois ele estaria se expondo mais neste local. Já o compartimento escuro, com menos área e recebendo menos luz, se torna atraente ao animal, pois parece ser mais seguro. Então, a permanência do animal em um ou outro destes compartimentos nos sugere possíveis alterações comportamentais relacionadas à ansiedade.

1.5 Locomoção e exploração em campo aberto

No aparato de campo aberto, podemos investigar uma série de comportamentos animais, já que este ambiente não conta com desafios ao rato, como choque ou outro animal. O campo aberto nos permite uma abordagem ampla e minuciosa de como o animal explora e se locomove nesta área. No campo aberto, semelhante ao que ocorre na caixa escura, existe um lugar de maior exposição do animal, que é o seu centro. As laterais do campo aberto são formadas por paredes, que se tornam uma proteção lateral ao animal que explora o campo aberto. Assim, se locomovendo pelas laterais, o rato tem um de seus lados protegidos; ao explorar o centro, nenhum dos lados é protegido, e isto é utilizado para investigar alterações cognitivas relacionadas a medo, ansiedade e depressão. Variações do campo aberto refinam ainda mais a investigação.

1.6 Objetivos do trabalho

Alguns dados da literatura, incluindo trabalhos do nosso grupo, mostram que a vitamina A, e alguns de seus derivados, são moléculas redox-ativas, ou seja, dependendo de algumas condições, podem reduzir ou oxidar outras biomoléculas. Dentre os efeitos observados, temos aumento na produção mitocondrial de radical superóxido, aumento nos níveis de marcadores de peroxidação lipídica e de carbonilação de proteínas, e modulação na atividade de enzimas antioxidantes, tais como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). Estes dados mostram que, em diferentes modelos experimentais, o tratamento com retinol (vitamina A) pode induzir pulsos de estresse

oxidativo, sendo que um pulso transiente na formação de espécies oxidantes é uma conseqüência deste tratamento *in vitro*.

Então, a partir dos resultados previamente publicados, hipotetizamos que uma suplementação com vitamina A pudesse induzir estresse oxidativo também *in vivo*. Objetivando analisar os efeitos desta suplementação sobre o ambiente redox *in vivo*, decidimos realizar tratamentos de curta e de moderada/longa exposição com vitamina A (na forma de palmitato de retinol - Arovit®) em doses terapêuticas e excessivas (de acordo com a literatura) em ratos Wistar machos adultos.

1.6.1 Objetivos específicos

Assim, analisamos os efeitos da suplementação diária com vitamina A via intragástrica nas doses de 1000, 2500, 4500 ou 9000 UI/kg/dia por 3, 7 ou 28 dias sobre:

1) Peroxidação lipídica, carbonilação de proteínas, estado redox de grupamentos sulfidril protéicos e não-protéicos nas estruturas cerebrais substância negra, estriado e hipocampo;
2) Modulação da defesas antioxidantes enzimáticas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) nas estruturas mencionadas acima;
3) Modificações comportamentais em caixa claro-escuro e em campo aberto, onde o comportamento tipo-ansiedade é observado no primeiro aparato e os comportamentos de exploração e locomoção livres são observados no segundo.

Parte II

2. MATERIAIS E MÉTODOS E RESULTADOS

Nesta parte do trabalho, apresentarei os resultados obtidos a partir de nossa pesquisa na forma de artigos científicos. Ao todo, foram publicados quatro artigos dentro do período de Mestrado, sendo que três dos quais estão anexados a esta Dissertação e serão apresentados a seguir. Em todos os trabalhos, o modelo experimental foi o mesmo: suplementação diária com vitamina A hidrossolúvel (palmitato de retinol) em doses terapêuticas (1000 e 2500 UI/kg/dia) e excessivas (4500 e 9000 UI/kg/dia) por dois períodos curtos (3 e 7 dias) e um longo (28 dias). O tratamento era sempre aplicado à noite, pois a vitamina A tem sua absorção aumentada quando administrada junto das refeições, e como os ratos são animais de hábitos noturnos, se alimentam principalmente à noite. Antes de sacrificar os animais e retirar as estruturas cerebrais substância negra, estriado e hipocampo, realizávamos observações em tarefas comportamentais (caixa claro-escuro e campo aberto) a fim de analisar alterações cognitivas e/ou motoras e sensoriais nestes animais. Embora não sejam testes comportamentais extremamente refinados, ou seja, não conseguem exatamente afirmar o que está alterado no animal após o tratamento, servem como bons indicadores de que rumo se tomar na investigação, caso ela prossiga. Os animais utilizados em um teste comportamental nunca eram utilizados para o outro teste, já que possíveis adaptações do animal a exposições freqüentes podem mascarar os efeitos do tratamento. Após as observações comportamentais, os animais eram sacrificados e as estruturas cerebrais retiradas cirurgicamente. Estas estruturas eram homogeneizadas em tampão fosfato, pH 7,4, e mantidas em congelamento de –80ºC até o dia das análises, que nunca pode exceder uma semana. Investigamos parâmetros de estresse oxidativo, como marcadores de dano oxidativo em biomoléculas (peroxidação lipídica, carbonilação de

proteínas e estado redox de grupamentos tióis) e atividade de enzimas antioxidantes (SOD, CAT, GPx).

Capítulo I

“Therapeutic vitamin A doses increase the levels of markers of oxidative insult in *substantia nigra* and decrease locomotory and exploratory activity in rats after acute and chronic supplementation”

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Artigo aceito para publicação no periódico Neurochemical Research (doi: 10.1007/s11064-007-9438-2

Neurochem Res
DOI 10.1007/s11064-007-9438-2

ORIGINAL PAPER

Therapeutic Vitamin A Doses Increase the Levels of Markers of Oxidative Insult in *Substantia Nigra* and Decrease Locomotory and Exploratory Activity in Rats after Acute and Chronic Supplementation

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Accepted: 9 July 2007

Abstract Vitamin A is known to regulate some central nervous system (CNS)-associated functions. Vitamin A at high doses has been demonstrated to be beneficial in the treatment of some diseases, for instance acute promyelocytic leukemia. However, vitamin A and its naturally occurring metabolites (retinoids) are known to alter neuronal function, inducing behavioral disorders. Here we provide an evidence to indicate that vitamin A supplementation, at both therapeutic and excessive doses, induces oxidative stress in the rat *substantia nigra.* Vitamin A supplementation induced lipid peroxidation, protein carbonylation, and oxidation of protein thiol groups, as well as change in catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activity. Surprisingly, locomotory and exploratory activity of rats were decreased after acute and chronic vitamin A supplementation. Therefore, we may conclude from our results that vitamin A supplementation is prooxidant to the rat *substantia nigra* and effective in altering behavior.

Keywords Vitamin A · Oxidative stress · Rat *substantia nigra* · Behavior · Lipid peroxidation · Protein carbonylation

Introduction

Vitamin A (also referred as retinol) is essential to both developing and adult central nervous system (CNS), immune system, epithelial proliferation and vision [1–3], among others. Vitamin A and retinoids modulate dopamine pathways and participate in locomotory behavior [4] and to activate D2 dopamine receptor promoter [5]. Additionally, retinoids regulate the dopamine-dependent signal transduction in dopaminergic cells, suggesting an important role of vitamin A upon these cells [6].

However, an increasing body of evidence suggests that vitamin A may induce neurotoxic effects in humans. Excessive vitamin A intake either acutely or chronically has been suggested to induce intra-cranial hypertension, headache, and irritability in adult humans [7, 8]. In addition, even the intake of low vitamin A doses during pregnancy has been associated to congenital malformations in the CNS [9]. The treatment with retinoids at therapeutic doses also has been demonstrated to induce cognitive disturbances, for instance depression, in mice [10], and humans [11].

Oxidative stress is a condition in which reactive molecules such as superoxide ($O_2^{\bullet-}$), peroxynitrite ($ONOO^-$), hydroxyl ($OH^{\bullet}$), and other radicals are produced in excess. It is a chemical alteration of the cellular environment imposed by exogenous chemicals (e.g., agrochemicals or pollutants), or resulted from an intrinsic alteration, such as that in antioxidant defenses [12]. Undoubtedly, reactive oxygen species (ROS) and reactive nitrogen species (RNS) participate in so many neurodegenerative diseases including, Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis [12, 13].

Vitamin A may be either anti [14] or prooxidant (see below), depending on dosage and cellular condition. Our group has demonstrated the prooxidant effects of retinol treatment upon cultured Sertoli cells, such as induction of lipid peroxidation and protein carbonylation, and alteration in the activity of antioxidant enzymes [15–19]. Furthermore, we found increased levels of oxidative stress in rat

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liver mitochondria exposed to retinol [20]. Interestingly, retinol induced activation of the Src/MEK/MAPK/CREB pathway through an oxidative-dependent manner [21].

Substantia nigra is particularly vulnerable to oxidative stress given the presence of some intrinsic prooxidant factors. Dopamine degradation, for instance, is a source of hydrogen peroxide (H_2O_2) via monoamine oxidase (MAO) action [22]. Moreover, *substantia nigra* presents a high iron and copper content, which facilitate the formation of hydroxyl ($OH^{\bullet}$) radical after reacting with H_2O_2 [12].

Oral vitamin A is therapeutically used at high doses to treat acute myeloid leukemia [see 23 for review]. In addition, the excessive intake of vitamin A as supplemented foods is a current concern regarding the consequences that may result from this procedure [24]. Then, based on previously reports indicating a prooxidant role of vitamin A in different experimental models, we decided to investigate the effects of acute and chronic vitamin A supplementation at either therapeutic (1,000 or 2,500 IU/kg) or excessive (4,500 or 9,000 IU/kg) doses on the redox status of rat *substantia nigra*, and on locomotory and exploratory activity of rats in an open field.

Experimental procedure

Animals

Adult male Wistar rats (*Rattus norvegicus*) (90 days old; 300 ± 20 g body weight) 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 7:00 a.m.), at a temperature-controlled colony room (23 ± 1°C). All behavioral testing was conducted during the light phase. 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.

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.

Drug administration

The animals were treated once a day during three different periods: 3, 7, or 28 days (i.e., acute and chronic vitamin A-supplementation effects were analyzed). All treatments were carried out at night (i.e., when animals usually 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 gavaged with vehicle (0.15 M NaCl), 1,000, 2,500, 4,500, or 9,000 IU/kg of retinol palmitate, orally, in a maximum volume of 0.8 mL during each period of interest. 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 *substantia nigra* was dissected out immediately after the rat was sacrificed and stored at –0°C for posterior analyses. *Substantia nigra* was 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* for 5 min to remove cellular debris. Supernatants were used to all the biochemical assays described herein. Results were normalized by the protein content using bovine serum albumin as standard [25].

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 measurement of lipid redox state, as previously described [26]. 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 nmol TBARS/mg protein.

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 [27]. 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.

Measurement of protein and non-protein thiol content

Other form to analyze oxidative alterations in proteins is to measure the level of protein thiol content. Briefly, sample 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 [28]. Free sulfhydryl

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(–SH) content was estimated in supernatants of 20% TCA precipitated homogenates by the same method. Results are expressed as μmol SH/mg protein.

Antioxidant enzyme activity estimations

Catalase (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm [29]. The results of CAT activity are expressed as U CAT/mg protein. Superoxide dismutase (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described [30]. The results of SOD are expressed as U SOD/mg protein. Glutathione peroxidase (GPx) activity was determined by measuring the rate of NAD(P)H oxidation in a spectrophotometer at 340 nm, as previously described [31]. The results of GPx are expressed as mM NADPH consumed/min/mg protein. A ratio between SOD and CAT activity (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. An imbalance between their activity is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminates in oxidative stress.

Behavioral task

The behavioral task occurred 15 h after the last treatment, and was performed between 14:00 and 16:00 hours (i.e., during the light phase). Briefly, the animals were gently placed in an open field and left free to explore it for 5 min in order to assess their locomotory and exploratory activity. The open field task was carried out in 60 × 40 cm^2 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 number of crossings of the black lines and rearings was counted. In behavioral tasks, rats were used only once.

Statistical analysis

Biochemical and behavioral results are expressed as mean ± SD and *p*-values were considered significant when $p < 0.05$. Differences in each experimental group were determined by the one-way ANOVA. Comparison between means was carried out using the post hoc Tukey's test.

Results

Vitamin A supplementation at 1,000 IU/kg increased lipid peroxidation in the *substantia nigra* of the rats that were treated for 3 days (Fig. 1a). However, we found a 3.6- to 4.0-fold increase in lipid peroxidation levels in the *substantia nigra* of the rats that were treated with vitamin A at any dose for 7 days. This effect was maintained in the rats that received vitamin A for 28 days (Fig. 1a). A 2.0- to 3.0-fold increase was found in the protein carbonylation levels in the *substantia nigra* of the rats that received vitamin A acutely or chronically (Fig. 1b). Vitamin A supplementation at any dose decreased (30–60%) the protein thiol content after all periods of exposition studied here (Fig. 1c). Vitamin A supplementation at 9,000 IU/kg for 3 days induced a decrease (42%) in the nigral non-protein thiol content (Fig. 1d). After 7 days of supplementation, 4,500 and 9,000 IU/kg vitamin A was able to decrease the nigral non-protein thiol content. After 28 days of supplementation, any dose tested decreased the non-protein thiol content in the rat *substantia nigra* (Fig. 1d).

SOD activity did not change in the *substantia nigra* of the rats that were treated with vitamin A for 3 days (Fig. 2a). However, vitamin A at 2,500, 4,500, and 9,000 IU/kg for 7 days induced a decrease (40%) in the

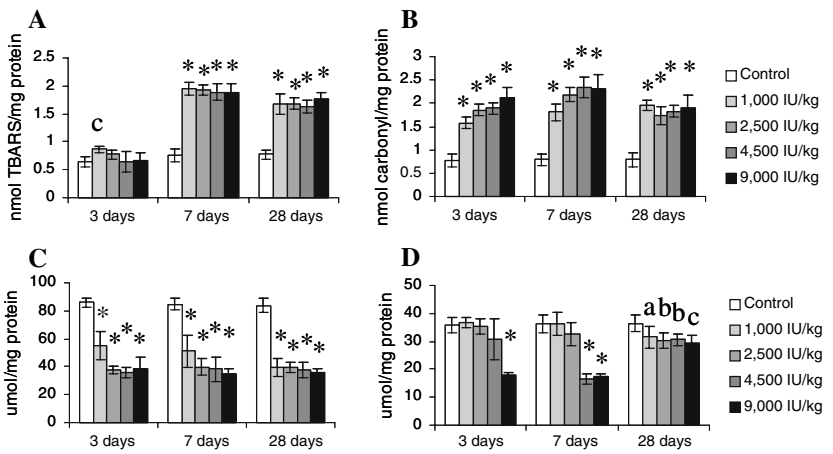

Fig. 1 Effects of acute and chronic vitamin A supplementation on lipid peroxidation (**A**), protein carbonylation (**B**), protein thiol content (**C**), and non-protein thiol content (**D**) the in adult rat *substantia nigra*. Data are mean ± SD of 7–12 animals per group performed in triplicate. Different from the respective control group $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.002$; $^{*}p < 0.0001$, as determined by one-way ANOVA followed by Tukey's test

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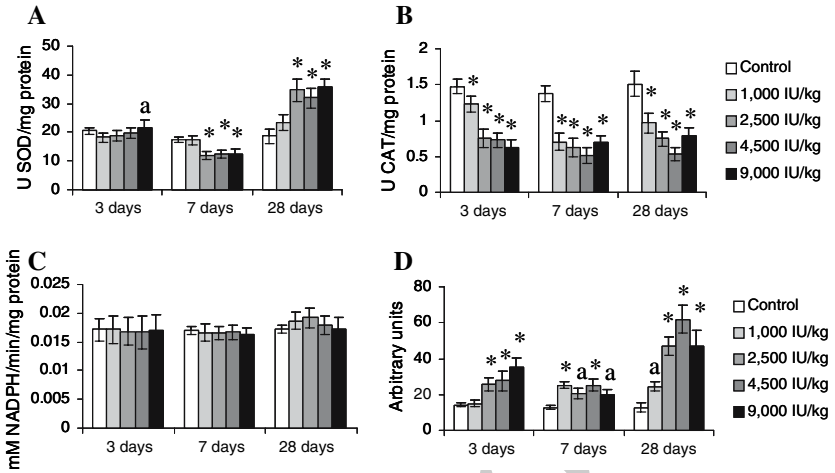

Fig. 2 Effects of acute and chronic vitamin A supplementation on superoxide dismutase (**A**), catalase (**B**), and glutathione peroxidase (**C**) activity. The SOD/CAT ratio is shown in **D**. Data are mean ± SD of 7–12 animals per group performed in duplicate or triplicate. Different from the respective control group, $^{a}p < 0.05$; $^{*}p < 0.0001$, as determined by one-way ANOVA followed by Tukey's test

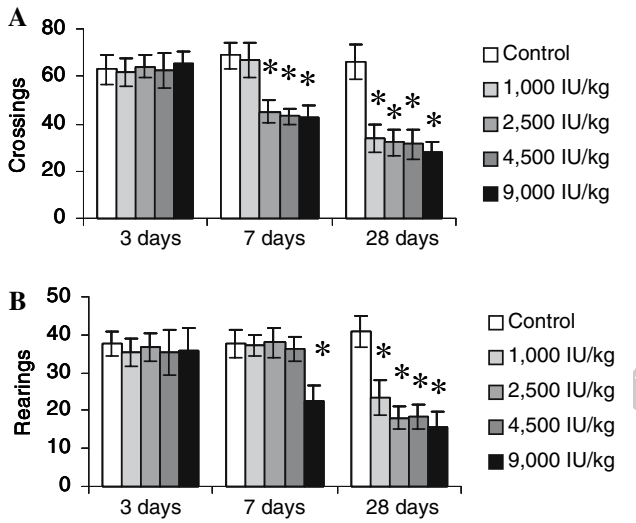

Fig. 3 Effects of acute and chronic vitamin A supplementation on locomotory (**A**) and exploratory (**B**) activity of rats in open field task. Data are mean ± SD of nine animals per group. Different from the respective control group, $^{*}p < 0.0001$, as determined by one-way ANOVA followed by Tukey's test

nigral SOD activity (Fig. 2a). Chronically, vitamin A supplementation at 2,500, 4,500, and 9,000 IU/kg increased SOD activity (Fig. 2a). Acute and chronic vitamin A supplementation decreased (1.2- to 2.8-fold) CAT activity (Fig. 2b). GPx activity (Fig. 2c) did not change after vitamin A supplementation. Finally, the SOD/CAT ratio was increased (1.3- to 6.0-fold) after acute or chronic vitamin A supplementation (Fig. 2d).

Locomotory and exploratory activity did not change in the rats that received vitamin A for 3 days (Fig. 3a). Surprisingly, supplementation with vitamin A at 2,500, 4,500, or 9,000 IU/kg for 7 days induced a decrease (1.3- to 1.5-fold) in locomotory activity (Fig. 3a). After 28 days of treatment, vitamin A at any dose tested induced a decrease (1.7- to 2.3-fold) in locomotion (Fig. 3a). Vitamin A supplementation at 9,000 IU/kg for 7 days decreased (1.7-fold) the number of rearings (Fig. 3b). Vitamin A at any dose for 28 days induced a decrease (1.9- to 2.4-fold) in the number of rearings performed by the rats in the open field (Fig. 3b).

Discussion

Our results show, for the first time, that vitamin A at both therapeutic and excessive doses alter the nigral redox environment after both acute and chronic supplementation. The brain as a whole is sensitive to oxidative stress due to its high content of peroxidizable fatty acids and relative decreased antioxidant defenses [12]. However, substantia nigra is particularly vulnerable to an oxidative insult due to: (1) its high content of iron and copper ions; (2) increased H_2O_2 production via MAO function during the degradation of dopamine (DA); and (3) DA is a source to DA-quinones, which react with thiol groups oxidizing it [32–35].

Here, we found that the level of lipid peroxidation (TBARS levels) increased after either acute (7 days) or chronic (28 days) vitamin A supplementation (Fig. 1a). In addition, an exacerbated level of protein carbonylation was also detected in this experimental model (Fig. 1b). Different from lipid peroxidation, protein carbonylation, and decreased protein thiol content (Fig. 1b, c, respectively) occurred earlier, indicating an increased vulnerability of nigral proteins to the oxidative insult induced in this experimental model. Increased TBARS and protein carbonylation levels, and decreased protein thiol content facilitate intra- and inter-molecular cross-links of proteins [36], which in turn induce conformational changes in proteins leading to increased hydrophobicity and aggregation of the protein is very likely to occur. Furthermore, these oxidative alterations on proteins favor the formation

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of protein aggregates, inducing generalized cellular dysfunction [37].

In situations of increased oxidative stress in *substantia nigra*, other than prooxidant molecules attack may also decreases its protein thiol content. It was demonstrated that DA-quinones covalently bind to thiol groups in proteins, oxidizing it [33, 34]. Consequently, this oxidation decreases the measurable content of protein thiol content (Fig. 1c). Previous reports suggested that lipid peroxidation participates in the non-specific DA oxidation, since DA is storage within acidic vesicles to avoid auto-oxidation, and loss of membrane integrity facilitates DA release to cytosol [33].

Non-protein thiol content, mainly represented by the reduced-form of glutathione (GSH), was decreased by vitamin A at some doses after 3 or 7 days of treatment (Fig. 1d). This indicates that: (1) there is a decrease in the reduced form of glutathione given the prooxidant circumstances imposed by vitamin A supplementation or (2) a possible action of a detoxifying system, such as glutathione-S-transferase (which uses GSH to conjugate to xenobiotics, eliminating them from the cell), upon vitamin A or its metabolites in *substantia nigra* and are conjugated with GSH to be exported from neuronal cells when in excess [38]. Non-protein thiol content was decreased by vitamin A at any dose after 28 days of supplementation, suggesting that long periods of exposition to vitamin A decrease an important non-enzymatic antioxidant defense.

In this work, we also show that vitamin A supplementation induced an imbalance in the ratio between SOD activity and CAT activity (SOD/CAT ratio) without any change in GPx activity (Fig. 1a–d). Increased SOD/CAT ratio suggests that there is an increased H_2O_2 production, since SOD metabolizes $O_2^{-\bullet}$ to H_2O_2, but CAT converts H_2O_2 to water at lower rates. In addition, increased H_2O_2 availability is favored since GPx activity did not change after vitamin A supplementation. This H_2O_2 excess may reacts with iron and/or copper, which are found in high contents in substantia nigra, and produces $OH^{\bullet}$ via Fenton reaction [12]. Actually, we found increased levels of oxidative damage markers in this experimental model (Fig. 1a–d), as mentioned above.

A behavioral analysis revealed that the animals that received vitamin A supplementation for 7 days walked and explored less a new place (the open field). Chronically, there is a drastic decreased in both locomotion and exploration of open field (Fig. 3a, b). These results indicate that vitamin A even at therapeutic doses (even considered high these doses are used therapeutically, as mentioned in the "Introduction" section) is effective in inducing a behavioral disturbance in adult rats. There are data reporting deleterious effects of excessive vitamin A use regarding behavior in humans [8, 39]. However, further studies would be useful to elucidate whether oxidative stress is a causative factor in the behavioral disturbances observed here.

We did not performed an analysis to investigate retinoids levels in plasma or *substantia nigra* because it is almost impossible to point to the retinoid responsible for the effects herein demonstrated, since there is a vast number of metabolites derived from vitamin A [40]. Moreover, case reports of vitamin A toxicity have shown serum retinol concentrations within normal limits [41–43], suggesting that serum retinol could not be a good parameter to analyze vitamin A toxicity.

In summary, we show that vitamin A, at therapeutic doses, is able to impairs the redox homeostasis of *substantia nigra* and to induce a disturbance in a nigral-related behavior. In some parameters, chronic vitamin A was deleterious to *substantia nigra*, showing no adaptation of the neuronal structure to the insult. Then, we suggest that vitamin A utilization at high doses, even therapeutically, must be rethought, mainly when administered to children or elderly, since some cognitive disturbances, which might be induced by vitamin A, are very difficult to be diagnosed in these stages of life.

Acknowledgments This work was supported by grants of CNPq and PROPESQ-UFRGS. M. R. de Oliveira and R. B. Silvestrin are recipients of a CNPq fellowship. T. Mello e Souza was supported by PROFIX (CNPq, Brazil).

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Journal : 11064 | Dispatch : 20-7-2007 | Pages : 6
Article No. : 9438 | ☐ LE | ☐ TYPESET
MS Code : NERE675 | ☑ CP | ☑ DISK

Capítulo II

“Vitamin A supplementation induces a prooxidative state in the striatum and impairs locomotory and exploratory activity of adult rats”

Marcos Roberto de Oliveira, Matheus Augusto de Bittencourt Pasquali, Roberta Bristot Silvestrin, Tadeu Mello e Souza, José Cláudio Fonseca Moreira

Artigo publicado no periódico Brain Research, volume 1169, p. 112-119 (2007).

BRAIN RESEARCH 1169 (2007) 112–119

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BRAIN RESEARCH

Research Report

Vitamin A supplementation induces a prooxidative state in the striatum and impairs locomotory and exploratory activity of adult rats

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ARTICLE INFO

Article history:
Accepted 5 July 2007
Available online 14 July 2007

Keywords:
Vitamin A
Striatum
Oxidative stress
Crossings
Rearings
Superoxide dismutase
Catalase

ABSTRACT

Although vitamin A has been reported to be essential to brain homeostasis, some central nervous system (CNS)-associated deleterious effects may be induced by vitamin A or by its metabolites. In this work, we investigated the effects of acute and chronic vitamin A supplementation at therapeutic (1000 or 2500 IU/kg/day) or excessive (4500 or 9000 IU/kg/day) doses on the redox state of the rat striatum. We found a 1.8- to 2.7-fold increase of lipid peroxidation in the striatum after acute or chronic supplementation (TBARS method). Therapeutic doses induced a 1.6- to 2.2-fold increase of protein carbonylation (dinitrophenylhydrazine (DNPH) derivatization). Vitamin A supplementation induced a 1.2- to 1.4-fold decrease of protein thiol content acutely and chronically. Superoxide dismutase (SOD) activity, assessed through the inhibition of epinephrine's autoxidation, was increased in a dose-dependent manner chronically. Acutely, both therapeutic and excessive vitamin A doses induced a 1.8- to 2.2-fold decrease of catalase (CAT) activity, as determined through the rate of decrease of hydrogen peroxide (H_2O_2). Glutathione peroxidase (GPx) activity did not change in this experimental model. Some vitamin A doses decreased the non-protein thiol content only chronically. Vitamin A supplementation decreased the striatal non-enzymatic antioxidant defenses (TRAP assay). Furthermore, our results show that vitamin A supplementation impaired the SOD/CAT ratio. Moreover, we observed a 1.6- to 2.0-fold decrease of locomotion and exploration in an open field after vitamin A supplementation. Therefore, our results suggest that vitamin A supplementation induces oxidative stress in the rat striatum and that it may be related to a metabolic impairment in such brain area.

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Abbreviations: CAT, catalase; CNS, central nervous system; GPx, glutathione peroxidase; GSH, glutathione reduced-form; H_2O_2, hydrogen peroxide; $O_2^{\bullet-}$, superoxide anion; PD, Parkinson's disease; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive species

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doi:10.1016/j.brainres.2007.07.008

1. Introduction

A vast amount of information has been gathered on the effect of vitamin A on central nervous system (CNS) function from cell culture to *in vivo* studies (Lane and Bailey, 2005). Undoubtedly, vitamin A exerts an essential role in both development and maintenance of the adult vertebrate brain homeostasis (De Luca, 1991; Mey and McCaferry, 2004), but its toxicity is undeniable (Cohlan, 1953; Adams, 1993; Holson et al., 1997).

Some studies demonstrate that vitamin A is essential to maintain striatal function (McCaffery and Dräger, 1994; Samad et al., 1997; Krezel et al., 1998; Wang and Liu, 2005). However, little is known about the redox effects of vitamin A upon the adult rat striatum. We demonstrated that vitamin A induces alterations in the redox environment of cultured Sertoli cells, a physiological target of retinol. Our results showed that lipid peroxidation, protein carbonylation, and modifications in the activities of antioxidant enzymes occur after exposition of cultured Sertoli cells to vitamin A (Moreira et al., 1997; Dal-Pizzol et al., 2000; Klamt et al., 2000; Dal-Pizzol et al., 2001; Frota et al., 2004). In isolated rat liver mitochondria, retinol induced swelling of the organelle with concomitant cytochrome *c* release from mitochondria (Klamt et al., 2005), a pro-apoptotic step (Hengartner, 2000). This retinol-dependent oxidant signal may participate also in the induction of cellular signaling pathways like Src/MEK/MAPK/CREB (Gelain et al., 2006). Murata and Kawanishi (2000) showed that vitamin A directly induces overproduction of superoxide anion ($O_2^{-\bullet}$) *in vitro*, resulting in DNA damage.

It was previously demonstrated that vitamin A easily crosses the blood–brain barrier (MacDonald et al., 1990), and that striatum metabolizes vitamin A (Valdenaire et al., 1998; Zetterström et al., 1999). Then we decided to investigate here whether vitamin A at either therapeutic (1000 and 2500 IU/kg) or excessive (4500 and 9000 IU/kg) doses is also able to exert its already known prooxidant effects in the adult rat striatum. In addition, locomotory and exploratory activities were also analyzed.

2. Results

2.1. Lipid peroxidation

Lipid peroxidation did not change in the striatum of the rats that received vitamin A supplementation at 1000 IU/kg/day for 3 days ($n=12$; $p=1.0$). Lipid peroxidation increased from 1.15±0.07 nmol TBARS/mg protein (control group; $n=12$) to 2.06±0.05 ($n=12$; $p<0.0001$), 2.74±0.14 ($n=12$; $p<0.0001$), or 2.87±0.13 ($n=12$; $p<0.0001$) nmol TBARS/mg protein in the striatum of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/kg/day, respectively, for 3 days (Fig. 1). Lipid peroxidation did not change in the striatum of the rats that received vitamin A supplementation at 1000 IU/kg/day for 7 days ($n=12$; $p=1.0$). Lipid peroxidation was increased from 1.25±0.07 nmol TBARS/mg protein (control group; $n=12$) to 2.07±0.05 ($n=12$, $p<0.0001$), 2.14±0.08 ($n=12$, $p<0.0001$), or 2.34±0.09 ($n=12$; $p<0.0001$) nmol TBARS/mg protein in the striatum of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/kg/day, respectively, for 7 days (Fig. 1). Chronically, we found that lipid peroxidation was increased from 1.19±0.08 nmol TBARS/mg protein (control group; $n=9$) to 1.97±0.05 ($n=12$, $p<0.0001$), 1.84±0.07 ($n=12$, $p<0.0001$), 2.12±0.12 ($n=12$, $p<0.0001$), or 1.84±0.07 ($n=12$, $p<0.0001$) nmol TBARS/mg protein, respectively, in the striatum of the rats that received vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day, respectively, for 28 days (Fig. 1).

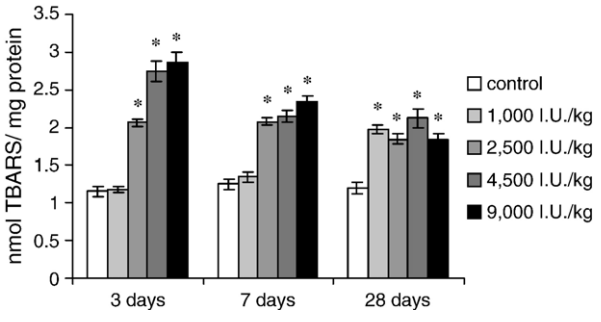

Fig. 1 – Effects of acute and chronic vitamin A supplementation on lipid peroxidation in rat striatum. Data are mean±S.E.M. of 9–12 animals per group performed in duplicate. *$p<0.0001$ (one-way ANOVA followed by Tukey's test).

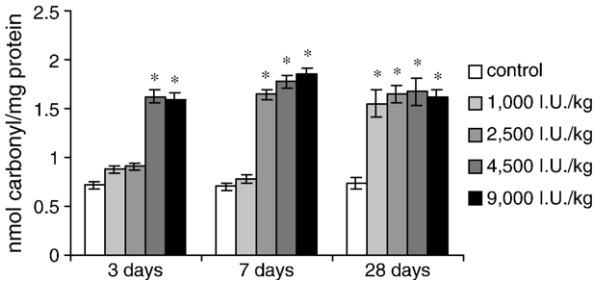

Fig. 2 – Effects of acute and chronic vitamin A supplementation on protein carbonylation in rat striatum. Data are mean±S.E.M. of 9–12 animals per group performed in duplicate. *$p<0.0001$ (one-way ANOVA followed by Tukey's test).

2.2. Protein carbonylation

Protein carbonylation did not change in the striatum of the rats that received vitamin A supplementation at 1000 or 2500 IU/kg/day for 3 days ($n=12$, $p=0.85$; $n=12$, $p=0.8$, respectively). However, we found that protein carbonylation increased from 0.71±0.04 nmol carbonyl/mg protein (control group; $n=12$) to 1.62±0.07 ($n=12$; $p<0.0001$), or 1.58±0.07 ($n=12$; $p<0.0001$) nmol carbonyl/mg protein in the striatum of the rats that received vitamin A supplementation at 4500 or 9000 IU/kg/day, respectively, for 3 days (Fig. 2). Protein carbonylation did not change in the striatum of the rats that were treated with vitamin A at 1000 IU/kg/day for 7 days ($n=12$; $p=0.98$). However, protein carbonylation increased from 0.69±0.03 nmol carbonyl/mg protein (control group; $n=12$) to 1.64±0.06 ($n=12$; $p<0.0001$), 1.77±0.06 ($n=12$; $p<0.0001$), or 1.85±0.06 ($n=12$; $p<0.0001$) nmol carbonyl/mg protein in the striatum of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/kg/day, respectively, for 7 days (Fig. 2). We found that protein carbonylation increased from 0.74±0.06 nmol carbonyl/mg protein (control group; $n=9$) to 1.54±0.14 ($n=12$; $p<0.0001$), 1.64±0.08 ($n=12$; $p<0.0001$), 1.67±

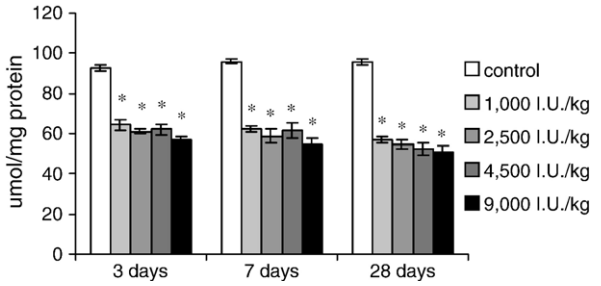

Fig. 3 – Effects of acute and chronic vitamin A supplementation on protein thiol content in rat striatum. Data are mean ± S.E.M. of 9–12 animals per group performed in duplicate. *$p<0.0001$ (one-way ANOVA followed by Tukey's test).

0.14 ($n=12$; $p<0.0001$), or 1.61±0.08 ($n=12$; $p<0.0001$) nmol carbonyl/mg protein in the striatum of the rats that received vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day, respectively, for 28 days (Fig. 2).

2.3. *Protein thiol content*

Protein thiol content was found decreased from 92.9±1.58 μmol/mg protein (control group; $n=12$) to 64.4±2.6 ($n=12$; $p<0.0001$), 61.1±1.5 ($n=12$; $p<0.0001$), 61.9±2.72 ($n=12$; $p<0.0001$), or 57.2±1.37 ($n=12$; $p<0.0001$) μmol/mg protein in the striatum of the rats that were treated for 3 days with vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day, respectively (Fig. 3). Protein thiol content decreased from 95.8±1.01 μmol/mg protein (control group; $n=12$) to 62.25±1.6 ($n=12$; $p<0.0001$), 58.9±3.23 ($n=12$; $p<0.0001$), 61.24±3.8 ($n=12$; $p<0.0001$), or 54.9±2.97 ($n=12$; $p<0.0001$) μmol/mg protein in the striatum of the rats that were treated for 7 days with vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day, respectively (Fig. 3). Chronically, protein thiol content was decreased from 95.7±1.6 μmol/mg protein (control group; $n=9$) to 57.1±1.4 ($n=12$; $p<0.0001$), 52.4±3.09 ($n=12$; $p<0.0001$), or 51.2±2.56 ($n=12$; $p<0.0001$) μmol/mg protein in the striatum of the rats that received vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day, respectively, for 28 days (Fig. 3).

2.4. *Superoxide dismutase, catalase, and glutathione peroxidase activities*

As demonstrated in Table 1, vitamin A supplementation induced several alterations in the activities of antioxidant enzymes. SOD activity did not change in the striatum of the rats that received vitamin A supplementation at 1000 IU/kg/day for 3 days ($n=12$; $p=0.91$). However, SOD activity was decreased from 15.7±0.6 U SOD/mg protein (control group; $n=12$) to 10.66±0.6 ($n=12$; $p<0.0001$), 9.91±0.36 ($n=12$; $p<0.0001$), or 8.88±0.43 ($n=12$; $p<0.0001$) U SOD/mg protein in the striatum of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/kg/day, respectively, for 3 days (Table 1). SOD activity did not change in the striatum of the rats that received vitamin A supplementation for 7 days ($n=12$ per group; $0.15<p\leq1.0$) (Table 1). However, we found that SOD activity was increased from 13.31±0.76 U SOD/mg protein (control group; $n=9$) to 16.85±0.97 ($n=12$; $p<0.05$), 19.31±1.02 ($n=12$; $p<0.0001$), 20.75±0.72 ($n=12$; $p<0.0001$), or 23.32±0.58 ($n=12$; $p<0.0001$) U SOD/mg protein in the striatum of the rats that received vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day, respectively, for 28 days (Table 1).

CAT activity was found decreased from 1.25±0.03 U CAT/mg protein (control group; $n=12$) to 0.64±0.03 ($n=12$; $p<0.0001$), 0.64±0.026 ($n=12$; $p<0.0001$), 0.6±0.05 ($n=12$; $p<0.0001$), or 0.67±0.042 ($n=12$; $p<0.0001$) U CAT/mg protein in the striatum of the rats that received vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day, respectively, for 3 days (Table 1). We observed a decrease in CAT activity from 1.28±0.04 U CAT/mg protein (control group; $n=12$) to 0.83±0.04 ($n=12$; $p<0.0001$), 0.72±0.05 ($n=12$; $p<0.0001$), 0.68±0.03 ($n=12$; $p<0.0001$), or 0.63±0.05 ($n=12$; $p<0.0001$) U CAT/mg protein in the striatum of the rats that received vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day, respectively, for 7 days (Table 1). However, CAT activity did not change in the striatum of the rats that received vitamin A supplementation for 28 days ($n=12$ per group; $0.25<p\leq1.0$) (Table 1).

GPx activity did not change in the striatum of the rats that received acute or chronic vitamin A supplementation ($n=9$ to each control group, $n=12$ to each treatment group; $0.19<p\leq1.0$) (Table 1).

Table 1 – Acute and chronic vitamin a supplementation effects on SOD, CAT, and GPx activities in the rat striatum

Group	SOD activity (U SOD/mg protein)			CAT activity (U CAT/mg protein)			GPx activity (NADPH mM/min/mg protein)		
	3 days	7 days	28 days	3 days	7 days	28 days	3 days	7 days	28 days
Control	15.7±0.6	13.8±0.3	13.31±0.76	1.25±0.03	1.28±0.04	1.16±0.03	0.03±0.0004	0.027±0.0006	0.027±0.0003
1000 IU/kg	15.1±0.8	13.6±0.6	16.85±0.97*	0.64±0.03**	0.83±0.04**	1.11±0.04	0.027±0.0005	0.027±0.0008	0.028±0.0007
2500 IU/kg	10.66±0.6**	13.9±0.6	19.31±1.02**	0.65±0.026**	0.72±0.05**	1.05±0.05	0.028±0.0007	0.027±0.0007	0.032±0.0022
4500 IU/kg	9.91±0.36**	12.0±0.5	20.75±0.72**	0.6±0.05**	0.68±0.03**	1.1±0.09	0.027±0.0008	0.026±0.0011	0.029±0.0013
9000 IU/kg	8.88±0.43**	11.9±0.8	23.32±0.58**	0.67±0.042**	0.63±0.05**	1.1±0.07	0.026±0.001	0.027±0.011	0.028±0.0008

Values are means±S.E.M. of 9–12 animals per group. *$p<0.05$, **$p<0.0001$, vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

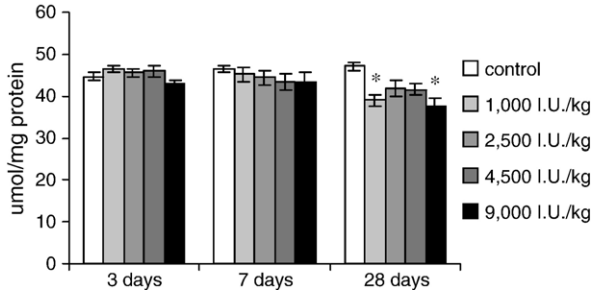

Fig. 4 – Effects of acute and chronic vitamin A supplementation on non-protein thiol content in rat striatum. Data are mean ± S.E.M. of 9–12 animals per group performed in duplicate. *$p=0.02$ (one-way ANOVA followed by Tukey's test).

2.5. *Non-protein thiol content*

As depicted in Fig. 4, non-protein thiol content did not change in the striatum of the rats that received vitamin A supplementation for 3 or 7 days ($n=12$ to each control group, $n=12$ to each treatment group; $0.76<p\leq1.0$). Non-protein thiol content did not change in the striatum of the rats that received vitamin A supplementation at 2500 or 4500 IU/kg/day for 28 days ($n=9$ animals to control group, $n=12$ to each treated group; $0.89<p\leq1.0$) (Fig. 4). Non-protein thiol content was decreased from 47.06 ± 1.05 μmol/mg protein (control group; $n=9$) to 38.95 ± 1.33 ($n=12$; $p=0.02$) or 37.64 ± 1.82 ($n=12$; $p=0.02$) μmol/mg protein in the striatum of the rats that received vitamin A supplementation at 1000 or 9000 IU/kg/day, respectively, for 28 days (Fig. 4).

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

The TRAP assay was used to detect variations on the level of non-enzymatic antioxidant defenses in rat striatum. After 3 days of supplementation, vitamin A did not induce alterations in the level of non-enzymatic antioxidant defenses in the rat striatum ($0.59<p\leq1.0$) (Fig. 5). However, we detected an increase in the emission of chemiluminescence of the samples of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/kg/day for 7 days ($n=12$ to each group; $p<0.0001$), which suggest that a decrease in the non-enzymatic antioxidant defense was induced after vitamin A supplementation. The samples of the animals that received vitamin A supplementation at 1000 ($n=12$; $p<0.01$), 2500 ($n=12$; $p<0.0001$), 4500 ($n=12$; $p<0.0001$), or 9000 ($n=12$; $p<0.0001$) IU/kg/day also emitted more chemiluminescence than the control ones ($n=9$) (Fig. 5). It is necessary to clarify that high-prooxidant-content samples will oxidize more luminol, emitting more chemiluminescence than high-antioxidant-content samples, giving rise to high values of percentage which are inversely proportional to its non-enzymatic antioxidant capacity. It is important to mention that the percentage values were obtained from the area under the curve originated from chemiluminescence values detected in the assay.

2.7. *SOD/CAT ratio*

As depicted in Fig. 6, we found that the SOD/CAT ratio increased from 12.75 ± 0.64 arbitrary units (control group; $n=12$) to 24.33 ± 1.93 ($n=12$; $p<0.0001$) or 17.3 ± 1.04 ($n=12$; $p<0.05$) arbitrary units in the striatum of the rats that received vitamin A supplementation at 1000 or 4500 IU/kg/day, respectively, for 3 days (Fig. 6). Vitamin A supplementation at either 2500 or 9000 IU/kg/day did not increase SOD/CAT ratio in the striatum ($0.25<p\leq1.0$). The SOD/CAT ratio was increased from 10.86 ± 0.47 arbitrary units (control group; $n=12$) to 20.43 ± 1.8 ($n=12$; $p<0.0001$), 18.04 ± 1.24 ($n=12$; $p<0.01$), or 20.5 ± 2.16 ($n=12$; $p<0.0001$) arbitrary units in the striatum of the animals that received vitamin A supplementation at 2500, 4500, or 9000 IU/kg/day for 7 days. Vitamin A supplementation at 1000 IU/kg/day for 7 days did not change the striatal SOD/CAT ratio in this experimental model ($n=12$; $p=0.05$) (Fig. 6). We found that the SOD/CAT ratio increased from 11.6 ± 0.81 arbitrary units (control group; $n=9$) to 18.7 ± 1.45 ($n=12$; $p<0.05$), 19.7 ± 1.9 ($n=12$; $p=0.004$), or 21.2 ± 1.54 ($n=12$; $p=0.001$) arbitrary units in the striatum of the rats that received vitamin A supplementation at 2500, 4500, or 9000 IU/kg/day, respectively, for 28 days (Fig. 6).

2.8. *Behavioral data*

Table 2 shows the effects of acute and chronic vitamin A supplementation on the locomotory (number of crossings) and

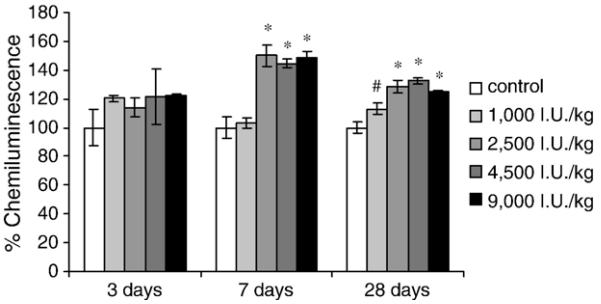

Fig. 5 – Effects of acute and chronic vitamin A supplementation on total radical-trapping antioxidant parameter in rat striatum. Data are mean ± S.E.M. of 9–12 animals per group performed in triplicate. #$p<0.01$; *$p<0.001$ (one-way ANOVA followed by Tukey's test).

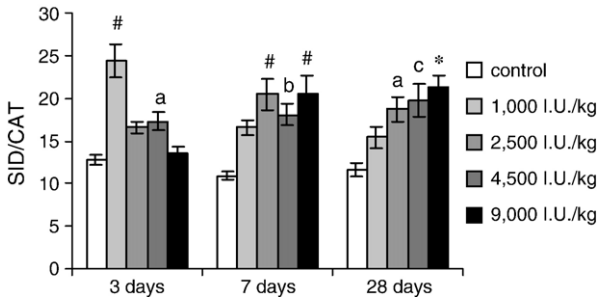

Fig. 6 – Effects of acute and chronic vitamin A supplementation on SOD/CAT ratio in rat striatum. Data are mean ± S.E.M. of 9–12 animals per group performed in duplicate. [a]$p<0.05$; [b]$p<0.01$; [c]$p=0.004$; *$p=0.001$; #$p<0.0001$ (one-way ANOVA followed by Tukey's test).

Table 2 – Acute and chronic vitamin a supplementation effects on the number of crossings and rearings in an open field

Group	Number of crossings			Number of rearings		
	3 days	7 days	28 days	3 days	7 days	28 days
Control	62.8±3.5	68.7±3.1	66.14±3.9	37.6±2.0	35.57±1.55	41±2.09
1000 IU/kg	61.7±3.3	66.8±3.6	33.85±2.95*	35.3±1.8	37±1.6	23.28±2.14*
2500 IU/kg	64.1±2.3	45±2.18*	32±2.43*	36.7±1.8	38±1.44	18±2.05*
4500 IU/kg	62.6±3.5	43.14±1.84*	31.3±2.8*	35.3±2.2	36.3±1.26	18.28±1.82*
9000 IU/kg	65.3±2.42	42.8±1.8*	27.2±2.92*	36±2.15	22.28±1.67*	15.42±2.52*

Values are means±S.E.M. of 9 animals per group. *$p<0.001$, vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

exploratory (number of rearings) behavior of rats. We did not observe alterations in the locomotory behavior of the rats that received vitamin A supplementation for 3 days ($n=9$ per group; $0.9<p\leq1.0$). In the rats that received vitamin A supplementation at 1000 IU/kg/day for 7 days, the number of crossings did not change. However, vitamin A supplementation at 2500, 4500, or 9000 IU/kg/day decreased the number of crossings from 68.7±3.1 (control group; $n=9$) to 45±2.18 ($n=9$; $p<0.0001$), 43.14±1.84 ($n=9$; $p<0.0001$), or 42.8±1.8 ($n=9$; $p<0.0001$), respectively, when the rats were treated for 7 days (Table 2). Chronic vitamin A supplementation decreased the number of crossings from 66.14±3.9 (control group; $n=9$) to 33.85±2.95 ($n=9$; $p<0.0001$), 32±2.43 ($n=9$; $p<0.0001$), 31.3±2.8 ($n=9$; $p<0.0001$), or 27.2±2.92 ($n=9$; $p<0.0001$) when the animals received vitamin A at 1000, 2500, 4500, or 9000 IU/kg/day, respectively, for 28 days (Table 2).

Table 2 shows the effects of acute and chronic vitamin A supplementation on the number of rearings performed by the rats in the open field apparatus. The number of rearings performed in the open field did not change in the rats that received vitamin A supplementation for 3 days ($n=9$ per group; $0.79<p\leq1.0$). The number of rearings was decreased from 35.57±1.55 (control group; $n=9$) to 22.28±1.67 ($n=9$; $p<0.0001$) in the rats that received vitamin A supplementation at 9000 IU/kg/day for 7 days. The other vitamin A doses did not affect the number of rearings in the open field ($n=9$ per group; $0.9<p\leq1.0$) when the rats were treated for 7 days. The number of rearings was decreased from 41±2.09 (control group; $n=9$) to 23.28±2.14 ($n=9$; $p<0.0001$), 18±2.05 ($n=9$; $p<0.0001$), 18.28±1.82 ($n=9$; $p<0.0001$), or 15.42±2.52 ($n=9$; $p<0.0001$), respectively, in the animals that received vitamin A supplementation at 1000, 2500, 4500, or 9000 IU/kg/day for 28 days (Table 2).

3. Discussion

Our results show that both acute and chronic vitamin A administration altered the striatal redox environment. Lipid peroxidation, protein carbonylation, and a decrease in protein thiol content occurred intensely when therapeutic or excessive vitamin A doses were administered. Moreover, antioxidant enzymes activities were also modulated after the treatment. A decrease in the non-enzymatic antioxidant content (represented by the TRAP assay) was also induced by vitamin A. Importantly, vitamin A supplementation drastically decreased locomotory activity, a striatum-associated behavioral function, in an open field.

Acutely (3-day period), vitamin A induced a decrease in both SOD and CAT activities (Table 1). This may increase $O_2^{-\bullet}$ availability, since SOD activity was decreased. Then, increased $O_2^{-\bullet}$ may allosterically inactivate CAT enzyme, decreasing its activity (Kono and Fridovich, 1982; Shimizu et al., 1984). Really, vitamin A is known to increase $O_2^{-\bullet}$ production, as previously demonstrated (Murata and Kawanishi, 2000; Klamt et al., 2005), and the decrease in both antioxidant enzymes activities may result in oxidative stress. Actually, as shown in Figs. 1–3, we found increased lipid peroxidation, protein carbonylation, and decreased protein thiol content, respectively, in the striatum of vitamin A-treated rats.

However, a 7-day vitamin A supplementation did not alter the SOD activity (Table 1). This indicates that $O_2^{-\bullet}$ production is not increased, since this free radical is the major allosteric activator of SOD activity (Halliwell and Gutteridge, 1999). However, we found decreased CAT activity (Table 1), which was found to result in an increase in the SOD/CAT ratio after vitamin A supplementation for 7 days (Fig. 6). As a consequence of increased SOD/CAT ratio, H_2O_2 availability might be increased, favoring the permanence of a prooxidant state in the striatum, since H_2O_2 is a source of hydroxyl radical ($OH^{\bullet}$) generation, the most powerful prooxidant molecule, via Fenton reaction (Halliwell, 2006). It may explain, at least in part, the increase in the levels of the markers of oxidative stress that we have found after 7 days with vitamin A supplementation (Figs. 1–3).

Chronically, vitamin A induced an increase in SOD activity (Table 1), suggesting that $O_2^{-\bullet}$ production is increased. However, CAT activity did not change after vitamin A supplementation for 28 days (Table 1). This indicates that H_2O_2 concentration is not sufficiently high to increase CAT activity, as previously described (Dröge, 2002). In addition, $O_2^{-\bullet}$ concentration, which inhibits CAT activity, as mentioned above, is decreased by increased SOD activity. In addition, we found impaired SOD/CAT ratio (Fig. 6) and increased oxidative damage (Figs. 1–3) chronically.

Vitamin A did not alter GPx activity in this experimental model (Table 1). A possible explanation to this is that when H_2O_2 production is increased above the K_m of GPx for H_2O_2, CAT is the main enzyme to metabolize H_2O_2 to water (Halliwell and Gutteridge, 1999). However, vitamin A treatment was found to decrease striatal CAT activity acutely, which might favor an acute increase in the H_2O_2 concentration. Further research would be necessary to elucidate this.

Increased protein carbonylation and excessive decrease in the protein thiol content may facilitate the arise of protein

aggregates, as a result of protein cross-links, and this is very likely to culminate in a widespread cellular dysfunction, which may accelerate neurodegenerative process (Shults, 2006). Additionally, increased oxidative damage to proteins might result in increased free iron, favoring the maintenance of the prooxidative state (Keyer and Imlay, 1996).

Protein thiol content (sulfhydryl) may have its levels diminished after oxidative insult at the dopaminergic axis by a singular way. Besides ROS or RNS attack on thiol groups, some prooxidant derivatives from both specific and non-specific dopamine oxidation might covalently bind to sulfhydryl groups, oxidizing them (Lotharius and Brundin, 2002).

Vitamin A supplementation induced a slightly, but significant decrease in the non-protein thiol content, which is represented mainly by GSH (Fig. 4). Accordingly, in a previous reported work, our group demonstrated that retinol (vitamin A) did not induce alterations in GSH content in cultured Sertoli cells (Gelain et al., 2006). Then it appeared that vitamin A did not induce strong alterations in the metabolism of glutathione, since we did not find alterations in GPx activity (Table 1), and vitamin A only induced a decrease in the non-protein thiol content after 28 days of supplementation (Fig. 4). Further research is needed to elucidate this phenomenon. However, vitamin A supplementation, mainly chronically, decreased the non-enzymatic antioxidant capacity of the striatum, since an increase in chemiluminescence was recorded (Fig. 5). Then, we suggest that acute and chronic vitamin A supplementation favor a prooxidant state in the striatum by, in addition to the effects described above, consuming the striatal non-enzymatic antioxidant defenses.

As previously mentioned, vitamin A is described as an antioxidant to some neurodegenerative processes (Prasad et al., 2002). Additionally, Zaidi and Banu (2004) reported that vitamin A is an antioxidant against immobilization stress. However, in that work, the authors used the whole brain to perform the investigation of the redox effects of vitamin A treatment against the stress that was induced. This methodology may give rise to misunderstandings, since brain structures may respond differently to the same insult, underestimating the oxidative damage that might occur in a given structure.

In humans, vitamin A, which is mainly used as retinol palmitate, is described to induce irritability, fatigue, depression, and anxiety, to cite a few (Myhre et al., 2003). Here, we demonstrated that acute and chronic retinol palmitate (vitamin A) supplementation did induce disturbances in both locomotory and exploratory behaviors in rats (Table 2). Importantly, vitamin A supplementation did not induce diarrhea in the animals. So this was not responsible for any redox effect seen here, since in diarrhea a loss of water-soluble antioxidants (for instance, vitamin C) may favor the permanence of a prooxidant state in the organism. It is difficult to affirm that these behavioral deficits are caused only by oxidative stress induced by vitamin A supplementation in the striatum. Further research is necessary to elucidate the mechanism by which vitamin A supplementation impairs rat behavior.

Together, the data reported here give rise to a new proposal of a toxic action of vitamin A on the adult striatum. Additionally, this study collaborate with literature showing that vitamin A doses lower than 8500 IU/kg/day, which was previously suggested as tolerable (Mactier and Weaver, 2005), impair behavior of adult rats in an open field. Since vitamin A are membranolytic (Roels et al., 1959), and also induces mitochondrial swelling (Rigobello et al., 1999; Klamt et al., 2005), at least in part, the oxidative stress herein demonstrated might be a consequence of a mitochondrial dysfunction and/or of an impairment in the energetic metabolism of striatum. Now, it is difficult to predict how useful a vitamin A therapy might be in Parkinson's disease (PD), since the oxidative insult imposed by vitamin A supplementation (including therapeutic doses) was demonstrated to be extremely high in striatum, a target of human PD.

4. Experimental procedures

4.1. *Animals*

We used a total of 177 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. Behavioral task was conducted during the light phase. 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. The Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul approved our research protocol.

4.2. *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. Vitamin A was prepared daily and it occurred by protecting from light.

4.3. *Drug administration*

The animals were treated once a day during three different periods: acutely (3 or 7 days), or chronically (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 (a total of 177 rats) were randomly separated between groups and were treated with vehicle (0.15 M saline; control group), 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 during each period of interest. Adequate measures were taken to minimize pain or discomfort.

4.4. *Preparations of the samples*

The animals were sacrificed by decapitation at 24 h after the last vitamin A administration. The striatum was dissected out in ice immediately after the sacrifice and stored at −80 °C for posterior biochemical analyses. The striatum 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 to all biochemical assays described herein. All the results were normalized by the protein content using bovine albumin as standard (Lowry et al., 1951).

4.5. *Thiobarbituric acid-reactive species (TBARS)*

As an index of lipid peroxidation, we detected striatal TBARS formation through a hot and acid reaction. This is widely adopted as a method for measuring 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. Results are expressed as nmol TBARS/mg protein.

4.6. *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 was read in a spectrophotometer at 370 nm. Results are expressed as nmol carbonyl/mg protein.

4.7. *Measurement of protein and non-protein thiol content*

Other form to analyze oxidative alterations in proteins is to measure the level of protein thiol content. 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 of incubation (Ellman, 1959). The free sulfhydryl (–SH) content was estimated in supernatants of 20% TCA precipitated homogenates by the same method. Results are expressed as μmol SH/mg protein.

4.8. *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. 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.

4.9. *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). Catalase (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm (Aebi, 1984). Glutathione peroxidase (GPx) activity was determined by measuring the rate of NAD(P)H oxidation in a spectrophotometer at 340 nm as previously described (Flohé and Günzler, 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.

4.10. *Behavioral task*

The behavioral task occurred 15 h after the last treatment, and was performed between 14:00 and 16:00 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. The number of crossings of the black lines and rearings were counted over this time. In behavioral analysis, rats were used only once.

4.11. *Statistical analyses*

Biochemical and behavioral data are 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 <0.05. Differences in experimental groups were determined by the one-way ANOVA followed by post hoc Tukey's test.

Acknowledgments

This work was supported by grants from CNPq, PROPESQ-UFRGS and FAPERGS. M.R. de Oliveira and R.B. Silvestrin are recipients of a CNPq fellowship. M.A.B. Pasquali is recipient of a FAPERGS fellowship. T. Mello e Souza was supported by PROFIX (CNPq).

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Capítulo III

“Oxidative stress in the hippocampus, anxiety-like behavior and decreased locomotory and exploratory activity of adult rats: effects of sub acute vitamin A supplementation at therapeutic doses”

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Artigo publicado no periódico NeuroToxicology, volume 28, p. 1191-1199 (2007)

Available online at www.sciencedirect.com
ScienceDirect
NeuroToxicology 28 (2007) 1191–1199
NeuroToxicology

Oxidative stress in the hippocampus, anxiety-like behavior and decreased locomotory and exploratory activity of adult rats: Effects of sub acute vitamin A supplementation at therapeutic doses

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Received 25 April 2007; accepted 23 July 2007
Available online 27 July 2007

Abstract

Vitamin A participates in the maintenance of normal hippocampal function during embryonic and postnatal stages of the vertebrate life. Some works demonstrated that vitamin A metabolites impair learning and induce a depression-like behavior in mice, among other effects. Since vitamin A has prooxidant effects on other experimental models, we decided to investigate whether vitamin A can induce oxidative stress in the adult rat hippocampus. We analyzed the sub acute effects of therapeutic (1000 and 2500 I.U./kg) or excessive (4500 and 9000 I.U./kg) vitamin A doses on the hippocampal redox state, as well as on levels of anxiety, and locomotory and exploratory activity. Vitamin A supplementation induced lipid peroxidation, protein carbonylation, and oxidation of the protein thiol content in the rat hippocampus in all periods analyzed. Increased superoxide dismutase (SOD) activity and decreased catalase (CAT) activity were also observed, which gives rise to an imbalance in the principal cellular enzymatic antioxidant system. Then, our results show, for the first time, that vitamin A induced oxidative stress in the adult rat hippocampus, is anxiogenic, and decreases locomotion in and exploration of an open field.

Keywords: Vitamin A; Oxidative stress; Hippocampus; Anxiety; Superoxide dismutase; Catalase

1. Introduction

Vitamin A (retinol) and its metabolites (retinoids) are essential to normal brain function (Lane and Bailey, 2005; Ross et al., 2000). During the prenatal life, absence of sufficient vitamin A may impair embryo's segmentation and growth, and stops normal vascularization (Maden et al., 1996; Wellik and DeLuca, 1995; White et al., 2000). Vitamin A continues to be important to other central nervous system (CNS)-related functions during adulthood, for instance to learning and memory (Chiang et al., 1998; Cocco et al., 2002). Furthermore, adult hippocampal neurogenesis seems to be regulated, at least in part, by retinoids (Takahashi et al., 1999).

Therapeutically, vitamin A at high doses (about 50,000–100,000 I.U./day) is used in the treatment of children and adults suffering from leukemia (Fenaux et al., 2001; Norum, 1993; Tsunati et al., 1990, 1991). In addition, vitamin A at moderate to high doses (at a maximum of 8500 I.U./(kg day)) is prescribed to very-low-birth-weight infants (see Mactier and Weaver, 2005 for a review). However, there are a number of deleterious effects that might result from excessive vitamin A intake, for instance necrotizing vasculitis, irritability, and depression to cite a few (Myhre et al., 2003; Paydas et al., 1998). Hippocampus seems to be a major target of the neurotoxicity imposed by retinoids. It was demonstrated that the vitamin A derivative 13-*cis*-retinoic acid (the active compound of Accutane™), when given chronically, decreases adult hippocampal neurogenesis and induces learning deficits (Crandall et al., 2004), and induces depression-like behavior in mice (O'Reilly et al., 2006) and in humans (Hazen et al., 1983; Hull and D'Arcy, 2003; Hull and Demkiw-Bartel, 2000; Josefson, 1998).

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0161-813X/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.neuro.2007.07.008

Oxidative stress may result from an overload of oxidants, particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS), with respect to the antioxidant defense system developed by cells to counteract oxidation. Oxidative insult may disrupt cell structures and functions, insofar as they are dependent on critical redox balance. Oxyradicals are suggested to be involved in several pathological conditions, including neurodegenerative diseases, for instance Alzheimer's disease (AD) and Parkinson's disease (PD) (Halliwell, 2006; Halliwell and Gutteridge, 1999).

Vitamin A is either antioxidant (Zaidi and Banu, 2004) or prooxidant (see below), depending on its concentration. Previously, we showed that vitamin A induces protein carbonylation and lipid peroxidation, and that antioxidant enzyme activity are modulated by retinol (Dal-Pizzol et al., 2000, 2001; Frota et al., 2004; Klamt et al., 2000; Moreira et al., 1997) or all-*trans* retinoic acid (Frota et al., 2006) treatments in cultured Sertoli cells. Rat liver mitochondria are vulnerable to retinol (Klamt et al., 2005) and retinoids (Rigobello et al., 1999), which may induce the release of pro-apoptotic factors from mitochondria.

The hippocampus is involved in mood disorders, such as anxiety and depression (Bannerman et al., 2003, 2004; Deacon and Rawlins, 2005; File et al., 2000). Oxidative stress may be associated with these and others cognitive disturbances (Floyd, 1999; Thiels and Klann, 2002), and hippocampus seems to be strongly affected by the deleterious effects of oxidative insult (Bonatto et al., 2005; Gabbita et al., 1998; Serrano and Klann, 2004). It was previously demonstrated that vitamin A crosses the blood–brain barrier easily (MacDonald et al., 1990). In addition, there are metabolizing enzymes and nuclear receptors in adult mammalian hippocampal cells (McCafferry et al., 2005; Zetterstrom et al., 1994). Then, we decided to investigate the effects of sub acute (3, 7, or 28 days) vitamin A supplementation either at therapeutic (1000 and 2500 I.U./kg) or excessive (4500 and 9000 I.U./kg) doses upon the redox environment of adult rat hippocampus, as well as on anxiety, and on locomotory and exploratory activities.

2. Experimental procedures

2.1. Animals

Adult male Wistar rats (90 days old; 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 (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. 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 three different periods: 3, 7, or 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), 1000, 2500, 4500, or 9000 I.U./kg of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) 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

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 hippocampus was dissected out in ice immediately after the rat was sacrificed and stored at −80 °C for posterior biochemical analyses. The homogenates were centrifuged ($700 \times g$, 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 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 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 dinitrophenylhidrazine (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 and non-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. Free sulfhydryl (–SH) content was estimated in supernatants of 20% TCA precipitated homogenates by the same method (Ellman, 1959). Results are expressed as μmol SH/mg protein.

2.5. Antioxidant enzyme activities estimations

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. 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. Glutathione peroxidase (GPx) activity were determined by measuring the rate of NAD(P)H oxidation in a spectrophotometer at 340 nm, as described (Flohé and Günzler, 1984), and the results are expressed as mM NADPH/(min 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). An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminates in oxidative stress.

2.6. Behavioral tasks

The behavioral tasks occurred after the last day of vitamin A supplementation. The animals were allowed to explore a light–dark arena and an open field only once. Behavioral tasks were carried out in a room with constant temperature (21 ± 2 °C) and light conditions (60-W light), except for the light–dark exploration task (see details below). Before session, the animals were allowed to adapt to the experimental room for at least 30 min. All tasks were performed between 14:00 and 16:00 h.

2.6.1. Light–dark exploration task

Anxiety is a well-known hippocampus-associated cognitive disorder (Deacon and Rawlins, 2005), and rodents are nocturnal animals preferring darker areas, then the decrease in the exploratory activity in the light area is taken as an index of anxiety. The dark–light box consists of two compartments: one light compartment (30 cm × 20 cm × 20 cm), with a transparent acrylic panel on one side to facilitate observation of the rat, also the surrounding walls and floor were painted white, and the dark compartment (15 cm × 20 cm × 20 cm) with the surrounding walls and floor painted black. The light compartment was illuminated under a 60-W light. The dark one received only part of the room illumination (at 20-W). The floor of the light compartment was divided into 12 equal squares by black lines and the dark compartment was divided into 9 equal squares by white lines. The two compartments were separated by a partition with an opening to allow passage from one compartment to the other. The rats were gently placed in the corner of the light compartment facing the wall opposite to the opening. The following parameters were recorded during 5 min: (1) latency time for the first crossing to the dark compartment, (2) the number of transitions between the light and the dark compartment, (3) the total time spent in the illuminated part of the cage, (4) number of crossings (horizontal activity) in each compartment, (5) number of rearings (vertical activity) in each compartment, (6) number of groomings in each part of the cage, (7) number of faecal boli in each compartment, and (8) the risk assessment behavior index (RA, i.e. the number of investigations of the light compartment by placing some but not all paws) was recorded. The apparatus was cleaned between each rat and urine and faeces were removed before wiping with a moist followed by a dry tissue. This regime was applied to avoid influence by odor on rat behavior.

2.6.2. Open field

The open field apparatus was applied to analyze free locomotion and exploration of animals. Exploration of the environment is a well-established function also dependent on hippocampal function (Lever et al., 2006) and we chose open field task because it is a task free of challenges (dividing walls as the dark–light box contains, for example) to the animal develop more naturally the exploration of the place. 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 gently placed in the same initial corner and were left free to explore the arena for 5 min. We counted both horizontal (number of crossings of the black lines) and vertical (number of rearings) activities performed during time.

2.7. 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 parameters

3.1.1. Lipid peroxidation

We found increased lipid peroxidation levels in the hippocampus of the rats that received vitamin A supplementation at 1000 I.U./kg for 3 days ($p < 0.0001$) (Fig. 1A). Vitamin A supplementation at 1000, 4500, or 9000 I.U./kg for 7 days induced a significant increase ($p < 0.0001$) in hippocampal lipid peroxidation levels (Fig. 1A). Vitamin A supplementation at 1000, 2500, or 4500 I.U./kg for 28 days increased lipid peroxidation in the rat hippocampus ($p < 0.05$) (Fig. 1A).

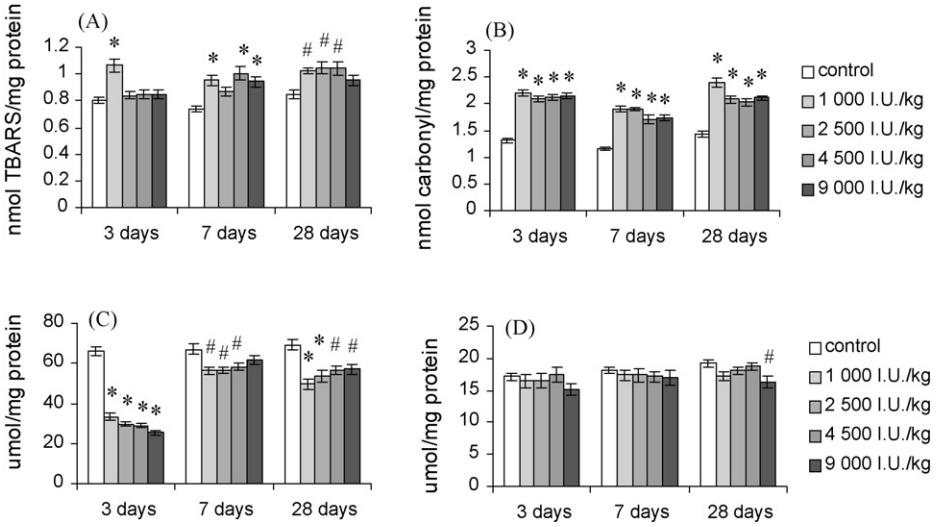

Fig. 1. Effects of vitamin A supplementation on lipid peroxidation (A), protein carbonylation (B), protein (C) and non-protein (D) thiol content in the rat hippocampus. Data are mean ± S.E.M ($n = 7$–12 per group). $^{\#}p < 0.05$; $^{*}p < 0.0001$ (one-way ANOVA followed by the post hoc Tukey's test).

3.1.2. Protein carbonylation

Interestingly, we found increased protein carbonylation levels ($p < 0.0001$) in the hippocampus of the rats that were treated with vitamin A supplementation at any dose for 3, 7, or 28 days (Fig. 1B).

3.1.3. Protein and non-protein thiol content

Vitamin A supplementation at any dose for 3 days induced a significant decrease in the hippocampal protein thiol content ($p < 0.0001$) (Fig. 1C). Vitamin A supplementation at 1000, 2500, or 4500 I.U./kg for 7 days induced a significant decrease ($p < 0.05$) in the hippocampal protein thiol content (Fig. 1C). Vitamin A at any dose tested induced a significant decrease in the protein thiol content of the rat hippocampus when given for 28 days (Fig. 1C).

Surprisingly, the non-protein thiol content was altered only in the hippocampus of the rats that were treated with vitamin A supplementation at 9000 I.U./kg for 28 days ($p < 0.05$) (Fig. 1D).

3.2. Antioxidant enzymes activities

3.2.1. SOD activity

Vitamin A supplementation at any dose for 3 days induced a significant increase ($p < 0.05$) in the SOD activity (Fig. 2A). SOD activity was found to be increased ($p < 0.0001$) in the hippocampus of the rats that received vitamin A supplementation at 2500, 4500, or 9000 I.U./kg for 7 days (Fig. 2A). We found increased SOD activity in the hippocampus of the rats that received vitamin A supplementation at any dose for 28 days (Fig. 2A).

3.2.2. CAT activity

Vitamin A supplementation at any dose for 3 or 7 days induced a significant decrease in the hippocampal CAT activity

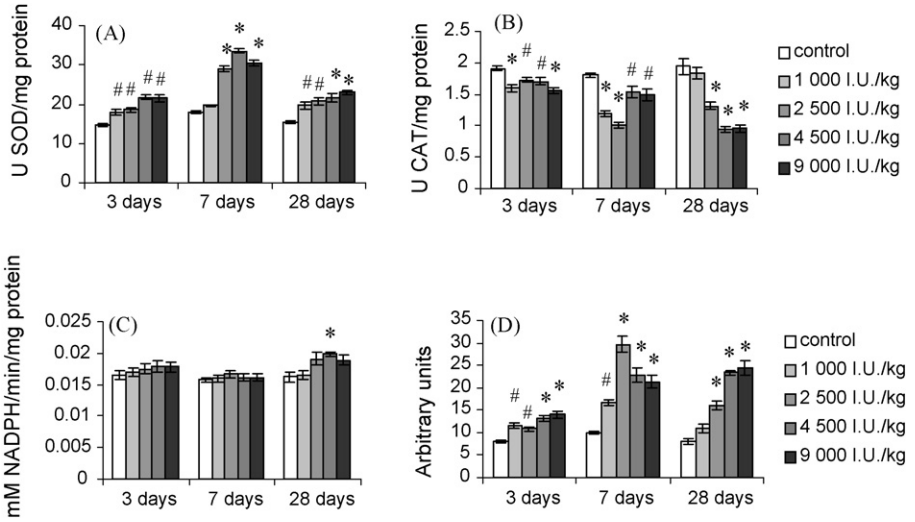

Fig. 2. Effects of vitamin A supplementation on SOD (A), CAT (B), and GPx activity (C) in the rat hippocampus. (D) The hippocampal SOD/CAT ratio. Data are mean ± S.E.M ($n = 7$–12 per group). $^{\#}p < 0.05$; $^{*}p < 0.0001$ (one-way ANOVA followed by the post hoc Tukey's test).

Table 1
Vitamin A-supplementation effects on rat behavior in the light–dark exploration task

Group	Time in the light compartment (s)			Latency (s)			Number of transitions		
	3 days	7 days	28 days	3 days	7 days	28 days	3 days	7 days	28 days
Control	91.1 ± 1.9	80.3 ± 2.9	87.6 ± 2.4	53 ± 4.3	44.4 ± 2.4	56 ± 2.7	4.7 ± 0.5	4.4 ± 0.5	7.3 ± 1.2
1000 I.U./kg	86.6 ± 5.8	71.1 ± 3.1	49 ± 1.1**	42.4 ± 1.9	34.4 ± 1.4	17.7 ± 3.5**	4.1 ± 0.9	4.4 ± 0.5	7.9 ± 1.8
2500 I.U./kg	84.1 ± 3.7	83.3 ± 3.3	33 ± 6.6**	30.7 ± 5.9*	28.6 ± 4.3*	13.7 ± 2.4**	4.7 ± 0.4	7.4 ± 1.1	2.8 ± 0.6*
4500 I.U./kg	74.8 ± 6.4	85.3 ± 1.8	57.7 ± 3.8**	15.7 ± 1**	18.6 ± 2**	18 ± 2.3**	7.4 ± 0.4	7.6 ± 0.6	2.9 ± 0.4*
9000 I.U./kg	77.8 ± 6.2	85.6 ± 4.9	52.3 ± 4.5**	17.7 ± 2**	9.8 ± 1**	15.7 ± 2.6**	6.6 ± 0.9	7.4 ± 0.4	3.7 ± 0.3

Values are means ± S.E.M. of nine animals per group. *$p < 0.05$, **$p < 0.0001$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

(Fig. 2B). We found decreased CAT activity also in the hippocampus of the rats that were treated with vitamin A at 2500, 4500, or 9000 I.U./kg for 28 days ($p < 0.0001$) (Fig. 2B).

3.2.3. GPx activity

We detected increased GPx activity only in the hippocampus of the rats that received vitamin A supplementation at 4500 I.U./kg for 28 days ($p < 0.0001$) (Fig. 2C).

3.2.4. SOD/CAT ratio

As depicted in Fig. 2D, vitamin A supplementation for 3, 7, or 28 days induced an imbalance in the hippocampal SOD/CAT ratio.

3.3. Behavioral tasks

3.3.1. Dark–light exploration task

The total time spent by the animals in the light compartment of the box was significantly decreased by any vitamin A dose after 28 days of supplementation ($p < 0.0001$) (Table 1). Moreover, we may observe that the animals that were treated with vitamin A supplementation at 2500, 4500, or 9000 I.U./kg for 3 or 7 days spent less time to leave for the first time (latency time) the light compartment. Vitamin A at any dose for 28 days induced this same behavior ($p < 0.0001$) (Table 1). The number of transitions between both compartments was decreased after 28 days of vitamin A supplementation at 2500 or 4500 I.U./kg ($p < 0.05$) (Table 1). The number of groomings was decreased after 28 days of vitamin A supplementation at any dose in both compartments ($p < 0.001$) (Table 2). The number of faecal boli was increased only by vitamin A at 1000 I.U./kg after 3 or 28 days of supplementation ($p < 0.05$) (Table 3).

Vitamin A supplementation induced significant decrease in the locomotory activity (number of crossings) of rats in both compartments of the dark–light exploration task. Vitamin A supplementation for 3 days at 4500 or 9000 I.U./kg induced a significant decrease in the number of crossings performed in the light compartment of the box ($p < 0.0001$) (Fig. 3A). We did not observe any effect regarding locomotion of the animals that received vitamin A for 7 days at any dose in the light compartment of the box (Fig. 3A). After 28 days of supplementation, vitamin A at any dose induced a decrease in the number of crossings in the light compartment

Table 2
Vitamin A supplementation effects on the number of groomings in the light–dark exploration task

Group	Light compartment			Dark compartment		
	3 days	7 days	28 days	3 days	7 days	28 days
Control	0.3 ± 0.1	0.5 ± 0.1	0.6 ± 0.2	2 ± 0.3	1.6 ± 0.2	2.2 ± 0.3
1000 I.U./kg	0.4 ± 0.1	0.6 ± 0.2	0*	1.8 ± 0.3	1.7 ± 0.3	0.7 ± 0.1*
2500 I.U./kg	0.4 ± 0.2	0.5 ± 0.2	0*	1.8 ± 0.2	1.7 ± 0.2	0.4 ± 0.1*
4500 I.U./kg	0.5 ± 0.2	0.4 ± 0.1	0*	1.5 ± 0.2	1.4 ± 0.2	0.5 ± 0.2*
9000 I.U./kg	0.2 ± 0.1	0.4 ± 0.2	0*	1.2 ± 0.1	1.3 ± 0.2	0*

Values are means ± S.E.M.of nine animals per group. *$p < 0.001$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

Table 3
Vitamin A supplementation effects on number of faecal boli in the light–dark exploration task

Group	Light compartment			Dark compartment		
	3 days	7 days	28 days	3 days	7 days	28 days
Control	1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1 ± 0.2	1.5 ± 0.4	1.3 ± 0.33
1000 I.U./kg	1.3 ± 0.4	1.2 ± 0.2	1.3 ± 0.1	2.5 ± 0.4*	1.7 ± 0.2	3.4 ± 0.6*
2500 I.U./kg	2.1 ± 0.4	1.2 ± 0.3	1.4 ± 0.2	1.9 ± 0.3	1 ± 0.3	2 ± 0.2
4500 I.U./kg	1.9 ± 0.3	1.2 ± 0.2	1.4 ± 0.3	1.1 ± 0.2	1.2 ± 0.2	2.9 ± 0.5
9000 I.U./kg	0.8 ± 0.2	1.2 ± 0.1	1.5 ± 0.2	1.6 ± 0.2	2.2 ± 0.4	2.2 ± 0.2

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

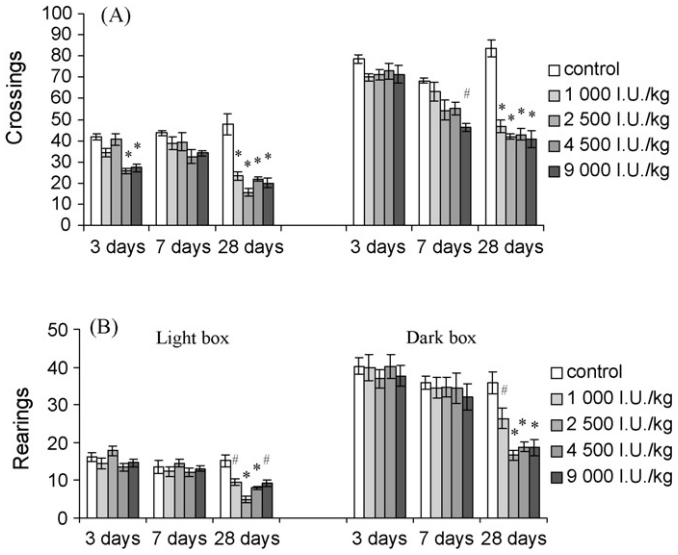

Fig. 3. Effects of vitamin A supplementation on crossings (A) and rearings (B) performed by the animals in the dark–light exploration task. Data are mean ± S.E.M ($n = 9$ per group). $^{\#}p < 0.05$; $^{*}p < 0.0001$ (one-way ANOVA followed by the post hoc Tukey's test).

($p < 0.0001$) (Fig. 3A). We did not observe any effect regarding locomotion of the animals that received vitamin A for 3 days at any dose in the dark compartment of the box (Fig. 3A). We observed that vitamin A supplementation for 7 days at 9000 I.U./kg induced a significant ($p < 0.05$) decrease in the locomotion of the rats in the dark compartment of the box (Fig. 3A). Surprisingly, vitamin A supplementation for 28 days at any dose decreased the locomotion performed by the animals in the dark compartment ($p < 0.0001$) (Fig. 3A).

The number of rearings was also affected by vitamin A supplementation. Number of rearings was significantly decreased by any vitamin A dose investigated in the light and in the dark compartment only after 28 days of vitamin A supplementation (Fig. 3B).

We also observed the number of times that the animals explore the light compartment of the box by putting its two anterior paws in that compartment without cross completely the door between the two compartments of the box (risk assessment). We did not find any change in this behavior in the animals that received vitamin A for 3 days (Fig. 4). The animals that received vitamin A supplementation at 2500, 4500, or 9000 I.U./kg for 7 days were found to avoid more intensely

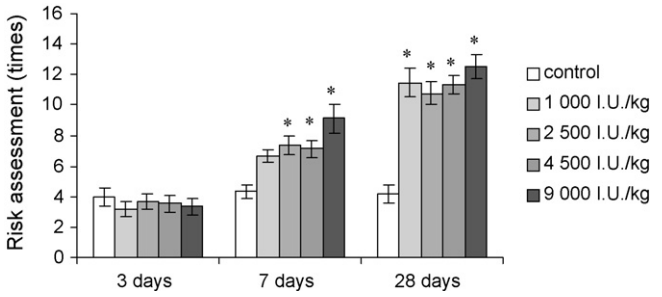

Fig. 4. Effects of vitamin A supplementation on the risk assessment in the dark–light exploration task. Data are mean ± S.E.M ($n = 9$ per group). $^{*}p < 0.0001$ (one-way ANOVA followed by the post hoc Tukey's test).

the transition to the light compartment than the control animals ($p < 0.0001$) (Fig. 4). We found increased risk assessment in the animals that were treated with vitamin A at any dose for 28 days ($p < 0.0001$) (Fig. 4).

3.3.2. Open field

Interestingly, we found that vitamin A supplementation also affects both locomotion in and exploration of an open field (Table 4). Neither locomotion nor exploration were changed in the animals that received vitamin A supplementation for 3 days. However, we observed decreased locomotory activity of the rats that received vitamin A supplementation at 2500, 4500, or 9000 I.U./kg for 7 days ($p < 0.0001$) (Table 4). Vitamin A at any dose decreased the locomotory activity of the rats that were treated for 28 days ($p < 0.0001$) (Table 4). Exploratory behavior in the open field was decreased in the animals that received vitamin A at 9000 I.U./kg for 7 days ($p < 0.0001$) (Table 4). Vitamin A supplementation at any dose decreased the exploratory activity of the rats that were treated for 28 days ($p < 0.0001$) (Table 4).

4. Discussion

Our results clearly show, for the first time, that vitamin A at different doses induced a prooxidant state in the adult rat hippocampus. In addition, behavioral alterations were also observed. According to our data, vitamin A may induce an anxiety-like behavior characterized by intolerance to the light compartment in the dark–light box and by a decreased number of grooming in both compartments. Additionally, locomotory and exploratory activities were decreased after vitamin A supplementation in both tasks. However, this is not due a gross

Table 4
Vitamin A supplementation effects on the number of crossings and rearings in the open field

Group	Crossings			Rearings		
	3 days	7 days	28 days	3 days	7 days	28 days
Control	62.8 ± 3.5	68.7 ± 3.1	66.1 ± 3.9	37.6 ± 2.0	35.5 ± 1.5	41 ± 2.0
1000 I.U./kg	61.7 ± 3.3	66.8 ± 3.6	33.8 ± 2.9*	35.3 ± 1.8	37 ± 1.6	23.2 ± 2.1*
2500 I.U./kg	64.1 ± 2.3	45 ± 2.1*	32 ± 2.4*	36.7 ± 1.8	38 ± 1.4	18 ± 2.0*
4500 I.U./kg	62.6 ± 3.5	43.14 ± 1.8*	31.3 ± 2.8*	35.3 ± 2.2	36.3 ± 1.2	18.2 ± 1.8*
9000 I.U./kg	65.3 ± 2.4	42.8 ± 1.8*	27.2 ± 2.9*	36 ± 2.15	22.2 ± 1.6*	15.4 ± 2.5*

Values are means ± S.E.M.of nine animals per group. $^{*}p < 0.001$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

motor alteration, since the animals walked normally, without presenting muscular weakness or tremor.

Vitamin A treatment was previously found to increase the superoxide anion ($O_2^{\bullet -}$) production *in vitro* (Murata and Kawanishi, 2000; Klamt et al., 2005). $O_2^{\bullet -}$ is a known CAT inhibitor (Kono and Fridovich, 1982; Shimizu et al., 1984). Here, we found that vitamin A supplementation induced a decrease in the CAT activity. In addition, we found increased SOD activity. Together, these results indicate a possible exacerbation in the $O_2^{\bullet -}$ concentration in this experimental model. Increased $O_2^{\bullet -}$ may favor SOD activity, since it is the major SOD allosteric activator (Halliwell and Gutteridge, 1999). Then, an impaired SOD/CAT ratio is very likely to occur.

An increased SOD activity (Fig. 2A) and a decreased CAT activity (Fig. 2B) by occurring simultaneously may result in an increased concentration of H_2O_2, since its production from $O_2^{\bullet -}$ by SOD is increased and its elimination by CAT is decreased. Secondly, an excess of H_2O_2 facilitates the production of hydroxyl radical ($OH^{\bullet}$), the most powerful oxidant molecule, through a reaction with iron or copper (Fenton chemistry) (Halliwell, 2006). Thus, impaired SOD/CAT is very likely to culminate in increased oxidative damage to biomolecules. Glutathione peroxidase (GPx) also metabolizes H_2O_2 to water, although other peroxides are also substrates to GPx. However, we did not find any change in hippocampal GPx activity of the rats that received vitamin A for 3 days (Fig. 2C). The increase in the GPx activity found in the hippocampus of the rats that received vitamin A for 28 days was not sufficient to decrease the oxidative damage observed (Fig. 1A–D). Therefore, our hypothesis that an imbalance in the SOD/CAT ratio might be responsible for the vitamin A supplementation-dependent oxidative alterations is reinforced.

Although essential to the maintenance of hippocampal function, vitamin A may be toxic to the hippocampus, as previously described (Crandall et al., 2004; McCafferry et al., 2005). Now, we show a new deleterious effect of vitamin A on hippocampus: induction of oxidative stress. Oxidatively modified proteins and lipids may contribute to the formation of neurofibrillary tangles, which are intracellular fibrillar aggregates of the microtubule-associated protein tau exhibiting hyperphosphorylation and oxidative modifications (Mattson, 2004). Neurofibrillary tangles are, together with plaques (extracellular deposits of amyloid β-peptide), molecular hallmarks of AD, a neurodegenerative disorder in which the involvement of ROS and RNS in hippocampal and cortical degeneration is undeniable (Castegna et al., 2002; Smith et al., 1997; Völkel et al., 2006).

Furthermore, our herein demonstrated data show that vitamin A was effective in altering rat behavior. In the light–dark task, the light compartment represents a risky place. Therefore, anxious rats prefer to stay in the dark compartment and may represent a defensive behavior (for instance, absence of grooming). Since the animals chronically submitted to any vitamin A dose spent less time relative to controls in the light compartment (Table 1) and, at the same time, showed a higher number of refusals for entering the light compartment (Fig. 4), we may conclude that they had higher levels of anxiety. This is reinforced by the fact that, even though being for a longer time in the dark compartment, they groomed less often in it (Table 2). Vitamin A at 4500 and 9000 I.U./kg for 3 days induced a decrease in locomotion, without altering the exploration or the time spent in the light compartment, indicating that the animals walked less, but explored as much as the controls. This is also an early sign of anxiety. Further studies are necessary to investigate the role of vitamin A in altering rat behavior, since oxidative stress is not the only one causative factor effective in inducing disturbances in behavior.

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 (Barua and Olson, 1986; Buck et al., 1991, 1993; Derguini et al., 1995; Idres et al., 2002; Napoli, 1999). Also, case reports of vitamin A toxicity have shown serum retinol concentrations within normal limits (Croquet et al., 2000; Ellis et al., 1986; Mills and Tanumihardjo, 2006), indicating that serum retinol is not a good measure of vitamin A status during toxicity. In conclusion, we suggest some caution regarding the use of vitamin A as an antioxidant or vitamin supplement, mainly to children and elderly, which are often users of vitamin supplementation or fortified foods.

Acknowledgements

This work was supported by grants of CNPq, PROPESQ-UFRGS and FAPERGS. M.R. de Oliveira and R.B. Silvestrin are recipients of a CNPq fellowship. T. Mello e Souza was supported by PROFIX (CNPq).

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Parte III

3. DISCUSSÃO

Neste trabalho, mostramos que suplementações aguda (3 e 7 dias) e crônica (28 dias) com palmitato de retinol (forma hidrossolúvel da vitamina A - Arovit®) em doses terapêuticas (1000 e 2500 UI/kg/dia) e excessivas (4500 e 9000 UI/kg/dia) foram capazes de alterar parâmetros de estresse oxidativo e comportamentais em ratos machos adultos. Aumento nos níveis de carbonilação de proteínas e de peroxidação lipídica, e diminuição na concentração de grupamentos tióis reduzidos foram observados. Além disso, o tratamento modificou a atividade de enzimas antioxidantes tais como SOD, CAT e GPx, esta última em poucos casos. A alteração nas atividades de SOD e de CAT levou a um desequilíbrio na relação SOD/CAT, pois, na maioria dos casos, o tratamento com palmitato de retinol induziu aumento na atividade da SOD e diminuição na atividade da CAT, o que favorece um aumento na permanência de H_2O_2 no seu sítio de produção, que pode ser mitocondrial (Mn-SOD) ou citosólico (Cu/Zn-SOD). O excesso de H_2O_2 facilita a produção de $OH^{\bullet}$, o mais instável dentre os radicais livres, ou seja, é aquele que tem o maior potencial de causar dano a biomoléculas, seja por redução ou por oxidação destas. Além dos dados de ambiente redox, encontramos que a suplementação com palmitato de retinol foi capaz de, principalmente cronicamente, induzir comportamento tipo-ansiedade e de diminuir as atividades locomotora e exploratória dos animais. Estes dados podem ser indicativos de que um estado de depressão está afetando os animais, tendo em vista que ansiedade e diminuição nas atividades comportamentais básicas como locomoção e exploração (estado letárgico) compõem a lista de alterações cognitivas e motoras que se estabelecem no processo de depressão.

A passagem da vitamina A pela barreira hematoencefálica (BH) ocorre naturalmente, existindo proteínas ligadoras de retinol em células deste tecido (MacDonald, Bok e Ong, 1990). Após cruzar a BH, a vitamina será distribuída entre as diferentes regiões cerebrais em quantidades semelhantes, sendo que todas estas regiões apresentam o aparato enzimático necessário para transformar retinol em retinóides biologicamente mais ativos (McCaferry e Dräger, 1994; Lane e Bailey, 2005; McCaffery, Zhang e Crandall, 2006), os quais atuam via ativação dos seus respectivos receptores nucleares (Napoli, 1999). Então, é provável que todas estruturas cerebrais analisadas neste trabalho tenham recebido quantidades aumentadas de vitamina A perante o tratamento aplicado. Uma quantificação de retinóides não foi usada porque isto poderia sugerir que algum retinóide, se em concentração elevada, seria o responsável pelos efeitos observados. Sabe-se que muitos retinóides desempenham ação membranolítica, por exemplo, não sendo apenas um o responsável por tal efeito (Goodall, Fisher e Lucy, 1980). Além disso, muitos retinóides são altamente instáveis, não sendo possível detectá-los em amostras de tecidos.

Na região da substância negra, encontramos uma impressionante diminuição nas formas reduzidas de grupamentos tióis protéicos (ou seja, aqueles grupamentos -SH de resíduos de cisteína) (Cap. I; Fig. 1C). Parte deste efeito pode ser mediado por ligação de semi-quinonas a estes grupamentos, conforme proposto por (Lotharius e Brundin, 2002). Estas semi-quinonas podem se originar da auto-oxidação da dopamina, uma catecolamina produzida em grandes taxas na substância negra. Fisiologicamente, a dopamina é armazenada em vesículas ácidas que impedem a auto-oxidação da catecolamina. No entanto, um excesso de moléculas lipofílicas, como os retinóides, na substância negra, pode diminuir a integridade das membranas de tais vesículas, facilitando a liberação não específica de dopamina para o citoplasma, um local bem menos ácido e que,

conseqüentemente, facilita a auto-oxidação de dopamina em semi-quinonas, as quais são bastante reativas com grupamentos sulfidril protéicos (Smythies e Galzigna, 1998). De fato, alguns autores já mostraram um efeito membranolítico da vitamina A e de alguns de seus derivados em diferentes modelos experimentais, conforme mencionado acima. Além disso, tanto a ligação inespecífica de semi-quinonas aos grupos tióis protéicos quanto aumento em intermediários do processo de peroxidação lipídica (espécies reativas ao ácido tiobarbitúrico – TBARS, por exemplo) facilitam agregação de proteínas no citoplasma dos neurônios dopaminérgicos, e isto é observado, freqüentemente, no eixo nigro-estriatal de pacientes com doença de Parkinson ou de Alzheimer (Montine *et al.*, 1997; Völkel *et al.*, 2006). Além disso, aumento nos níveis de carbonilação de proteínas pode também diminuir a integridade celular, aumentando a taxa de morte de neurônios envolvidos no controle dos movimentos, que depende, em parte, da homeostasia da substância negra e do estriado.

No estriado, a suplementação com palmitato de retinol (após 7 e 28 dias) induziu uma diminuição na capacidade antioxidante não-enzimática, que pode ser composta por vitaminas e agentes redutores como a glutationa reduzida (GSH) (Cap. II; Fig. 5). No entanto, não observamos diminuição na concentração de grupamentos tióis reduzidos não-protéicos, o que pode ser explicado por uma ausência na variação da atividade de GPx, enzima que, em conjunto da glutationa redutase (GR), remove o H_2O_2 produzido. Para isto, GSH é consumido pela GPx, sendo regenerado, às custas de NADPH, de novo em GSH (Halliwell, 2006). Assim como na substância negra, encontramos, no estriado dos animais tratados com palmitato de retinol em qualquer dose, e após todos os períodos analisados, uma diminuição considerável na concentração de grupamentos tióis reduzidos em proteínas (Cap. II; Fig. 3). A elevada concentração de dopamina, nestes tecidos, pode favorecer a oxidação, ou mesmo outra reação que diminua a leitura destes grupamentos no

espectrofotômetro, já que aumento nas taxas de dopamina livre no citosol favorece formação de semi-quinonas, moléculas com elevada afinidade aos grupos tióis protéicos.

O hipocampo também se mostrou sensível ao tratamento utilizado em relação ao ambiente redox. No entanto, observamos uma diminuição drástica nos níveis de grupamentos sulfidril protéicos somente agudamente (Cap. III; Fig. 1C). Nos períodos de 7 e de 28 dias de suplementação com palmitato de retinol, os níveis de sulfidril protéicos aumentaram nos animais tratados quando comparados com aqueles animais que receberam a vitamina por 3 dias, mas continuaram menores que os níveis encontrados no controles. A peroxidação lipídica e a carbonilação de proteínas aumentaram em relação ao controle em todos os períodos analisados e em qualquer dose utilizada (Cap. III; Fig. 1A e B). Novamente, não encontramos qualquer adaptação ao insulto oxidativo provocado pela suplementação. Mais uma vez, também encontramos desequilíbrio na relação SOD/CAT, ou seja, o tratamento induz um estado facilitador de produção de H_2O_2, que pode favorecer aumento na formação de $OH^{\bullet}$ via reação de Fenton ou de Haber-Weiss (Cap. III; Fig. 2D; Halliwell, 2006).

Interessantemente, foi observado que, durante os tratamentos diários, alguns animais se comportavam de maneira passiva em relação ao tratamento, o qual é, de certa forma, desconfortável, já que o animal recebe no seu trato digestório uma cânula de metal longa, mas sem capacidade de perfuração. Assim, foi decidido investigar alguns parâmetros comportamentais como atividades locomotora e exploratória (campo aberto) e comportamento tipo-ansiedade (caixa claro-escuro) neste estudo. Foi verificado que, principalmente após o tratamento crônico (28 dias), um estado de letargia e de ansiedade se estabelecia nos animais tratados com palmitato de retinol em qualquer dose (Cap. III;

Tabela 4). Isto foi observado por meio de contagem, na caixa claro-escuro: do número de vezes que o animal realizava “groomings” (atividade de limpeza que o animal realiza quando está tranqüilo); número de cruzamentos (“crossings”) entre linhas demarcadas no chão da caixa; número de comportamentos exploratórios (“rearings” – atividade na qual o animal fica sobre suas duas patas posteriores observando o meio); tempo que o animal demorava em deixar o campo claro pela primeira vez; número de fezes em cada compartimento; número de passagens evitadas entre um compartimento e outro, e número de transições entre um compartimento e outro. O teste da caixa claro-escuro se baseia no fato de que o animal normal prefere o ambiente escuro e menor para permanecer por mais tempo, pois este o exporia menos aos riscos do ambiente. Assim, verificamos que os animais tratados com palmitato de retinol em qualquer dose, e principalmente após 28 dias de tratamento, permaneciam mais tempo no ambiente escuro, além de saírem mais rápido do ambiente claro em relação aos animais controle. Mesmo no ambiente que protegeria o animal, o número de locomoções e de explorações foi menor, além de o número de “groomings” também ser menor neste ambiente em relação aos animais controle (Cap. III; Tabelas 1, 2, 3 e 4; Fig. 3). O número de passagens evitadas pelos animais que estava no ambiente escuro foi maior entre aqueles tratados com a vitamina que nos animais controle (Cap. III; Fig. 4). Todos estes resultados sugerem, fortemente, que se estabeleceu um comportamento tipo-ansiedade entre os animais tratados em longo prazo com palmitato de retinol em todas as doses investigadas. No campo aberto, diminuição nas atividades locomotora e exploratória também foram verificadas, sugerindo, além de ansiedade, comportamento tipo-depressão (Cap. III; Tabela 4).

Um aumento na produção de espécies reativas ou de radicais livres pode influenciar de diferentes maneiras no destino celular. Um pulso de produção de espécies reativas pode,

por exemplo, disparar o processo apoptótico dependente de NFkB (Fink e Cookson, 2005), além de este pulso também ser necessário para induzir desligamento de citocromo c a partir da cardiolipina, com conseqüente liberação deste fator pró-apoptótico para o citosol estimulando ativação de pró-caspase-9 via apoptossomo (Hengartner, 2000; Zanzami e Kroemer, 2001). No entanto, se o estado pró-oxidante se mantiver, ou ainda for aumentado, poderá ocorrer inibição da apoptose, pois as caspases, enzimas efetoras da apoptose, são sensíveis à oxidação, já que seu sítio ativo conta com um resíduo de cisteína, a qual apresenta um grupamento tiol reduzido (Halliwell e Gutteridge, 1999). Se este resíduo sofrer oxidação, a caspase terá sua atividade inibida, com conseqüente diminuição na capacidade de a célula manter o processo apoptótico. Assim, morte celular por necrose passa a ocorrer, levando o tecido a sofrer um processo inflamatório. De fato, nos processos neurodegenerativos, existe um aumento considerável na taxa de morte celular via necrose, o que pode explicar a manutenção da inflamação observada (Berg, Youdim e Riederer, 2004; Halliwell, 2006). Então, a partir dos dados encontrados neste trabalho, recomendamos cautela em relação ao uso de vitamina A na forma hidrossolúvel como agente antioxidante em indivíduos normais ou em tratamento com esta vitamina, ou mesmo em pacientes sofrendo de distúrbios neurodegenerativos, uma vez que tal composto pode facilitar, ou mesmo manter, um processo pró-oxidante que pode participar de um processo inflamatório já existente.

Trabalhos mostram que tanto o palmitato de retinol, quanto um derivado da vitamina A utilizado cronicamente no tratamento de acnes, são capazes de induzir déficits nas capacidades de aprendizado e de memória e, além disso, depressão e ansiedade tanto em animais quanto em humanos (McCaffery *et al.*, 2003; McCaffery, Zhang e Crandall, 2006). Recentemente, uma meta-análise mostrou que as formas hidrossolúveis de vitamina

A comercializadas seriam mais tóxicas que aquelas solúveis em lipídios (Myhre *et al.* 2003). No entanto, ainda não é claro o mecanismo de tal toxicidade. Neste trabalho, utilizamos uma forma hidrossolúvel de vitamina A, o Arovit®, o qual é facilmente encontrado em drogarias, ou seja, está disponível a todos os indivíduos que necessitam de tratamento com o mesmo. Infelizmente, o uso inadvertido desta droga ainda existe, embora o número de informações mostrando que a toxicidade é elevada e facilmente atingida. Terapeuticamente, o tratamento alternativo de leucemia pró-mielocítica com altas doses de vitamina A em diferentes formas, inclusive palmitato de retinol, tem se mostrado eficaz no parâmetro objetivado (Tsunati *et al.*, 1990; Tsunati *et al.*, 1991; Norum, 1993; Fenaux, Chomienne e Degos, 2001). No entanto, doses semelhantes, e até mesmo mais baixas, mostraram-se indutoras de efeitos indesejáveis, tais como aumento na irritabilidade dos pacientes (Myhre *et al.*, 2003). Além disso, foi sugerido que doses de palmitato de retinol de até 8500 UI/kg/dia seriam bem toleradas por bebês prematuros que necessitassem ganhar peso (Mactier e Weaver, 2005).

No entanto, Crandall e colaboradores (Crandall *et al*., 2004) mostraram que, em camundongos, o tratamento com ácido retinóico 13-*cis* diminuía a progressão do ciclo celular em uma região hipocampal que ainda prolifera durante a fase adulta do animal. Além disso, este tratamento, que se baseava em uma dose terapêutica da droga, induziu aumento nos níveis de morte celular naquela mesma região. Conseqüentemente, a capacidade de aprendizado dos animais tratados foi diminuída, tendo em vista que o hipocampo é uma região plástica cerebral justamente para receber dados constantemente, o que faz parte das atividades de aprendizado e de memória cerebrais (McCafferry, Zhang e Crandall, 2005). Em 2006, O'Reilly e colaboradores (O'Reilly *et al*., 2006) observaram

que, após tratamento durante 28 dias também com ácido retinóico 13-*cis* em dose terapêutica, os animais apresentaram comportamentos tipo-depressão, com diminuição na reação ao estímulo estressante (teste de suspensão pela cauda). No que diz respeito ao estudo utilizando animais, a droga mais utilizada é o retinóico 13-*cis*. No entanto, em humanos é grande o número de trabalhos mostrando que palmitato de retinol apresenta inúmeros efeitos colaterais envolvendo perturbações cognitivas, além, é claro, dos efeitos mostrando sua toxicidade por meio de técnicas clássicas de investigação toxicológica (Geelen, 1979; Hazen *et al.* 1983; Adams, 1993; Holson *et al.* 1997; Hull e D'Arcy, 2003). Mesmo assim, o ambiente redox de regiões cerebrais isoladas nunca havia sido investigado previamente em modelos experimentais *in vivo*. Além disso, há grande confusão, na literatura, no que diz respeito à dosagem utilizada, pois as unidades citadas são várias, dificultando a escolha por uma metodologia ou outra. Então, decidimos estudar doses que, embora elevadas, são utilizadas em tratamentos como os já descritos (leucemia, bebês prematuros, distúrbios dermatológicos) mais o uso inadvertido da vitamina A, o qual excede em muito as dosagens recomendadas. Além disso, a escolha pelos períodos de tratamento (3, 7 e 28 dias) buscava evidenciar se as estruturas analisadas poderiam se adaptar ao insulto gerado. Em alguns tratamentos menos graves, a vitamina A na forma de palmitato de retinol é indicada por períodos curtos de tempo, inclusive alguns menores que uma semana. Mesmo em períodos curtos de exposição à droga, encontramos alterações significativas no que diz respeito ao ambiente redox das estruturas analisadas. É importante salientar que, dentro do período máximo de suplementação (28 dias), não houve qualquer adaptação à situação deletéria induzida. Talvez, em um período maior de exposição à droga, seria possível observar alguma adaptação, que poderia ser representada por aumento nas atividades de CAT e/ou de GPx.

Estes dados, incluindo as avaliações dos parâmetros redox sensíveis e dos comportamentais, foram demonstrados pela primeira vez no presente trabalho. Em um estudo prévio, os autores encontraram que vitamina A em doses mais baixas (em torno de 25000 UI) seria um agente antioxidante para o sistema nervoso de ratos submetidos a estresse (Zaidi e Banu, 2004). No entanto, estes autores realizaram análises com o cérebro total homogeneizado, ou seja, não estudaram estruturas isoladas. A maior crítica a esta metodologia é a de diferenças região-específicas podem ser perdidas quando misturadas a outras estruturas cerebrais. Além disso, como foi descrito acima, diferenças na forma de vitamina A administrada podem influenciar, fortemente, nos resultados observados. Sendo assim, este trabalho contribuiu com a literatura mostrando dados ainda não antes publicados, além de alertar ao uso inadvertido de vitamina A, ou de seus derivados, já que esta forma de tratamento (via intragástrica, palmitato de retinol hidrossolúvel) pode dar origem a diversos metabólitos da vitamina A, incluindo formas muito instáveis e, portanto, muito reativas, as quais podem induzir distúrbios nos níveis molecular, celular e funcional da estrutura onde são produzidos.

Sabendo que estresse oxidativo não é o único fator causador de distúrbios comportamentais, um co-tratamento com antioxidantes clássicos, como as vitaminas E e C simultaneamente, nos ajudaria a entender a importância da alteração redox do meio nas observações cognitivas e motoras observadas. Não podemos, inclusive, descartar a hipótese de que os retinóides produzidos a partir deste tratamento, nas regiões específicas que controlam aqueles comportamentos observados, possam estar agindo via receptor nuclear, a forma clássica de atuação da vitamina A.

Não realizamos uma quantificação dos níveis de retinóides porque, conforme citado acima, o número destas moléculas que podem se originar do tratamento utilizado é bastante

grande (Napoli, 1999), e algumas não são estáveis o suficiente para serem detectadas pela metodologia. Além disso, mesmo que um retinóide seja encontrado em concentração elevada na circulação ou em determinada região cerebral, isto não significa, diretamente, que aquele retinóide é o responsável pela toxicidade evidenciada. Outros trabalhos mostraram que, mesmo em situações de hipervitaminose A, a concentração de retinol, na circulação, não estava acima dos valores normais, ou seja, este parâmetro nem sempre está associado com o processo de toxicidade (Ellis *et al.,* 1986; Croquet, Pilette e Lespine, 2000; Mills e Tanumihardjo, 2006).

Então, com base nos resultados apresentados, mostramos que a vitamina A, em doses que antes eram tidas como seguras ao organismo humano, induziram estresse oxidativo em diferentes regiões cerebrais e distúrbios cognitivos e/ou motores em ratos adultos. Doses elevadas de vitamina A, e de alguns de seus retinóides (como o ácido retinóico 13-*cis*), são utilizadas terapeuticamente por períodos muito variados, desde dias até meses. Como conseqüência dos tratamentos crônicos, já foram encontrados efeitos colaterais envolvendo a função cerebral humana em adultos, tais como irritabilidade, depressão e ansiedade. No entanto, tem-se um grande problema quando doses elevadas destas drogas são aplicadas como tratamento a bebês prematuros. Nestes pacientes, o diagnóstico de alterações cognitivas é mais complexo, e poderá surgir num estágio diferente de desenvolvimento da criança, inclusive durante sua fase adulta de vida, já que a vitamina A desempenha um importante papel no desenvolvimento do SNC, e uma perturbação de sua homeostasia pode induzir alterações cognitivas futuras, muitas vezes sem reversão.

4. CONCLUSÕES

A partir dos resultados obtidos no presente trabalho, podemos concluir que:

1) A suplementação com vitamina A, tanto nas doses consideradas terapêuticas quanto naquelas excessivas – geralmente atingidas em tratamentos prolongados, ou devido ao uso inadvertido da vitamina – é capaz de induzir aumento nos marcadores de estresse oxidativo mesmo em períodos agudos de tratamento – 3 dias. Além disso, não foi observada qualquer adaptação ao insulto induzido, já que os marcadores de peroxidação lipídica, de carbonilação de proteínas e de estado redox de grupamentos tióis não retornaram a valores de controle em 28 dias de tratamento;

2) O tratamento foi capaz de modular a atividade de enzimas antioxidantes nas estruturas cerebrais analisadas, com um aumento marcante na atividade de SOD, e uma diminuição na atividade de CAT, sem alterar (em quase todos os casos) a atividade da GPx. Tais resultados podem indicar um aumento na produção de $O_2^{\bullet-}$, com conseqüente metabolização em H_2O_2 pela SOD, que parece não ser metabolizado a água em taxas normais devido à baixa atividade da CAT. Assim, uma facilitação na reação de Fenton pode ser esperada, onde íons como Fe^{2+} ou Cu^{2+}, ao reagirem com o H_2O_2, podem originar o radical $OH^{\bullet}$, o mais deletério dos radicais livres;

3) A suplementação crônica com vitamina A foi capaz de induzir um comportamento tipo-ansiedade nos animais tratados, desde doses terapêuticas até as excessivas. Além disso, um estado de letargia foi observado no campo aberto, onde os animais podem explorar o meio livremente. Todos estes resultados podem indicar aumento nos níveis de ansiedade dos animais, mas também podem indicar depressão.

4) Em suma, mostramos que a suplementação com vitamina A na forma hidrossolúvel (palmitato de retinol – Arovit®) foi capaz de induzir um estado pró-oxidante em todas as três estruturas cerebrais de rato apresentadas nesta Dissertação: substância negra, estriado e hipocampo. É importante salientar que não houve adaptação ao insulto induzido, pois, em alguns casos, o dano oxidativo encontrado após três dias de suplementação é muito semelhante ao observado após 28 dias de suplementação. Também é interessante mencionar que o motivo que favorece o dano agudo nem sempre é o mesmo que favorece aquele crônico, ou seja, a disfunção observada cronicamente pode ser resultado de uma perturbação à homeostasia de organelas celulares, por exemplo. Ainda, mostramos que esta suplementação induziu diminuição nas capacidades locomotora e exploratória dos animais, além de induzir comportamento tipo-ansiedade. Este trabalho abre uma larga margem para futuros estudos considerando vitamina A e SNC, além de outros tecidos animais, é claro.

5. PERSPECTIVAS

Os resultados obtidos no presente trabalho abrem espaço para pesquisas mais apuradas no que diz respeito à variação no comportamento dos animais, já que são muitos os parâmetros moleculares que podem estar alterados, resultando em comportamento tipo-ansiedade, por exemplo. Além disso, ainda resta investigar se os efeitos encontrados aqui são dependentes da ligação de vitamina A, ou de seus retinóides, aos respectivos receptores nucleares. Com principais perspectivas de continuação deste trabalho, temos:

1) Analisar as taxas de morte celular na estruturas investigadas neste trabalho, incluindo outras já em fase de estudos, como córtex cerebral frontal e cerebelo, já que diminuição na quantidade de células neuronais pode fazer parte do mecanismo de alteração comportamental. Além disso, pretendemos diferenciar morte celular induzida por inflamação (via extrínseca) daquela dependente de liberação de citocromo c a partir de mitocôndrias (via intrínseca);

2) Quantificar o imunoconteúdo de alfa e beta sinucleínas, já que o acúmulo das sinucleínas é favorecido em situações de estresse oxidativo, com conseqüente alteração comportamental em animais e em humanos. O conteúdo do receptor D2 será investigado porque sua expressão pode ser modulada por retinol ou retinóides, e tanto um excesso na quantidade deste receptor, quanto uma diminuição nestes níveis, são observados em patologias nervosas.

3) Investigar o funcionamento da cadeia transportadora de elétrons nas estruturas já investigadas neste trabalho, além de outras também

importantes para uma melhor compreensão dos efeitos gerados através da suplementação com vitamina A;

4) Investigar o comportamento tipo-depressão nos animais tratados com vitamina A por meio de técnicas específicas de abordagem deste distúrbio cognitivo. Além disso, pretendemos analisar as capacidades de aprendizado e de memória dos animais tratados com vitamina A em relação aos animais controle.

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