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PROGRAMA DE PÓS-GRADUAÇÃO EM
CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Efeitos do tratamento com palmitato de retinol para ratas Wistar durante a gestação e amamentação sobre parâmetros de estresse oxidativo no sistema nervoso central e sobre avaliações comportamentais dos filhotes

CARLOS EDUARDO SCHNORR

Porto Alegre, fevereiro de 2011

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“A ciência nunca resolve um problema sem criar pelo menos outros dez”

George Bernard Shaw

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RESUMO

A vitamina A é um nutriente essencial o qual deve ser obtido na dieta em quantidades adequadas para a manutenção da saúde. Ela é necessária para diversos processos biológicos, incluindo a reprodução, o sistema imune, a visão, assim como a manutenção da diferenciação celular. De fato, a vitamina A é importante para o desenvolvimento e manutenção da homeostase do cérebro adulto, mas a exposição ao excesso de vitamina A tem sido relacionado a déficits cognitivos e defeitos congênitos, incluindo anomalias morfológicas neuronais. Adicionalmente, pequenas variações no status nutricional materno têm sido demonstradas como sendo capazes de afetar parâmetros fisiológicos e metabólicos no feto. As evidências sugerem um papel importante do estresse oxidativo nestes eventos. Recentemente, nós também demonstramos que a suplementação com vitamina A é capaz de alterar parâmetros comportamentais e induzir um estado pró-oxidante em muitos tecidos de ratos Wistar machos adultos. Então, o objetivo deste trabalho foi investigar os efeitos da suplementação com vitamina A em ratas gestantes e lactantes sobre o comportamento e tecidos das mães e dos filhotes, especialmente sobre o sistema nervoso central. Ratas Wistar (7 por grupo) foram suplementadas oralmente com palmitato de retinol (2.500, 12.500 e 25.000 UI/kg/dia) ou salina (controle) durante toda a gestação e amamentação. Ninhadas tratadas apresentaram alterações comportamentais no teste de Homing e ambos, mães e ninhadas, apresentaram alterações comportamentais nos parâmetros do teste de Campo Aberto. A suplementação com vitamina A durante a gravidez e amamentação não apresentou efeitos tóxicos severos em parâmetros reprodutivos, mas apresentou modulação de atividades enzimáticas (catalase, superóxido dismutase e glutationa-S-transferase) e também aumento nos marcadores de estresse oxidativo (lipoperoxidação, carbonilação proteica e mudanças no conteúdo de tióis reduzidos), especialmente no estriado e hipocampo das mães e das ninhadas. Em conclusão, nós sugerimos alguma cautela a respeito da suplementação com vitamina A durante a gestação e amamentação, pois o estresse oxidativo pode causar distúrbios em diversos fenômenos biológicos, incluindo sinalização neuronal e neurotransmissão, as quais podem induzir diversos déficits comportamentais. Entretanto, a completa extensão das modulações redox apresentadas neste estudo não é conhecida e novos estudos são necessários para avaliar seus efeitos sobre a saúde e o bem-estar.

ABSTRACT

Vitamin A is an essential nutrient that must be provided in the diet in sufficient amounts for the maintenance of health. It is required for several life processes, including reproduction, the immune system, vision, as well as the maintenance of cellular differentiation. Indeed, vitamin A is important for both development and maintenance of adult brain homeostasis, but excessive vitamin A exposure has been linked to cognitive impairments and congenital defects, including neuronal malformations. Additionally, subtle variations in the status of maternal nutrition had been demonstrated to affect physiological and metabolic parameters in the fetus. Evidence suggests a key role for oxidative stress in these events. Recently, we also demonstrated that vitamin A supplementation is able to alter behavioral parameters and induce a pro-oxidant state in many tissues of adult male Wistar rats. Thus, the aim of the present work was to investigate the effects of vitamin A supplementation in pregnant and nursing rats on maternal and offspring behavior and tissues, specially the central nervous system. Wistar female rats (7 per group) were orally supplemented with retinyl palmitate (2500, 12500 and 25000 IU/kg/day) or saline (control) throughout pregnancy and nursing. Treated offspring showed behavioral alterations in the Homing test and both, dams and offspring, showed alterations in the open field test scores. Vitamin A supplementation during pregnancy and nursing showed no overtly toxic effects on reproductive parameters, but showed modulation of enzymatic activities (catalase, superoxide dismutase and glutathione-S-transferase) and also increased oxidative damage markers (lipoperoxidation, protein carbonilation, reduced thiol content changes), specially in maternal and offspring striatum and hippocampus. In conclusion, we suggest some caution regarding vitamin A intake during pregnancy and breastfeeding, since oxidative stress can disturb several biological phenomena, including neuronal signaling and neurotransmission, which may induce several behavioral deficits. However, the full extent of the redox modulations showed in this study is not well known and further research are needed to ascertain its effects on overall health and well-being.

LISTA DE ABREVIATURAS

BH – barreira hemato-encefálica
CAT – catalase
GPx – glutationa peroxidase
GST – glutationa-S-transferase
LOAEL – menor dose teratogênica
RBP - proteínas ligantes de retinol
RDA - consumo diário recomendado
SNC – sistema nervoso central
SOD – superóxido dismutase

SUMÁRIO

1. INTRODUÇÃO

1.1 Vitamina A

A vitamina A pertence ao grupo das vitaminas lipossolúveis. Por definição, as vitaminas não são sintetizadas *de novo* em quantidades suficientes pelo nosso organismo, mas devem ser obtidas através da dieta. No caso, a vitamina A pode ser obtida nos alimentos tanto como pré-vitamina A, na forma de retinol ou éster de retinol, provenientes de alimentos de origem animal, ou como pró-vitamina A proveniente de alimentos de origem vegetal, na forma de carotenóides que serão convertidos a vitamina A no organismo (Bellovino, 2003). Entretanto, a vitamina A pré-formada é absorvida e utilizada de forma mais eficiente pelo nosso organismo, possuindo taxas de absorção entre 70-90% enquanto a pró-vitamina A possui taxas de absorção entre 20–50%, dependendo do status de vitamina A de cada indivíduo e outros fatores nutricionais e não-nutricionais (Penniston e Tanumihardjo, 2006).

A vitamina A e os retinóides, compostos naturais e sintéticos possuindo uma estrutura química ou propriedades funcionais similares a vitamina A, são importantes em diversos processos biológicos, incluindo a reprodução, a regulação do sistema imune e a visão, assim como a manutenção do crescimento e da diferenciação celular (Wasserman e Corradino, 1971). De particular importância para a saúde pública é o papel da vitamina A durante períodos de grande crescimento proliferativo e durante o desenvolvimento dos tecidos, como na gestação, no período neonatal e na infância.

Atualmente, entretanto, pouco se conhece sobre o padrão de aquisição dos diferentes retinóides pelo embrião, ou seu papel exato durante o desenvolvimento embriônico. Satre e colaboradores (1992) em seu estudo mostraram que o retinol cruza a placenta e alcança o embrião, em um fluxo bidirecional que favorece o feto, e que parece envolver, pelo menos em parte, as holo-proteínas ligantes de retinol (RBP)

maternas. Portanto, o retinol ligado a RBP é tido como o retinóide fisiologicamente mais importante transportado da mãe para o feto, assim como dentro do feto. Entretanto, Quadro e colaboradores (2005) mostraram que a RBP materna parece não ser capaz de cruzar a placenta. Em seu estudo eles revelaram que, para adentrar na circulação fetal, o retinol materno ligado a RBP é liberado na interface materno-fetal, onde ele liga-se ao RBP do tecido placentário e pode ser secretado na circulação fetal.

Por outro lado, enquanto a rota do retinol-RBP materno é a principal contribuinte para o desenvolvimento fetal, a rota do éster de retinol tem sido apontada como a principal responsável pelo acúmulo das reservas de retinóides fetal (Quadro et al., 2005). Além disso, Quadro e colaboradores (2005) também demonstraram que, na ausência de RBP maternal, o éster de retinol circulante, que é encontrado em níveis significativamente elevados, pode representar a principal fonte disponível para o desenvolvimento embrionário. Outros estudos apontam ainda que a RBP embrionária exerce um papel chave na distribuição de vitamina A durante o desenvolvimento quando o suporte materno de vitamina A é inadequado (Leung et al., 2004).

Além disso, próximo ao final da gestação, o status nutricional e o hábito alimentar materno continuam importantes para maximizar a vitamina A transferida para o feto em preparação para o parto e para a lactação (Underwood, 1994). É importante salientar que, durante o período neonatal, o leite materno é muitas vezes a única fonte de vitamina A para o neonato. Portanto, a capacidade de obter os requerimentos do infante depende muitas vezes da concentração e do volume consumido, sendo que ambos são influenciados pelo status nutricional e hábito alimentar da mãe.

1.2 Deficiência

A deficiência de vitamina A é considerada pela Organização Mundial da Saúde um grande problema de saúde pública. Ela é considerada endêmica em países pobres, mas sabe-se que a deficiência de vitamina A subclínica afeta também populações nutricionalmente desfavorecidas em países desenvolvidos e países industrializados (Quadro et al., 2005). Atualmente, estima-se que pelo menos 100 milhões de crianças abaixo dos 5 anos no mundo sejam deficientes em vitamina A (Zhang et al., 2007). O leite materno, os alimentos enriquecidos ou fortificados, e suplementação vitamínica estão entre as intervenções mais comuns para combater a deficiência (WHO, 2009).

A hipovitaminose A é capaz de aumentar significativamente as taxas de mortalidade materna, de reabsorção e mortalidade fetal, de morbidade e mortalidade infantil, assim como diminuição do peso ao nascimento (Wasserman e Corradino, 1971; Clagett-Dame e DeLuca, 2002). Além disso, pode provocar também falha na segmentação e crescimento embrionário, interrupção na vascularização, retardo no crescimento, metaplasia esquamosa glandular e epitelial, assim como uma série de defeitos congênitos afetando o sistema ocular, esquelético, cardíaco, respiratório, urogenital e nervoso central, inclusive provocando a degeneração dos neurônios motores (Ross et al., 2000; Mark et al., 2006).

1.3 Excesso

Assim como a deficiência, o excesso de vitamina A também é conhecido por uma série de efeitos deletérios para a saúde. As intoxicações agudas com a vitamina A ocorrem quando um indivíduo ingere grandes quantidades da vitamina em um curto período de tempo, mas casos de intoxicação aguda são relativamente raros. Por outro lado a intoxicação crônica parece ser um problema mais grave, como Moise e colaboradores (2007) mostraram em sua revisão bastante recente. Entre os inúmeros

efeitos, são observados o ressecamento, ulceração e descamação da pele, anorexia, anemia e perda óssea. O excesso de vitamina A pode também provocar dano ao fígado, causando fibrose e ativação de células hepáticas estreladas, que perdem sua capacidade de armazenar éster de retinol e passam a secretar colágeno, causando cirrose. Além disso, observa-se a estimulação da formação e da atividade de osteoclastos, levando a reabsorção óssea e hipercalcemia. Adicionalmente, a administração de retinóides em excesso para jovens e crianças pode resultar em retardo no crescimento.

Existem também inúmeras evidências para efeitos teratogênicos relacionados ao excesso de vitamina A e diversos dos seus metabólitos. Sabe-se que o excesso de vitamina A é capaz de afetar estruturas de uma forma bastante similar a deficiência, provocando anormalidades nas estruturas craniofaciais, esqueléticas, cardíacas, tímicas e o sistema nervoso central (SNC) (Ross et al., 2000). Em humanos, estas evidências estão muito bem documentadas para os efeitos teratogênicos de retinóides naturais e sintéticos utilizados em testes clínicos, como o ácido trans-retinóico e o ácido 13-cis retinóico, assim como seus oxo-derivados, mas as evidências diretas de que o excesso de vitamina A em humanos, na forma de retinol ou éster de retinol, tenha provocado efeitos teratogênicos são limitadas (Moise et al., 2007; WHO, 2009).

Entretanto, embora a deficiência de vitamina A seja decididamente um problema maior, principalmente nos países em desenvolvimento, e esteja recebendo muitas iniciativas em termos de saúde pública, a ingestão de excesso de vitamina A pode ser um problema crescente e parcialmente negligenciado. Atualmente, alguns autores estimam que mais de 75% da população em países desenvolvidos esteja consumindo quantidades de vitamina A acima do consumo diário recomendado (RDA), através do consumo de alimentos que contém vitamina A, principalmente na forma de palmitato de retinol (Penniston e Tanumihardjo, 2006). Adicionalmente, indícios apontam que em

alguns países, como os Estados Unidos, aproximadamente 25% dos adultos ingerem rotineiramente suplementos contendo vitamina A e aproximadamente 5% destes ingerem suplementos contendo apenas vitamina A (Rothman et al., 1995). Além disso, os retinóides são muito consumidos como drogas terapêuticas, possuindo uma eficácia comprovada no tratamento de diversos distúrbios dermatológicos e na prevenção/tratamento de câncer, especialmente da leucemia pro-mielocística (Inomata et al., 2005).

1.4 Sistema nervoso central

A vitamina A, em quantidades adequadas, é muito importante tanto para o desenvolvimento quanto para a manutenção da homeostasis do SNC durante a vida adulta (McCaffery et al., 2003; Lane e Bailey, 2005). No SNC, a vitamina A é importante para todo o processo de diferenciação celular e apoptose, este último de grande importância durante o desenvolvimento. Além disso, quantidades adequadas de vitamina A são essenciais para a manutenção tanto da função estriatal quanto da neurogênese hipocampal do adulto (Takahashi et al., 1999; Wang e Liu, 2005; McCaferry et al., 2006). Durante a vida adulta, a vitamina A também é importante para um grande número de funções relacionadas ao SNC, como o aprendizado, a memória, e a função locomotor/exploratória (Chiang et al., 1998; Cocco et al., 2002). Entretanto, o excesso de vitamina A tem sido relacionado a inchaço cerebral (*pseudotumor cerebri*), tontura, irritabilidade, ansiedade e depressão, chegando a ser relacionada com o aumento dos índices de suicídio (Allen e Haskell, 2002; Hull e D'Arcy, 2003).

De fato, o aparato molecular, incluindo receptores nucleares desta vitamina, é encontrado, em diferentes níveis, em todas as regiões cerebrais de mamíferos adultos (Zetterström et al., 1999). A vitamina A e os retinóides ultrapassam facilmente a

barreira hemato-encefálica (BH) e o SNC dos mamíferos possui todo o aparato molecular responsável pela produção e manutenção do ácido all-trans-retinóico nos neurônios, através da atividade da retinal desidrogenase e da ação das RBP celular (Zetterström et al., 1999; Duester, 2000). Portanto, o SNC é totalmente capaz de transportar e metabolizar moléculas de retinóides, aumentando rapidamente suas concentrações.

1.5 Recomendações

Uma ingestão adequada de vitamina A é essencial para a saúde, incluindo a saúde do embrião e do feto. Portanto, existe um interesse muito grande por parte de diversas instituições internacionais em estabelecer recomendações quanto ao consumo normal diário para crianças, adultos de diversas faixas etárias e para gestantes. Atualmente, o consumo diário recomendado (RDA) para homens adultos atualmente é de aproximadamente 3.330 UI e para mulheres de 2.670 UI de vitamina A, valor baseado no peso corporal médio (Trumbo et al., 2001).

Entretanto, sabe-se que durante a gestação é necessária uma quantidade superior de vitamina A para dar suporte aos processos reprodutivos maternos, incluindo o crescimento fetal e o desenvolvimento, assim como é necessário repor a perda no leite materno durante a lactação (Underwood, 1994). Apesar disso, hoje a maior parte das instituições internacionais considera que este aumento durante a gestação é pequeno e que pode ser facilmente obtido através de uma dieta balanceada e pelas reservas maternas de mulher bem nutridas (IVACG, 1998; WHO, 2009). Portanto, o RDA durante a gestação parece não ser muito diferente daquele anterior a gestação.

Existem também recomendações quanto à quantidade máxima de vitamina A que pode ser consumida de forma segura por gestantes e a quantidade que representaria

um risco teratogênico. Embora atualmente não exista um verdadeiro consenso, muitos estudos e instituições internacionais têm concordado que parece segura a administração de 10.000 UI/kg/dia para mulheres férteis, independente de seu status de vitamina A, durante qualquer período da gestação (IVACG, 1998; Dolk et al, 1999; Ross et al, 2000; WHO, 2009). Outros apontam que não existem evidências suficientes e consistentes para que a suplementação com vitamina A em doses de até 25.000 UI/dia possa ser consideradas teratogênica, mas que 50.000 UI/dia poderia representar um risco significante (Mills et al., 1997; Miller et al., 1998; Ritchie et al., 1998).

1.6 Espécies Reativas (radicais livres)

Um radical livre pode ser definido como qualquer espécie química (seja um átomo, um metal de transição ou uma molécula) que possua um ou mais elétrons desemparelhados no seu orbital molecular externo. Classicamente, os radicais livres são descritos com uma notação para a espécie química seguida de um ponto, o qual indica o elétron desemparelhado, por exemplo, o radical livre ânion superóxido: $\bullet O_2^-$. Quimicamente, este elétron desemparelhado confere uma reatividade relativamente alta a esta molécula, pois existe uma grande tendência desta molécula em adquirir um segundo elétron para este orbital (Halliwell, 2006).

Outra característica importante dos radicais livres é sua capacidade de sustentar reações em cadeia, onde uma molécula reduzida perde seu elétron para o radical livre, e aquela reduzida passa a ser em si um novo radical, podendo reagir com outro composto químico, sucessivamente. De uma forma geral, as reações que envolvem radicais livres podem ser divididas em: reações de iniciação; reações de propagação; e reações de terminação. Nas reações de iniciação, um radical livre é formado a partir de espécies químicas não-radicais (portanto estáveis): $AB + C \rightarrow \bullet A + D + E$. Nas reações de

propagação, um radical livre, também chamado de centro da reação, reage com uma molécula estável, resultando em outro radical livre, ou centro da reação: •A + CD → AC + •D. 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 (•H) é o mais reativo dos radicais livres. Por outro lado, o radical livre de ocorrência mais comum é o ânion superóxido ($\bullet O_2^-$), produzido quando uma molécula de oxigênio é parcialmente reduzida (recebe apenas um elétron). Dentro da célula, encontram-se diversas fontes de formação de radicais livres como, por exemplo, a cadeia transportadora de elétrons mitocondrial, onde a redução parcial do oxigênio forma $\bullet O_2^-$. Entretanto, apesar da mitocôndria ser considerada a fonte mais importante de produção de radicais livres endógenos, existem outras fontes que podem formar estas moléculas oxidantes como, por exemplo, a cascata do ácido araquidônico no citosol e algumas isoenzimas citocromo P-450 (Halliwell, 2006).

1.7 Defesas antioxidantes

Os radicais livres são capazes de provocar dano oxidativo a diversos componentes celulares, tais 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. Estas são as chamadas defesas antioxidantes, que podem ser tanto enzimáticas quanto não-enzimáticas (Halliwell, 2006). Entre as defesas enzimáticas podemos citar as enzimas superóxido dismutase, catalase,

glutationa peroxidase e glutationa-S-transferase. O tripeptídio glutationa (na forma reduzida, GSH) e as vitaminas (como o ácido ascórbico, e a vitamina E, por exemplo) representam as principais defesas antioxidantes não- enzimáticas.

Todas as enzimas são consideradas como defesas antioxidantes primárias, ou seja, elas atuam diretamente sobre a molécula do radical livre, antes que este possa vir a oxidar uma biomolécula. A enzima superóxido dismutase (SOD), que apresenta quatro classes, atua sobre o radical $\bullet O_2^-$**,** transformando-o em peróxido de hidrogênio (H_2O_2) e oxigênio através da seguinte reação: $\bullet O_2^- + \bullet O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. Já a enzima catalase (CAT), atua sobre o H_2O_2 gerado, transformando-o em água por meio da reação: $2H_2O_2 \rightarrow 2H_2O + O_2$. Por outro lado, as enzimas glutationa peroxidase (GPx) e glutationa-S-transferase (GST) também atuam sobre o H_2O_2, no entanto, por meio de um mecanismo diferente (Boveris, 1998).

1.8 Estresse oxidativo

Estresse oxidativo é o termo utilizado para descrever as situações onde a formação de radicais livres excede a capacidade de transformação destas moléculas em outras não oxidantes através das defesas antioxidantes. Nestas situações, costuma-se dizer que houve um desequilíbrio entre a formação de radicais livres e a atuação das defesas antioxidantes. O estresse oxidativo pode ocorrer por diversos motivos, como a produção excessiva de radicais livres, ou a inativação de enzimas, como a SOD e a CAT, onde as enzimas perdem sua característica nativa devido a reações destas com as próprias moléculas oxidantes, perdendo sua função como, por exemplo, a inativação da catalase por $\bullet O_2^-$ (Shimizu et al., 1984).

Atualmente, o estresse oxidativo tem sido continuamente apontado como fator importante envolvido em diversas condições patológicas em humanos, incluindo

doenças neurodegenerativas, como a doença de Alzheimer e a doença de Parkinson (McEligot et al, 2005; Halliwell, 2006; Upham e Trosko, 2009; Forman et al, 2010). Adicionalmente, o estresse oxidativo tem sido sugerido como fator chave entre diversos estímulos durante o período perinatal, como pequenas variações no status nutricional materno, por exemplo, e o fenômeno da reprogramação fetal (Allen e Balin, 1989; Myatt, 2010). A reprogramação fetal, por sua vez, também é capaz de induzir respostas permanentes, que podem produzir mudanças a longo-prazo na fisiologia e metabolismo dos tecidos, podendo predispor um indivíduo a diversas doenças durante a vida adulta (Lau e Rogers, 2004; Gluckman et al., 2005). Portanto, o estresse oxidativo pode estar envolvido direta ou indiretamente na iniciação e progressão de diversas patologias.

Além disso, a vitamina A é também uma molécula redox-ativa, a qual nosso grupo tem demonstrado ser capaz de induzir um estado pró-oxidante em concentrações não muito superiores aos níveis fisiológicos em diferentes modelos experimentais in vitro (Moreira et al., 1997; Dal-Pizzol et al., 2001; Frota et al., 2004; Zanotto-Filho et al., 2008). Efeitos pró-oxidantes do tratamento com vitamina A incluem aumento na peroxidação lipídica e na carbonilação proteica, dano ao DNA e modificação na atividade de enzimas antioxidantes em cultura de células de Sertoli (Dal-Pizzol et al., 2000, 2001). Recentemente, nós também demonstramos que a suplementação com vitamina A em doses clínicas induz um estado pró-oxidante em diferentes tecidos, como o pulmão e o coração, assim como em diferentes regiões do sistema nervoso central de ratos Wistar adultos (De Oliveira e Moreira, 2007; De Oliveira et al., 2007a,b, 2008). Além disso, a suplementação com vitamina A induziu comportamentos do tipo ansiedade e diminuição nas atividades locotomora e exploratória em animais tratados por 28 dias (De Oliveira et al., 2007a,b, 2008).

2. OBJETIVOS

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. Estes dados mostram que, em diferentes modelos experimentais, o tratamento com vitamina A pode induzir pulsos de estresse oxidativo, inclusive no SNC de ratos Wistar adultos suplementados com doses terapêuticas de palmitato de retinol.

Então, a partir dos resultados previamente publicados, hipotetizamos que a vitamina A, em doses de equivalência aproximada com aquelas doses consideradas seguras para a suplementação de mulheres férteis, independente do seu status nutricional, pudesse induzir estresse oxidativo sobre o SNC e demais tecidos dos filhotes de ratas Wistar suplementadas durante a gestação e lactação com palmitato de retinol (Arovit®).

2.1 Objetivos específicos

Assim, neste trabalho analisamos os efeitos da suplementação diária com vitamina A, via intragástrica, nas doses de 2.500, 12.500 ou 25.000 UI/kg/dia durante a gestão e lactação sobre os seguintes parâmetros:

1) Peroxidação lipídica, carbonilação de proteínas, tióis reduzidos totais e potencial antioxidante redutor total no fígado, rim, coração, plasma e nas estruturas cerebrais (hipocampo e estriado) das mães e dos seus filhotes, assim como nos tecidos reprodutivos (útero e ovários) das mães;

2) Modulação das defesas antioxidantes enzimáticas: superóxido dismutase (SOD), catalase (CAT) e glutationa-S-transferase (GST) nos mesmos tecidos e estruturas mencionadas acima;

3) Modificações comportamentais nos testes de Homing, com o objetivo de avaliar o desenvolvimento do aprendizado olfativo pelos filhotes, e Campo Aberto, para avaliar atividade locomotor/exploratória, assim como comportamentos do tipo-ansiedade nas mães e nos filhotes.

3. MATERIAIS E MÉTODOS

Os materiais e métodos utilizados nesta dissertação estão redigidos em detalhes nos artigos científicos que compõem o item 4 (RESULTADOS). A seguir os métodos são apresentados de forma resumida.

3.1 Modelo Experimental

Ratas Wistar suplementadas durante toda a gestação e lactação com vitamina A (palmitato de retinol), em doses com equivalência aproximada com doses consideradas seguras para serem administradas durante a fase reprodutiva para humanos, independente do seu status de vitamina A. O tratamento foi sempre administrado no período noturno do ciclo, pois se sabe que a absorção da vitamina A é favorecida quando administrada junto às refeições. Como ratos são animais de hábitos noturnos, alimentam-se principalmente no início do período noturno. Durante todo o tratamento os animais foram diariamente monitorados quanto a sinais clínicos de toxicidade e diversos parâmetros de toxicidade reprodutiva foram analisados ao longo do estudo.

3.2 Testes Comportamentais

Os testes comportamentais foram conduzidos em diferentes fases do desenvolvimento dos filhotes e ao final do tratamento para as mães e para os filhotes a fim de analisar alterações cognitivas e/ou motoras nestes animais associadas ao tratamento. Embora os testes selecionados não sejam testes muito específicos, são testes muito aplicados em estudo de teratologia comportamental e servem como bons indicadores para a existência de efeitos adversos associados ao tratamento.

3.3 Análises Bioquímicas

Após as observações comportamentais, os animais foram sacrificados, as estruturas de interesse removidas cirurgicamente, homogeneizadas em tampão fosfato, pH 7,4, e mantidas em congelamento de -80°C até o dia das análises. Investigamos parâmetros redox, 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 e GST).

4. RESULTADOS

Os resultados desta Dissertação estão formatados em dois artigos científicos: um publicado e outro submetido para publicação.

Artigo I

“Vitamin A supplementation in rats under pregnancy and nursing induces behavioral changes and oxidative stress upon striatum and hippocampus of dams and their offspring”

Artigo publicado no periódico Brain Research, volume 1369, p. 60-73 (2011).

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BRAIN RESEARCH

Research Report

Vitamin A supplementation in rats under pregnancy and nursing induces behavioral changes and oxidative stress upon striatum and hippocampus of dams and their offspring

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ABSTRACT

Vitamin A is important for both development and maintenance of adult brain homeostasis. However, excessive vitamin A exposure has been linked to cognitive impairments and may induce congenital defects, including neuronal malformations. Recently, we demonstrated that vitamin A supplementation is able to alter behavioral parameters and induce a pro-oxidant state in hippocampus and striatum of adult male rat. Thus, the aim of the present work was to investigate the effects of vitamin A supplementation in pregnant and nursing rats on maternal and offspring striatum and hippocampus. Wistar female rats (7 per group) were orally supplemented with retinyl palmitate (2500, 12,500 and 25,000 IU/kg/day) or saline (control) throughout pregnancy and nursing. Homing test was performed at postnatal days (PND) 5 and 10 for offspring, while open field test (OFT) was carried out at PND19 and 20 for dams and offspring, respectively. Redox parameters were evaluated at PND21 for both. Vitamin A supplementation during pregnancy and nursing increased superoxide dismutase/catalase (SOD/CAT) ratio and oxidative damage in maternal and offspring striatum and hippocampus. Additionally, supplementation induced behavioral alterations. In conclusion, we suggest some caution regarding vitamin A intake during pregnancy and breastfeeding, since oxidative stress can disturb several biological phenomena, including neuronal signaling and neurotransmission, which may induce several behavioral deficits.

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Abbreviations: CNS, central nervous system; ROS, reactive oxygen species; RNS, reactive nitrogen species; AD, Alzheimer's disease; PD, Parkinson's disease; GD, gestational day; PND, post natal day; OFT, open field test; CAT, catalase; SOD, superoxide dismutase; GST, glutathione S-transferase; TRAP, total reactive antioxidant potential; TBARS, thiobarbituric acid reactive species; LOAEL, lowest observed adverse effect level; $\bullet O_2^-$, superoxide radical; H_2O_2, hydrogen peroxide; •OH, hydroxyl radical; TCA, trichloroacetic acid; DNPH, dinitrophenylhidrazine; AAPH, 2,2azobis[2-amidinopropane]; AUC, area under curve; GSH, glutathione; CDNB, chloro-dinitro benzene; ANOVA, analysis of variance

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1. Introduction

Vitamin A performs important roles in both development and maintenance of adult vertebrate brain homeostasis (De Luca, 1991; Lane and Bailey, 2005; McCafferry et al., 2005). Insufficient vitamin A availability during prenatal life may impair embryonic segmentation and growth, and also stop vascularization process (Maden et al., 1996; Wellik and DeLuca, 1995; White et al., 2000). Throughout adulthood, vitamin A remains to be important to other central nervous system (CNS)-related functions, for instance learning and memory (Chiang et al., 1998; Cocco et al., 2002). Furthermore, vitamin A and its related retinoids easily penetrate into blood–brain barrier, and mammalian CNS contains the molecular apparatus responsible for the production and maintenance of all-trans-retinoic acid in neurons, through retinal dehydrogenases and cellular retinoid binding proteins action (Duester, 2000; MacDonald et al., 1990; Zetterström et al., 1999). Thus, the CNS is able to transport and metabolize retinoid molecules and may rapidly increase their concentrations.

Moreover, strong evidences suggest that over 75% of people in developed nations may routinely ingest vitamin A above the recommended dietary allowance (Penniston and Tanumihardjo, 2006). Additionally, in some countries, like United States of America (USA), about 5% take a vitamin A supplement while 25% of adults ingest supplements containing vitamin A (Rothman et al., 1995). Lastly, vitamin A has been largely consumed as a prescription drug in retinoid therapies with demonstrated efficacy, such as in several dermatological conditions and cancer treatment/chemoprevention, especially in acute promyelocytic leukemia (Moise et al., 2007; Napoli, 1999).

The potential excessive vitamin A intake may induce a serious threat to the brain development and vitamin A homeostasis, since it has long been demonstrated to be teratogenic in many natural and synthetic preparations and induce a spectrum of congenital defects similar to that of vitamin A deficiency, including neuronal malformations (Adams, 1993; Cohlan, 1953; Collins and Mao, 1999). Additionally, excessive vitamin A intake has been linked to several CNS-associated disturbances, including headache, pseudotumor cerebri and confusion, as well as cognitive impairments, such as irritability, anxiety and depression (Fenaux et al., 2001; Allen and Haskell, 2002; Myhre et al., 2003). On the other hand, vitamin A supplementation, like retinyl palmitate in doses as high as 10,000 IU/daily (200 IU/kg/Day), seems to be safe by many authors to fertile women, at any time during pregnancy, independently of their vitamin A status, and others suggest higher levels of safety (Dolk et al., 1999; IVACG, 1998; Ross et al., 2000). According to this contradictory data, retinoid research in pregnancy is of great value to truly elucidate this confused panel.

Furthermore, vitamin A is also a redox-active molecule and has been demonstrated to play a potential pro-oxidant effect in concentrations slightly above the physiologic levels in different *in vitro* experimental models (Moreira et al., 1997; Dal-Pizzol et al., 2001; Frota et al., 2004; Zanotto-Filho et al., 2008). Pro-oxidant effects of vitamin A treatment include increased lipid peroxidation, protein carbonylation, DNA damage and modified activity of antioxidant enzymes in cultured Sertoli cells (Dal-Pizzol et al., 2000, 2001). Recently, we have shown that vitamin A supplementation at clinical doses induced a pro-oxidant state in different rat brain regions like the hippocampus, striatum, and frontal cortex (De Oliveira and Moreira, 2007; De Oliveira et al., 2007a,b, 2008). Interestingly, vitamin A treatment also increased reception of advanced glycation endproducts immunocontent in rat cerebral cortex (Dal-Pizzol et al., 2000). Moreover, vitamin A supplementation induced anxiety-like behavior and decreased both locomotory and exploratory activities in adult male Wistar rats under a 28-day treatment (De Oliveira et al., 2007a,b, 2008). According to the previously reported works from our group and others, the best recommendation is caution when vitamin A supplementation is the choice in treating human.

Oxidative stress may result from an overload of oxidants, particularly reactive oxygen species (ROS) and reactive nitrogen species (RNS), and when the cells' antioxidant defense system is unable to counteract uncontrolled oxidation disrupts cell structures and functions. In addition, oxyradicals are suggested to be involved in several pathological conditions, including neurodegenerative diseases, like Alzheimer's disease (AD) and Parkinson's disease (PD) (Halliwell, 2006; Halliwell and Gutteridge, 1999).

Moreover, vitamin A metabolism is essential to maintain striatal function and for adult hippocampal neurogenesis, which seems to be regulated, at least in part, by retinoids (Valdenaire et al., 1998; Zetterström et al., 1999; McCaffery and Dräger, 1994; Samad et al., 1997; Krezel et al., 1998; Takahashi et al., 1999; Wang and Liu, 2005). Additionally, the hippocampus is also involved in mood disorders, such as anxiety and depression, and vitamin A is also known to participate in locomotory and exploratory behavior (Bannerman et al., 2003, 2004; Deacon and Rawlins, 2005; File et al., 2000).

Therefore, based on previous reports indicating a prooxidant role of vitamin A in a variety of experimental models, we have decided to investigate in the present work if the vitamin A supplementation is also able to exert its described prooxidant effects in maternal and offspring rat striatum and hippocampus. Additionally, behavioral parameters evaluation was also targeted.

2. Results

2.1. *Reproductive data*

No treatment-related clinical symptoms of toxicity were found in mothers throughout the treatment period. One of the mothers at 12,500 IU/kg/day was euthanized on lactation day 4 because it became moribund. Their pups died due to deterioration of maternal condition. The examination of the moribund female and her litter showed no treatment-related abnormality. No gross malformations were observed in pups at post natal day (PND) 0. Incidences of gross lesions were not found during necropsy in dams and pups of the retinyl palmitate-treated groups. Body weight gain in gestation or lactation, gestation length, delivery index, the number of pups delivered, the number of implants and the sex ratio of the litters in retinyl palmitate-treated groups showed no

Table 1 – Reproductive data.

	Retinyl palmitate (IU/kg/day)			
	0 (control)	2500	12,500	25,000
No. of dams	7	7	7	7
Gestation weight gain (%)	16.8±4.1	15.5±3.1	17.2±3.4	18.1±3.8
Lactation weight gain (%)	12.4±2.6	15.4±6.1	14.6±6.2	15.4±3.1
Gestation length (days)	21	21	21	21
No. of implantations	9.5±1.7	10.5±3.3	10.7±1.5	8.6±2.6
Delivery index (%)	97.2±5.6	88.2±17.3	70.8±25.5	83.3±18.9
No. of pups delivered	9.3±1.9	9±2.8	7.5±2.5	7±2.5
Sex ratio of pups	0.464	0.404	0.538	0.541
Viability index (%)				
Day 0	95.3±5.6	95.9±6.9	95.8±10.2	96.2±8.9
Day 7	89.2±9.2	91.8±9.3	69.3±40.6	98.2±4.1
Day 14	97.7±4.6	98.3±4.1	95±10	98±4.5
Day 21	95±10	98.6±3.4	97.2±5.6	97.8±5
Pup weight (g)				
Day 0	5.9±0.4	6.0±0.4	5.7±0.8	6.4±0.5
Day 7	11.5±1.7	11.4±1.5	10.6±0.6	12.5±1.5
Day 14	17±3.5	20.5±2.8	18.4±0.1	22.3±3.9
Day 21	30.2±4.6	32.5±3.3	30.9±4.1	34.9±4.8

Gestation weight gain (%) = [(weight on PND0 – weight on GD0)/weight on GD0)] × 100.
Lactation weight gain (%) = [(weight on PND21 – weight on PND0)/weight on PND0)] × 100.
Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
Sex ratio of pups = no. of male pups/total no. of pups.
Viability index on postnatal day 0 (%) = (no. of live pups delivered/total no. of pups delivered) × 100.
Viability index on postnatal day 7 (%) = (no. of live pups on postnatal day 7/no. of live pups delivered) × 100.
Viability index on postnatal day 14 (%) = (no. of live pups on postnatal day 14/no. of live pups on postnatal day 7 after cull) × 100.
Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live pups on postnatal day 14 after cull) × 100.

treatment-related changes (Table 1). During nursing, the pups exhibited no treatment-related clinical symptoms. Litter data revealed that the viability index on PND7 decreased slightly in the 12,500 IU/kg/day group, although no treatment-related reduction in body weights was observed. This was due to the loss of a whole litter as described before.

2.2. Behavior tasks

2.2.1. Homing test

Offspring of retinyl palmitate treated dams showed no significant alteration in the frequency of correct and incorrect performance on homing test in PND5 and PND10 (Table 2). On the other hand, the time spent over the homing area in offspring of treated dams on PND5 increased at all doses when compared to offspring of control dams (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,48]}=24.62$, $p<0.0001$) (Fig. 1A). However, on PND10 there was no difference between male offspring from retinyl palmitate treated dams and control dams; but, in female offspring palmitate supplementation spent less time over the homing area at 25,000 IU/kg/day ($F_{[3,48]}=5.342$, $p=0.0029$) (Fig. 1B). Additionally, male offspring spent more time in homing area at 2500 and 25,000 IU/kg/day when compared to female offspring on PND5 (according to two-way ANOVA the sex difference affect the result, $F_{[1,48]}=25.31$, $p<0.0001$ and one-way ANOVA with Tukey's post hoc comparisons showed detailed differences) and on PND10 only at 25,000 IU/kg/day ($F_{[1,48]}=33.07$, $p<0.0001$).

2.2.2. Open field test

Retinyl palmitate treated dams showed significant alterations on open field test (OFT) scores (Fig. 2). The number of crossings decreased in treated dams at 25,000 IU/kg/day (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,24]}=3.618$, $p=0.0276$) (Fig. 2A), but the number of center entries and rearings did not change (Figs. 2B and C, respectively). The number of groomings decreased in treated dams at 12,500 IU/kg/day ($F_{[3,24]}=4.104$, $p=0.0174$) (Fig. 2D). The number of freezings also increased in treated dams at 12,500 IU/kg/day ($F_{[3,24]}=3.022$, $p=0.0494$) (Fig. 2E). However, the number of fecal boli did not change at all doses (Fig. 2F).

Offspring of retinyl palmitate treated dams also showed significant alterations on OFT scores (Fig. 3). The number of crossings decreased in male treated offspring at 12,500 and 25,000 IU/kg/day (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,48]}=5.098$, $p=0.0038$), but not in females (Fig. 3A). The number of center entries decreased in both treated offspring sex at all doses ($F_{[3,48]}=11.81$, $p<0.0001$) (Fig. 3B). The number of rearings decreased in treated males at 12,500 and 25,000 IU/kg/day ($F_{[3,48]}=6.520$,

Table 2 – Proportion of male and female pups with correct and incorrect performance in the homing test on PND5 and PND10.

	Retinyl palmitate (IU/kg/day)							
	0 (control)		2500		12,500		25,000	
	Correct	Incorrect	Correct	Incorrect	Correct	Incorrect	Correct	Incorrect
PND5								
Males	6/24 (25)	18/24 (75)	6/18 (33)	12/18 (67)	6/26 (23)	20/26 (77)	12/27 (44)	15/27 (56)
Females	6/21 (29)	15/21 (71)	12/27 (44)	15/27 (56)	2/16 (12)	14/16 (88)	10/25 (40)	15/25 (60)
PND10								
Males	21/24 (83)	4/24 (17)	16/18 (89)	2/18 (11)	20/26 (77)	6/26 (23)	21/27 (78)	6/27 (22)
Females	17/21 (81)	4/21 (19)	23/27 (85)	4/27 (15)	10/14 (71)	4/14 (29)	16/23 (70)	7/23 (30)

Data are presented as proportions between the number of successful or unsuccessful pups per total offspring tested (percentage off in parenthesis).

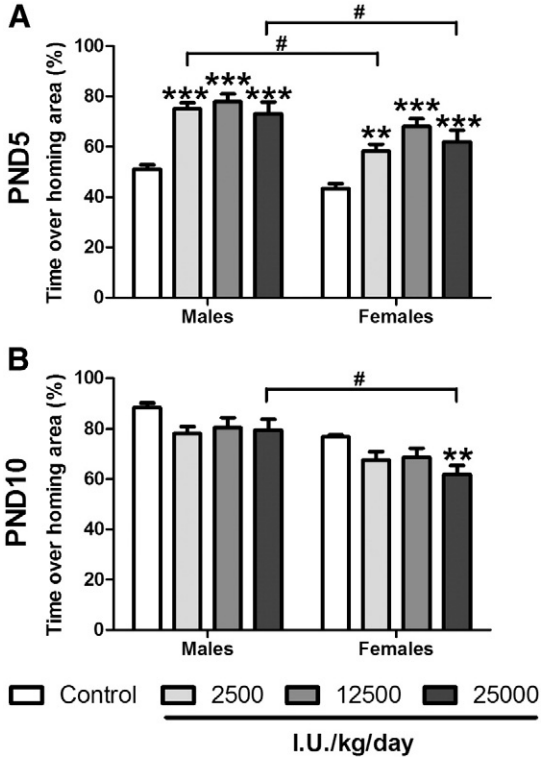

Fig. 1 – Homing test at postnatal days (PND) 5 and 10. The time spent over the homing area in PND5 (A) and 10 (B) was analyzed in both male and female pups from control and retinyl palmitate treated dams. Each animal was monitored for 3 min in homing test apparatus and results are expressed as percentual of the total time spent in both areas. Data are presented as mean ± S.E.M. (n = 7 litters per group). P≤0.01 when different from control group. *** P≤0.001 when different from control group. # p≤0.05 when males different from females at the same dose.**

p=0.0009) (Fig. 3C). The number of groomings decreased in treated males at 12,500 and 25,000 IU/kg/day ($F_{[3,48]}$=4.708, p=0.0058), but in females decreased only at 25,000 IU/kg/day (Fig. 3D). The number of freezings increased in both treated offspring sex at 25,000 IU/kg/day ($F_{[3,48]}$=8.755, p<0.0001) (Fig. 3E), but the number of fecal boli did not change at all doses (Fig. 3F).

2.3. *Biochemical results*

2.3.1. *Maternal striatum*

Striatum of retinyl palmitate treated dams showed significant alterations on the redox parameters analyzed (Table 3). Catalase (CAT) activity decreased in treated dams at 12,500 and 25,000 IU/kg/day ($F_{[3,24]}$=3.478, p=0.0316), but superoxide dismutase (SOD) activity did not change at all doses. However, SOD/CAT ratio increased at 25,000 IU/kg/day ($F_{[3,24]}$=3.373, p=0.0349). Glutathione-S-transferase (GST) activity increased in treated dams at 12,500 and 25,000 IU/kg/day ($F_{[3,24]}$=5.756, p=0.0041), but total reactive antioxidant potential (TRAP) and reduced thiol content did not change at all retinyl palmitate treated dams. Lipoperoxidation increased in treated dams at 25,000 IU/kg/day ($F_{[3,24]}$=26.75, p<0.0001) while protein carbonylation increased at 12,500 and 25,000 IU/kg/day ($F_{[3,24]}$=6.544, p=0.0022).

2.3.2. *Maternal hippocampus*

Hippocampus of retinyl palmitate treated dams also showed significant alterations on the redox parameters analyzed (Table 3). CAT activity and SOD activity did not change at all doses, but SOD/CAT ratio increased at 25,000 IU/kg/day ($F_{[3,24]}$=3.106, p=0.0484). GST activity and TRAP did not change at all doses, but total reduced thiol content decreased at 12,500 and 25,000 IU/kg/day ($F_{[3,24]}$=4.377, p=0.0136). Lipoperoxidation increased only at 25,000 IU/kg/day ($F_{[3,24]}$=3.517, p=0.0304) and protein carbonylation increased at 12,500 and 25,000 IU/kg/day ($F_{[3,24]}$=5.508, p=0.0050).

2.3.3. *Offspring striatum*

Striatum of offsprings from retinyl palmitate treated dams showed significant alterations on the redox parameters analyzed (Table 4). CAT activity decreased in treated males at 12,500 and 25,000 IU/kg/day (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,48]}$=6.171, p=0.0012), but SOD activity did not change in both sexes at all doses. SOD/CAT ratio increased only in males at 25,000 IU/kg/day ($F_{[3,48]}$=2.934, p=0.0427) and GST activity increased in treated males at 2500 and 25,000 IU/kg/day, but increased in females only at 25,000 IU/kg/day ($F_{[3,48]}$=11.92, p<0.0001). TRAP decreased in both sexes at 12,500 and 25,000 IU/kg/day ($F_{[3,48]}$=11.24, p=0.0001). Total reduced thiol content decreased only for males at 25,000 IU/kg/day ($F_{[3,48]}$=3.124, p=0.0344) and lipoperoxidation increased in both sexes at the same dose ($F_{[3,48]}$=8.970, p=0.0001). Protein carbonylation increased in males at 2500 and 25,000 IU/kg/day, but only in females at 25,000 IU/kg/day ($F_{[3,48]}$=5.008, p=0.0039).

2.3.4. *Offspring hippocampus*

Hippocampi of offsprings from retinyl palmitate treated dams showed significant alterations on the redox parameters analyzed (Table 5). CAT activity decreased in both sexes at all retinyl palmitate doses (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,48]}$=15.57, p<0.0001), but SOD activity did not change at all doses. SOD/CAT ratio increased in males at all retinyl palmitate doses, but only increased in females at 12,500 and 25,000 IU/kg/day ($F_{[3,48]}$=11.98, p<0.0001). GST activity did not change at all doses. TRAP and total reduced thiol content did not change. Lipoperoxidation increased in both sexes at all retinyl palmitate doses ($F_{[3,48]}$=16.34, p<0.0001), but protein carbonylation only increased at 12,500 IU/kg/day in males and 25,000 IU/kg/day in females ($F_{[3,48]}$=5.056, p=0.0040).

3. Discussion

Vitamin A exerts important roles in both development and the adult brain, but excessive vitamin A intake may be teratogenic in humans (De Luca, 1991; Lane and Bailey, 2005; McCaferry et al., 2005). Although the evidence of such effects for retinyl palmitate supplementation in humans is limited, there is a growing concern about the safety of retinyl palmitate supplementation during pregnancy and breastfeeding (Dolk et al.,

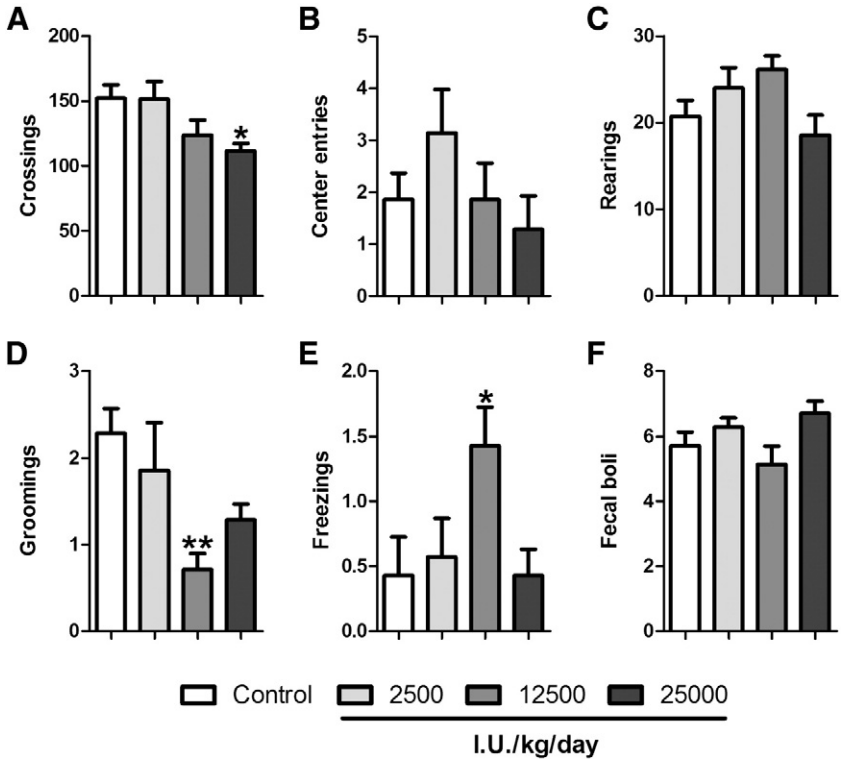

Fig. 2 – Maternal open field test. The number of total crossings (A), the number of visits to the center of arena (B), the number of rearings (C), the number of groomings (D), the number of freezings (E), and the number of fecal boli (F) were analyzed in control and retinyl palmitate treated rats. The open field test was performed at postnatal day 19. Each animal was monitored for 5 min in open field apparatus. Data are presented as mean ± S.E.M. (n = 7 per group). *P ≤ 0.05 when different from control group. **P ≤ 0.01 when different from control group.

1999; IVACG, 1998; Miller et al., 1998; Mills et al., 1997; Ross et al., 2000). In general, human data regarding retinyl palmitate supplementation effects during pregnancy and breastfeeding are mostly in observational and epidemiological studies based in morphological endpoints. However, biochemical changes induced by prenatal insults that lead to physiological deficits of organ function may not always be accompanied by detectable anatomical abnormalities (Lau and Rogers, 2004).

Hence, in the last few decades, considerable attention has been drawn to functional teratology, an extension beyond the investigation of morphological examinations to include the evaluations of functional integrity of organ systems. In this work we have proposed an evaluation of the functional integrity in organs system of mothers and their offspring by redox evaluation of several enzymatic and non-enzymatic parameters. The redox profile is important because ROS are generated in cells by several pathways and there has been much speculation regarding the role of free radicals during development (Allen and Balin, 1989; Hitchler and Domann, 2007). According to the free radical theory of development, it is the influence of the balance between the production and removal of ROS/RNS (Hitchler and Domann, 2007).

We show in the present work, for the first time, vitamin A supplementation at 2500, 12,500 and 25,000 IU/kg/day during pregnancy and nursing to rats inducing a prooxidant state in maternal and offspring hippocampus and striatum. In addition, behavioral alterations were also observed in the homing and open field tests. These doses were used in order to evaluate the effects of equivalent doses to those stated as safe for humans during pregnancy and breastfeeding upon dams and their offspring. Additionally, the doses investigated in this work are all lower than 163,000 IU/kg/day, the lowest observed adverse effect level (LOAEL) of retinyl palmitate in rats, established in segment II developmental toxicity testing (Ritchie et al., 1998).

The brain is sensitive to oxidative stress due to its high content of peroxidizable fatty acids and relative decreased antioxidant defenses (Halliwell and Gutteridge, 1999). Clearly, in maternal striatum and hippocampus, lipid peroxidation occurred when dams received retinyl palmitate supplementation. In addition, protein carbonylation also increased in these maternal tissues and was present at lower doses then lipid peroxidation, as did decreased protein thiol content in the hippocampus. These molecular changes could indicate an increased vulnerability of nigral proteins to the oxidative insult induced in this experimental model. In offspring striatum and hippocampus, retinyl palmitate supplementation also increased lipid peroxidation and protein carbonylation; however, reduced thiol content was found only in male offspring striatum.

Increased lipoperoxidation, protein carbonylation levels, and decreased total thiol content make it easier for intra- and intermolecular cross-links of proteins, which in turn induce conformational changes leading to increased hydrophobicity and aggregation (Goetz and Gerlach, 2004). Furthermore, these oxidative alterations on proteins favor the formation of protein aggregates, inducing generalized cellular dysfunction (Mattson

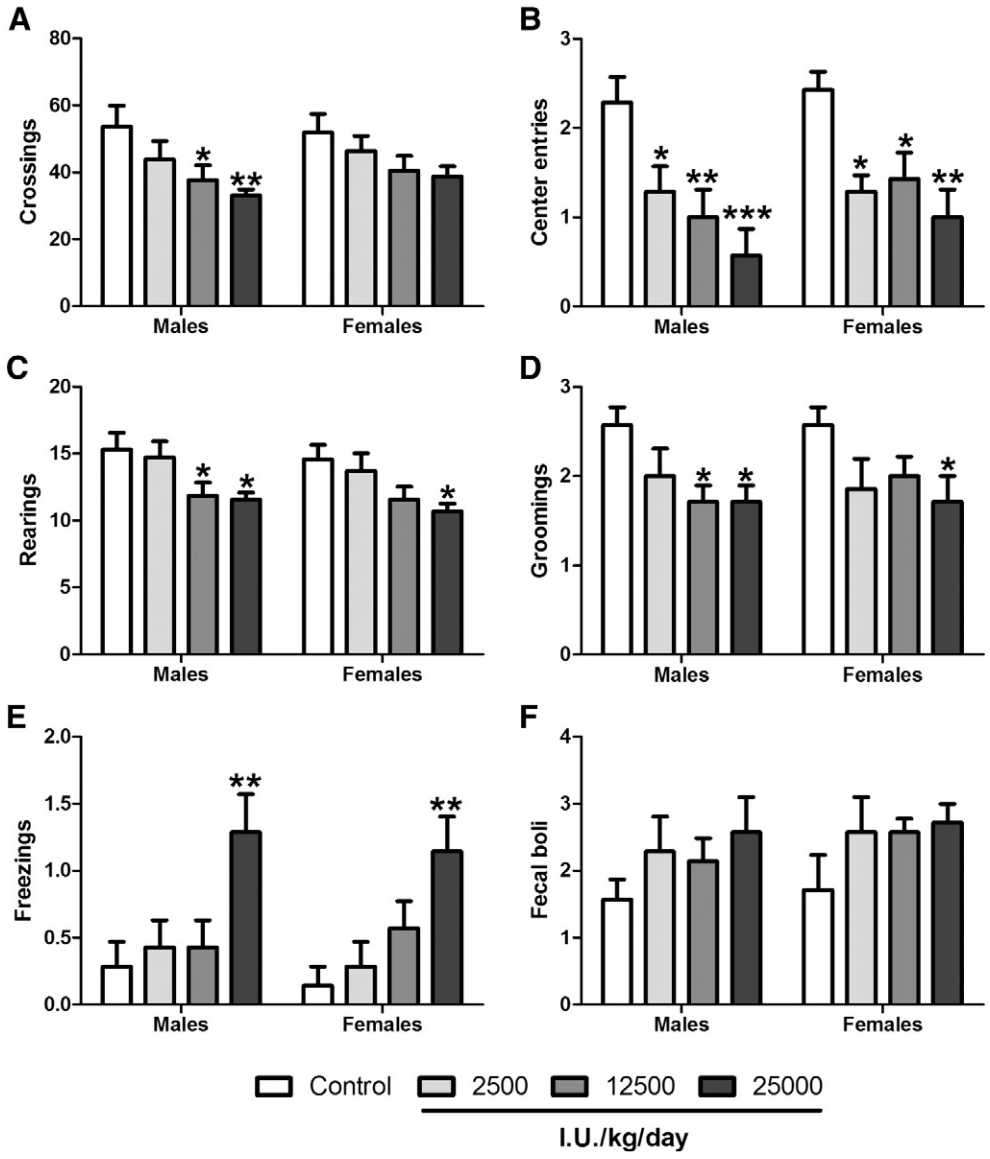

Fig. 3 – Offspring open field test. The number of total crossings (A), the number of visits to the center of arena (B), the number of rearings (C), the number of groomings (D), the number of freezings (E), and the number of fecal boli (F) were analyzed in control and retinyl palmitate treated pups for both male and female offspring. The open field test was performed at postnatal day 20. Each animal was monitored for 5 min in open field apparatus. Data are presented as mean ± S.E.M. (n = 7 litters per group). *P ≤ 0.05 when different from control group. **P ≤ 0.01 when different from control group. ***P ≤ 0.001 when different from control group.

and Magnus, 2006). Additionally, increased oxidative damage to proteins might result in increased free iron, favoring the maintenance of the prooxidative state (Keyer and Imlay, 1996).

In addition, total reduced thiol content presents an important intracellular nonenzymatic defense in the CNS, mainly by the action of glutathione molecules. In this way, the observed reduction on reduced thiol content in the present work indicates a possible decrease on reduced glutathione, given the prooxidant circumstances imposed by vitamin A supplementation. Another possibility is the action of a detoxifying system, such as GST, which needs GSH to conjugate with xenobiotics, eliminating them from the cell (Fang et al., 2002). Indeed, GST activity increased in maternal and offspring striatum of retinyl palmitate treated animals. There is an indication of oxidative activation of this enzyme that also detoxifies endogenous electrophiles, which are usually the consequence of free-radical damage and may be an important participant in the mechanism of free-radical damage repair (Aniya et al., 1993; Ketterer and Meyer, 1989; Wu et al., 2004). Additionally, we also found a decreased TRAP in the retinyl palmitate treated animals in these same tissues. The total reactive antioxidant potential is representative of the non-enzymatic capability of the tissue in preventing oxidative damage. A wide range of molecules, including uric acid, vitamin E, vitamin C and also glutathione, are active free-radical scavengers (Halliwell, 1996).

In this work we also found modulated antioxidant enzyme activity in maternal and offspring hippocampus and striatum,

Table 3 – Effects of retinyl palmitate treatment during pregnancy and nursing upon maternal redox parameters.

	Retinyl palmitate (IU/kg/day)			
	0 (control)	2500	12,500	25,000
No of dams examined	7	7	7	7
Striatum				
CAT activity (U CAT/mg protein)	1.40±0.11	1.24±0.24	1.11±0.21*	1.13±0.19*
SOD activity (U SOD/mg protein)	13.55±1.68	13.82±1.15	13.94±1.31	15.36±1.15
SOD/CAT ratio (arbitrary units)	9.76±1.79	11.51±2.28	13.09±3.22	14.04±3.22*
GST activity (nmol/min mg protein)	65.35±7.63	85.93±19.37	88.39±21.87*	100.27±10.61*
TRAP (arbitrary units)	0.0102±0.001	0.0092±0.001	0.0085±0.001	0.0080±0.001
Total thiol content (mmol SH/mg protein)	67.07±7.94	58.03±8.04	56.49±8.48	55.56±7.89*
TBARS level (nmol MDA/mg protein)	1.11±0.17	1.19±0.15	1.18±0.25	1.91±0.19*
Carbonyl level (nmol carbonyl/mg protein)	0.4935±0.04	0.5139±0.05	0.6116±0.07*	0.6027±0.07*
Hippocampus				
CAT activity (U CAT/mg protein)	1.42±0.36	1.13±0.31	1.07±0.29	0.98±0.33
SOD activity (U SOD/mg protein)	12.82±1.29	13.89±1.97	14.27±1.92	14.35±1.66
SOD/CAT ratio (arbitrary units)	9.57±2.87	11.25±2.26	12.81±3.56	14.62±3.59*
GST activity (nmol/min mg protein)	69.35±4.01	65.45±6.92	67.54±6.08	70.79±16.14
TRAP (arbitrary units)	0.0102±0.002	0.0101±0.001	0.0091±0.001	0.0092±0.002
Total thiol content (mmol SH/mg protein)	77.62±7.01	72.29±4.47	68.15±7.02*	66.83±5.73*
TBARS level (nmol MDA/mg protein)	1.36±0.14	1.48±0.20	1.59±0.16	1.66±0.21*
Carbonyl level (nmol carbonyl/mg protein)	0.2699±0.03	0.2743±0.03	0.3321±0.06*	0.3461±0.05*

Data are presented as mean±S.E.M. (n=7 per group).
* $P \leq 0.05$ when different from control group.

indicating again that reactive oxygen species may be produced in excess during vitamin A supplementation. Vitamin A supplementation increased SOD activity in maternal striatum, offspring hippocampus, and in male offspring striatum, which may indicate increased superoxide radical ($\bullet O_2^-$) production, since it is the major SOD allosteric activator (Halliwell and Gutteridge, 1999). Furthermore, we found decreased CAT activity in the same tissues. Increased $\bullet O_2^-$ may allosterically

Table 4 – Effects of retinyl palmitate treatment during pregnancy and nursing upon offspring striatum redox parameters.

	Retinyl palmitate (IU/kg/day)			
	0 (control)	2500	12,500	25,000
No of litters examined	7	7	7	7
CAT activity (U CAT/mg protein)				
Male	1.43±0.18	1.18±0.24	1.16±0.14*	0.99±0.13*
Female	1.36±0.17	1.18±0.17	1.19±0.28	1.17±0.26
SOD activity (U SOD/mg protein)				
Male	14.23±1.39	14.38±2.08	14.83±2.87	15.51±2.43
Female	15.70±1.45	15.37±2.52	13.56±2.54	14.47±2.37
SOD/CAT ratio (arbitrary units)				
Male	10.13±1.99	12.79±3.77	12.94±2.76	16.01±3.94*
Female	11.71±2.05	13.38±3.51	11.95±3.29	13.12±4.27
GST activity (nmol/min mg protein)				
Male	33.55±6.71	43.93±8.05*	41.80±7.93	50.80±7.01*
Female	37.13±5.14	43.67±8.52	42.77±5.26	51.23±6.35*
TRAP (arbitrary units)				
Male	0.0103±0.002	0.0088±0.001	0.0067±0.001*	0.0072±0.002*
Female	0.0112±0.002	0.0085±0.002	0.0080±0.002*	0.0078±0.002*
Total thiol content (mmol SH/mg protein)				
Male	64.25±9.55	58.88±9.81	54.25±5.33	52.79±8.50*
Female	64.07±11.01	60.64±8.82	56.82±10.13	57.43±7.34
TBARS level (nmol MDA/mg protein)				
Male	1.46±0.19	1.68±0.19	1.63±0.33	1.96±0.32*
Female	1.55±0.23	1.61±0.42	1.61±0.27	2.08±0.21*
Carbonyl level (nmol carbonyl/mg protein)				
Male	0.4918±0.04	0.5838±0.05*	0.5312±0.06	0.5747±0.05*
Female	0.4532±0.06	0.5276±0.06	0.5194±0.09	0.5422±0.09*

Data are presented as mean±S.E.M. (n=7 per group).
* $P \leq 0.05$ when different from control group.

Table 5 – Effects of retinyl palmitate treatment during pregnancy and nursing upon offspring hippocampus redox parameters.

	Retinyl palmitate (IU/kg/day)			
	0 (control)	2500	12,500	25,000
No of litters examined	7	7	7	7
CAT activity (U CAT/mg protein)				
Male	1.75±0.18	1.11±0.30*	1.14±0.43*	1.02±0.32*
Female	1.59±0.28	1.18±0.28*	1.09±0.34*	0.85±0.16*
SOD activity (U SOD/mg protein)				
Male	16.37±1.76	17.67±1.75	16.82±1.49	18.09±1.29
Female	16.56±2.07	17.12±1.95	17.69±1.83	17.67±1.29
SOD/CAT ratio (arbitrary units)				
Male	9.47±1.62	17.03±5.16*	18.93±3.89*	17.04±3.09*
Female	10.84±3.01	15.67±5.89	17.92±6.43*	21.34±4.22*
GST activity (nmol/min mg protein)				
Male	42.26±8.12	56.20±11.65	50.69±10.31	53.34±10.89
Female	46.69±11.61	51.62±9.42	53.52±12.97	52.77±15.41
TRAP (arbitrary units)				
Male	0.0103±0.001	0.0101±0.002	0.0105±0.003	0.0110±0.003
Female	0.0101±0.001	0.0105±0.003	0.0101±0.002	0.0088±0.001
Total thiol content (mmol SH/mg protein)				
Male	52.54±10.80	47.15±4.59	44.67±6.77	44.28±7.52
Female	50.96±4.68	48.43±11.55	46.03±6.06	45.31±4.66
TBARS level (nmol MDA/mg protein)				
Male	1.46±0.23	1.82±0.17*	1.84±0.17*	1.98±0.20*
Female	1.51±0.23	1.79±0.16*	1.99±0.24*	1.91±0.14*
Carbonyl level (nmol carbonyl/mg protein)				
Male	0.3406±0.03	0.3672±0.04	0.4032±0.05*	0.3998±0.04
Female	0.3407±0.08	0.3376±0.04	0.3811±0.04	0.4030±0.05*

Data are presented as mean±S.E.M. (n=7 per group).
* $P \leq 0.05$ when different from control group.

inactivate CAT enzyme, decreasing its activity (Kono and Fridovich, 1982; Shimizu et al., 1984). In truth, vitamin A is known to increase $\bullet O_2^-$ production, as previously demonstrated (Murata and Kawanishi, 2000; Klamt et al., 2005).

These enzymatic modulations yielded an increase in the SOD/CAT ratio after vitamin A supplementation in almost all tissues analyzed. As a consequence of increased SOD/CAT ratio, hydrogen peroxide (H_2O_2) availability might be increased, since SOD metabolizes $\bullet O_2^-$ to H_2O_2, but CAT converts H_2O_2 to water at lower rates. Since H_2O_2 via the Fenton reaction is a source of hydroxyl radical (•OH) generation, the most powerful prooxidant molecule, this indicates a prooxidant state in all CNS tissues (Halliwell, 2006). Thus, impaired SOD/CAT is very likely to culminate in increased oxidative damage to biomolecules. Therefore, our hypothesis is that the imbalance in SOD/CAT ratio can be from a product of the vitamin A supplementation-dependent oxidative alterations observed in this work.

Herein, we show that vitamin A supplementation at different doses during pregnancy and nursing is effective in inducing a behavioral disturbance in dams and their offspring in the homing test and OFT. Previously, we have demonstrated that vitamin A supplementation induced anxiety, since rats' exploratory activity diminished in the OFT apparatus (De Oliveira et al., 2007b). In addition, vitamin A (mainly as retinyl palmitate) is also shown to induce human behavioral alterations, such as irritability, fatigue, depression, and anxiety (Myhre et al., 2003).

The identification of the mother is critical for survival and development of mammals. Infant rats rapidly learn to identify, orient, approach and prefer the maternal odor naturally within the nest (Sullivan et al., 1989; Leon, 1992; McLean et al., 1999; Roth and Sullivan, 2005). In rats, the molecular basis of infant olfactory learning involves a complex chain of events (Langdon et al., 1997; Nakamura et al., 1987; Rangel and Leon, 1995; Sullivan and Wilson, 2003). In this work we observed that female rats from retinyl palmitate-treated offspring displayed increased time spent over the homing area at PND5, but decreased at PND10 in the homing test. The immature brain at PND5 seems to be more vulnerable to the prooxidative insult of retinyl palmitate supplementation probably due to its larger proportion of sensitive immature cells (Ikonomidou and Kaindl, 2010). Additionally, the maternal preference in males appears to be more resistant to environmental intervention than in females. As shown by PND10 no behavioral effects were observed for males, but females showed effects at the higher dose at the same time. Moreover, the higher maternal behavior usually demonstrated by the male pups instead of female pups may account for the differences observed in the homing test (Melniczek and Ward, 1994; Moore et al., 1997). The effect of gender could also be attributed to differences in sexual hormones, but further investigation is needed to clarify the nature of observed sexual effect in this test.

Additionally, vitamin A supplementation reduced rearings and center entries in the OFT, and we also found a reduced number of crossings in male offspring. Furthermore, the treatment reduced grooming, but increased freezing scores in offspring of both sexes. Vitamin A supplementation also

reduced locomotory activity in dams at 25,000 IU/kg/day, but at 12,500 IU/kg/day reduced grooming and increased freezing scores. These alterations indicate a decreased exploratory activity in retinyl palmitate treated offspring and a decreased locomotory activity in dams and male offspring. However, this was not due a gross motor alteration, since the animals walked normally without presenting muscular weakness or tremor. In addition, these results suggest an anxiety-like behavior in vitamin A treated dams and offspring, which seems to be a preliminary step to depression in several experimental models, which has also been observed in humans (Burroughs and French, 2007).

The linking between oxidative stress and behavioral changes has been extensively investigated in various animal models. Oxidative stress plays an important role in the development of cognitive impairment in sepsis (Cassol-Jr et al., 2010). Antioxidant therapy with N-acetylcisteine and desferroxamine, as an additive to chloroquine, prevented cognitive impairment, confirming the importance of oxidative stress in cerebral malaria-associated cognitive sequellae (Reis et al., 2010). Hyperactivity in the amphetamine model of mania in rats also has been shown to be linked to oxidative stress (Steckert et al., 2010). Moreover, oxidative stress is believed to contribute to cognitive and behavioral deficits after ischemia, anoxia, carbon monoxide poisoning, traumatic brain injury, and in Alzheimer's disease (Dal-Pizzol et al., 2010). Finally, recent studies (including our own) have shown direct involvement of oxidative stress with anxiety-like behavior and with locomotory/exploratory deficit in rodents (Salim et al., 2010; Hovatta et al., 2005; Gingrich, 2005; Masood et al., 2008; Souza et al., 2007; Bouayed et al., 2007; de Oliveira et al., 2007). However, the linking between oxidative stress and behavioral changes found in this work remains to be elucidated by further investigation.

In summary, our data suggest that vitamin A supplementation during pregnancy and nursing was able to modify striatal and hippocampal redox parameters and the subsequent behavior in rats. Notably, the doses administrated in this work were approximately equivalent to presumed doses safe for humans during pregnancy and breastfeeding. Unfortunately, it is still difficult to indicate the vitamin A metabolite responsible for the observed effects, given the vast number of vitamin A existing metabolites (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 during pregnancy and breastfeeding; especially, in vitamin supplementation or fortified foods. This oxidative stress is able to disturb several biological phenomena, including neuronal signaling and neurotransmission, which may induce several behavioral deficits. Additionally, exposure to stress early in life can induce an increased vulnerability to mood disorders later in life (Heim and Nemeroff et al., 2001; Sanchez et al., 2001). Indeed, the origin of many adult diseases such as depression, anxiety, or impulse control disorders, can be found in infancy (Kaffman and Meaney, 2007; Swain et al., 2007).

4. Experimental procedures

All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996). Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

4.1. *Animal and housing conditions*

Male and female Wistar rats (*Rattus novergicus*) from our breeding colony were used in the present study. The animals were caged in groups of five animals with free access to water and standard commercial food (CR1 lab chow, Nuvilab, Curitiba, Brazil) and were kept on a 12 h light–dark cycle (7:00–19:00 h) at 23±1 °C. These conditions were maintained throughout the experiments. The nulliparous females, with 90 days and 200–250 g, were daily checked for their estrous cyclicity for 2 weeks, by direct vaginal smear examination in light microscope, before mating. Thereafter the females were selected in their sexual receptive phase of the estrous cycle (proestrous) and caged overnight with a single mature male (1F:1M). In the morning, the presence of a vaginal plug and/or viable sperm shown in a vaginal smear was regarded as successful mating. The day which a vaginal plug was detected and/or the presence of sperm in the vaginal smear was designated as gestation day 0 (GD0). The dams were allowed to litter naturally and the date of birth was defined as postnatal day 0 (PND0).

4.2. *Treatment*

The pregnant females were randomly divided into 4 groups of treatment: control, 2500, 12,500 and 25,000 IU/kg/day of retinol palmitate (Arovit®; a water-soluble form of vitamin). Treatment was orally performed, with a metallic gastric tube (gavage) in a maximum volume of 0.5 mL. Control group received NaCl 0.9%. The rats were treated once a day for the entire period of gestation and nursing (21 days of gestation and 21 days of nursing). They were always treated at night in order to ensure maximum vitamin A absorption, since it is better absorbed during or after a meal. Each female and its litter were separated into a cage at parturition and maintained according to conditions described earlier.

4.3. *Chemicals*

Arovit® (retinol palmitate, a commercial water-soluble form of vitamin A) was purchased from Roche, Rio de Janeiro, RJ, Brazil. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A administration solutions were prepared daily, protected from light exposure and temperature.

4.4. *Reproductive data*

All female rats were observed for clinical symptoms of toxicity and mortality once a day throughout the study. Body weights of the dams were assessed on GDs 0, 7, 14 and 20 and lactant days (LDs) 0, 7, 14 and 21, and body weight gain was calculated. Rats

that died during the administration period were autopsied and simply examined. On PND0, pups of both sexes were counted, weighed and checked for the presence of external malformations and/or stillbirths. During the nursing period, the pups were examined daily for clinical signs and mortality. Litter sizes were determined on PND0. Litters were weighed on PNDs 0, 7, 14 and 20, and body weight gain was calculated. Viability indexes of pups were calculated in each litter on PNDs 0, 7, 14 and 21. And at terminal necropsy, females were confirmed for pregnancy by counting the number of implantation sites in uterine horns.

4.5. *Behavior tasks*

The behavioral tasks were always performed between 10 a.m. and 4 p.m. (i.e., during the light phase) in specifically designed behavioral facilities illuminated with bright light from two, 40-W fluorescent overhead lights each. The homing test was performed for all offspring (males and females pups) at PND5 and PND10. The OPT was performed for all dams at PND19 and their offspring at PND20.

4.5.1. *Homing test*

The homing test exploits the strong tendency of the immature pup to maintain body contact with the dam and the siblings, which requires adequate sensory (olfactory) and motor skills as well as the associative and discriminative skills that allow the pup recognize the mother's odor among others (Bignami, 1996). The homing test apparatus is a plastic cage with similar 23structure to housing cages (34 cm length × 24 cm height × 40 cm width) and is divided in a half by a 2-cm wide neutral zone running the cage's length. Into each area, 300 mL of fresh or nest bedding is placed in adjacent corners. All the pups were gently placed on the division between the areas over home (nest bedding) and clean bedding. The animals were observed for 3 min and if they entered the home area with all 4 paws the test was counted as correct. If the animal did not enter the homing area the test was marked as incorrect. Correct tests were also measured for the time spent over fresh and homing area (Adams et al., 1985; Schlumpf et al., 1989). Time spent over home area was expressed as percentual of the total time spent in both areas. Following each test, the cage was cleaned with 30% ethanol to remove trace odors.

4.5.2. *Open field test*

One of the most traditional and widely used methods for the assessment of the locomotive and explorative behavior as well as the emotional state in rodents is the OFT, which plays many varieties (Tobach, 1969; Prut and Belzung, 2003). Because it is a relatively simple technique and gives quantitative information on a broad range of responses, it has been frequently used in teratologic studies (Cagiano et al., 1990; Di Giovanni et al., 1993). The OFT apparatus consists of a circular arena surrounded by 40-cm high walls. Two black circumferences divide its white floor into 3 concentric circles, with diameters of 20 cm, 50 cm, and 80 cm. Several radial lines cross the outer circles dividing them into sixteen equal cells in the periphery, eight in the medial circle, and four in the center. All the animals were gently placed in the periphery of the arena to freely explore it for 5 min. Then, they returned to their home cages. The number of crossings, center entries, rearings, groomings, freezing and fecal boli was registered. Following each test, the cage was cleaned with 30% ethanol to remove trace odors.

4.6. *Tissue extraction and sample preparation*

The animals were euthanized by decapitation 24 h after the last treatment. Maternal and offspring hippocampi and striatum were immediately dissected out in ice and stored at −80 °C for later biochemical analyses. All tissues were homogenized in 1 mM phosphate buffer (pH 7.0) and centrifuged (3000 ×g, 5 min) to remove cellular debris. Supernatants were used for all biochemical assays described. All the results were normalized by the protein content using bovine albumin as standard (Lowry et al., 1951).

4.7. *Redox profile in CNS structures*

4.7.1. *Lipid peroxidation*

The formation of thiobarbituric acid reactive species (TBARS) was quantified by an acid-heating reaction with thiobarbituric acid. It is a widely adopted parameter for measure oxidative damage on lipids, as previously described by Draper and Hadley (1990). The samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and centrifuged (10,000 ×g 10 min). Supernatant was mixed with 0.5 mL of 0.67% thiobarbituric acid and heated in a boiling water bath for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm. Results were expressed as nmol TBARS/mg protein.

4.7.2. *Protein carbonylation*

The formation of carbonyl groups was used as a parameter for oxidative damage to proteins, based on the reaction with dinitrophenylhidrazine (DNPH), as previously described by Levine et al. (1990). Proteins were precipitated by the addition of 20% TCA and re-solubilized in DNPH. Then, the absorbance was read in a spectrophotometer at 370 nm. Results were expressed as nmol carbonyl/mg protein.

4.7.3. *Total thiol content*

The total thiol content in its reduced form was measured as an estimative of redox status, since it is present in proteins as well as glutathione molecules, and is played as an intracellular redox buffer. As previously described by Ellman (1959), an aliquot of the sample was diluted in SDS 0.1%. Then, was added 0.01 M 5,5dithiobis-2-nitrobenzoic acid in ethanol. The intense yellow color was developed and read in a spectrophotometer at 412 nm after 60 min. Results were expressed as nmol SH/mg protein.

4.7.4. *Total reactive antioxidant potential*

The total reactive antioxidant potential (TRAP) was used as an index of non-enzymatic antioxidant capacity. As previously described by Lissi et al. (1992), this assay is based on the peroxyl radical (generated by AAPH solution, 2,2azobis[2-amidinopropane], with luminol) quenching by sample compounds. Sample addition decreases the luminescence proportionately to its antioxidant potential. The results were transformed in percentual and the area under the curve (AUC) was quantified as described by Dresch et al. (2009) by using GraphPad Software (San Diego, CA, USA — version 5.00).

The AUC are inversely proportional to antioxidant capacity, which is higher with lower AUC values, and is lower with higher AUC values. Therefore, we express the results as the inverse value (1/AUC) to make it easier to comprehend.

4.7.5. *Antioxidant enzyme activities*

The activities of two important antioxidant enzymes were analyzed: catalase (CAT) and superoxide dismutase (SOD). CAT (EC 1.11.1.6; CAT) activity was evaluated by observing the rate of decrease in hydrogen peroxide (H_2O_2) absorbance in a spectrophotometer at 240 nm. SOD (EC 1.15.1.1, SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm (Aebi, 1984; Misra and Fridovich, 1972). CAT activity is expressed as units CAT/mg protein and SOD activity as Units SOD/mg protein. To better understand the effect of vitamin A supplementation upon these free radical-detoxifying enzymes we applied a ratio between SOD and CAT activities (SOD/CAT), two enzymes that work in sequence to reduce the superoxide anion to water.

4.7.6. *Glutathione-S-transferase activity*

Glutathione S-transferase (GST, E.C. 2.5.1.18) activity was determined spectrophotometrically at 340 nm by measuring the formation of the conjugate of GSH (glutathione) with CDNB (chloro-dinitro benzene) as previously described by Habig and Jakoby (1981). Enzyme activity was determined by mixing buffer GSH 20 mM with the sample. The reaction started by CDNB 20 mM addition was carried out at 30 °C, and monitored spectrophotometrically for 3 min. Corrections of the spontaneous reaction were made by measuring and subtracting the rate in the absence of enzyme. Results are expressed as nmol of CDNB conjugated with glutathione/min/mg protein.

4.8. *Statistical analysis*

Body weights, body weight gains, gestation length, numbers of implants and pups delivered, delivery index and viability indices of pups were analyzed by the one-way analysis of variance (ANOVA) to determine if any statistical differences existed among the groups. If the ANOVA presented a significant result, Dunnett's test was performed to detect any significant differences between the treated groups and their corresponding controls. The litter was used as a unit for statistical evaluation for the data of body weights and viability index of pups. The sex ratios of pups were analyzed by Chi^2 test.

Differences in OFT scores and biochemical parameters in hippocampi and striatum between control and retinyl palmitate treated dams were determined with one-way ANOVA. For post-hoc comparisons, the Duncan's test was conducted. The number of correct and incorrect performances in the homing test was compared among groups using a Chi^2 test. A two-way (ANOVA), with drug exposure and sex difference as factors, was used to analyze differences in the time spent over the homing area, differences in OFT scores and biochemical parameters in offspring hippocampus and striatum. For post-hoc comparisons, the Bonferroni test was conducted when exposure factor was significantly and one-way ANOVA with Tukey's post hoc comparisons when sex difference was significantly different among groups. For the time spent over the homing area, OFT scores and biochemical analysis the litter was used as a unit for statistical evaluation with distinction between males and females.

Both behavioral and biochemical results are expressed as means ± standard error of the mean (S.E.M.); differences were considered significant when $p \leq 0.05$. All analyses were performed by using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA — SPSS version 15.0) software, and GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA — version 4.02) software.

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Artigo II

“The effects of vitamin A supplementation to rats during gestation and while breastfeeding upon redox parameters: increased oxidative stress and redox modulation in mothers and their offspring”

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The effects of vitamin A supplementation to rats during gestation and while breastfeeding upon redox parameters: increased oxidative stress and redox modulation in mothers and their offspring

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Abbreviations[1]

Abbreviations: AAPH, 2,2-azobis[2-amidinopropane]; ANOVA, analysis of variance; AUC, area under curve; CAT, catalase; CDNB, chloro-dinitro benzene; DNPH, dinitrophenylhidrazine; DTNB, 5,5-dithionitrobis 2-nitrobenzoic acid; GD, gestational day; GSH, glutathione; GST, glutathione S-transferase; H2O2, hydrogen peroxide; LD, lactant day; LOAEL, low observed adverse effect level; PND, postnatal day; RDA, recommended dietary allowance; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SVAD, subclinical vitamin A deficiency; TBARS, thiobarbituric acid reactive species; TCA, trichloroacetic acid; TRAP, total reactive antioxidant potential; VAD, vitamin A deficiency.

ABSTRACT

Vitamin A is an essential nutrient required in adequate amounts for reproduction and development. Subtle variations in the status of maternal nutrition may affect physiological and metabolic parameters in the fetus. Evidence suggests a key role for oxidative stress in these events. Literature is controversial about the effects of Vitamin A supplementation. Here, we studied the effects of Vitamin A supplementation on female Wistar rats during gestation and while breastfeeding on oxidative stress parameters of maternal and offspring tissues. Rats received daily doses of Vitamin A at 2500, 12500 and 25000 I.U./kg. We observed an increase of oxidative damage markers in the reproductive tissues and plasma of dams. The activity of glutathione-S-transferase was modulated by Vitamin A supplementation. It was found to be increased in the liver of dams and decreased in the kidneys of mothers and offspring. In pups, supplementation decreased the total antioxidant potential of the liver along with decreased superoxide dismutase/catalase activity ratio in the kidney. The levels of lipoperoxidation were increased in male offspring, but decreased in female pups. Collectively, the results suggest that excessive vitamin A intake during gestation and lactation might be toxic for mothers with adverse effects for the developing offspring.

Keywords: Retinol palmitate, oxidative stress, teratogenesis, reproduction, development

1. INTRODUCTION

Vitamin A is an essential nutrient that belongs to the fat soluble family of vitamins and, by definition, must be provided in the diet in sufficient amounts for the maintenance of health. It is required for several life processes, including reproduction, the immune system, vision, as well as the maintenance of cellular differentiation (Wasserman and Corradino, 1971). Of particular importance for public health is the role of vitamin A during periods of great proliferative growth and tissue development, as in gestation. Vitamin A is very important for fetal development and parturition (Clagett-Dame and DeLuca, 2002; Ross et al, 2000). In addition, breast milk is the only source of vitamin A to meet the infant requirements in neonatal period (Underwood, 1994).

On the other hand, there is strong evidence that states that a large intake of food that contain vitamin A, much of it as preformed vitamin A, more than 75% of people may be routinely ingesting more than the recommended dietary allowance (RDA) (Penniston and Tanumihardjo, 2006). Additionally, in some countries, like the United States, about 25% of adults ingest supplements containing vitamin A and about 5% take supplements of vitamin A alone (Rothman *et al*, 1995). Finally, vitamin A as prescription drugs in retinoid therapies had demonstrated efficacy for the treatment of several dermatological conditions and for the treatment/chemoprevention in cancer, especially in acute promyelocytic leukemia (Moise *et al*, 2007; Napoli, 1999).

This excessive vitamin A intake poses a serious treat for safe reproduction and development that might produce a spectrum of congenital defects at similar structures and developmental stages as in vitamin A deficiency (Collins and Mao, 1999). Teratogenic effects are observed for both natural and synthetic retinoids used in clinical

trials. Additionally, teratogenic effects are observed for retinyl palmitate supplementation in rats (Ritchie *et al*, 1998; Ross *et al*, 2000). The same is assumed to be true for humans although there is no strong evidence that excessive vitamin A (retinol or retinyl esters) has teratogenic effects (Dolk *et al*, 1999; Miller *et al*, 1998).

Furthermore, recent data shows that changes in maternal nutrition status, that might be subtle variations in the normal ranges, may affect the fetus and infant development (Langley-Evans, 2009). These changes can elicit permanent responses that produce long-term changes in tissue physiology and metabolism, predisposing an individual to several diseases in adult life (Gluckman *et al*, 2005; Lau and Rogers, 2004). Oxidative stress has been implicated as a key link between these stimuli and the remodeling response (Allen and Balin, 1989; Myatt, 2010). In addition, our previous results showed that vitamin A may also promote oxidative stress and modulate redox-dependent processes in living systems (Dal-Pizzol *et al*, 2001; Da Rocha, 2010; De Oliveira *et al*, 2009; Gelain *et al*, 2006, 2008; Pasquali *et al*, 2009).

Moreover, oxidative stress, reactive oxygen species (ROS) and reactive nitrogen species (RNS) have major biological functions and have been implicated in disease initiation and progression of many lethal and debilitating syndromes in humans (Davis *et al*, 2001; Forman *et al*, 2010; Gross, 1995; McEligot *et al*, 2005; Upham and Trosko, 2009). In this way, the aim of this work was to investigate the effects of vitamin A supplementation to rats during gestation and breastfeeding upon redox parameters of mothers and their offspring.

2. MATERIALS AND METHODS

All experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996). Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

2.1 Animals and housing conditions

Male and female Wistar rats (*Rattus norvegicus*) from our breeding colony were used throughout this study. The animals were caged in groups of five with free access to water and standard commercial food (CR1 lab chow, Nuvilab, Curitiba, Brazil) and were kept on a 12h light–dark cycle (7:00–19:00 h) at 23±1 °C. These conditions were maintained throughout the experiments. The nulliparous females, 90 days old and weighing 200-250g, were checked daily for 2 weeks for their estrous cyclicity by direct vaginal smear examination in light microscope, before mating. Thereafter the females were selected in their sexual receptive phase of the estrous cycle (proestrous) and caged with a single mature male (1F:1M) overnight. In the morning, the presence of a vaginal plug and/or viable sperm present in the vaginal smear was regarded as successful mating. The day on which the presence of a vaginal plug and/or the presence of sperm in the vaginal smear were detected was designated gestation day 0 (GD0). Each female was separated into a cage at GD20 and maintained together with their litter according to the protocol previously described above. The dams were allowed to litter naturally and the date of delivery was defined as postnatal day 0 (PND0). The number of pups per dose group were: control group, 6 dams, 55 pups; 2500 I.U./kg/day, 7 dams, 64 pups; 12500 I.U./kg/day, 7 dams, 51 pups; and 25000 I.U./kg/day, 6 dams, 44 pups.

2.2 Treatment

The pregnant females were randomly divided among groups and treated once a day for the entire period of gestation and lactation (21 days of gestation and 21 days of lactation). The pregnant and posterior lactant females were treated with 2500, 12500 or 25000 I.U./kg/day of retinol palmitate (Arovit®; a water-soluble form of vitamin, prepared in saline) orally, via a metallic gastric tube (gavage) in a maximum volume of 0.5 mL (control rats receive this volume of saline). The control group received a saline solution (NaCl 0.9%). The treatment was performed at night, everyday at 8:00 P.M., in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. Adequate measures were taken to minimize pain or discomfort.

2.3 Chemicals

Arovit® (retinol palmitate 150000 I.U./mL, a commercial water-soluble form of vitamin A) was purchased from Roche, Rio de Janeiro, RJ, Brazil. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A administration solutions were prepared daily and protected from light exposure and temperature.

2.4 Observations

All female rats were observed for clinical symptoms of toxicity and mortality once a day throughout the study. Body weights of the dams were assessed on GDs 0, 7, 14 and 20 and lactation days (LDs) 0, 7, 14 and 21, and body weight gain was calculated. Rats that died during the administration period were autopsied and simply examined. On PND0, pups of both sexes were counted, weighed and checked for the presence of external malformations and/or stillbirths. During the lactation period, the pups were examined daily for clinical signs and mortality. Litter sizes were determined on PND0.

Litters were weighed on PNDs 0, 7, 14 and 20, and body weight gain was calculated. On PND 3 they were observed for pinna detachment, on PND 11 for incisor eruption, on PND 15 for eye opening and on PND 20 for head, body and tail length. Viability indexes of pups were calculated in each litter on PNDs 0, 7, 14 and 21. And at terminal necropsy, females were confirmed for gestation by counting the number of implantation sites in uterine horns.

2.5 Tissues extraction and samples preparation

The animals were sacrificed by decapitation 24h after the last vitamin A administration. Blood samples were collected for analysis and the plasma was separated immediately. The livers, kidneys and hearts from all of the animals plus the uteri and ovaries of the dams were immediately dissected out in ice and stored at -80°C for later biochemical analyses. All tissues were homogenized in 1 mM phosphate buffer (pH 7.0) and centrifuged (3000 *x* g, 5 min) to remove cellular debris. The supernatants were used for all biochemical assays described. All the results were normalized by the protein content using bovine albumin as standard (Lowry *et al*, 1951).

2.6 Biochemical analysis

2.6.1 Lipid peroxidation

The formation of thiobarbituric acid reactive species (TBARS) was measured during an acid-heating reaction as an index of lipid peroxidation. This is a widely adopted parameter for the measure of lipid redox state, as previously described (Draper and Hadley, 1990). The samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid, then heated in a boiling water bath for 25 min. TBARS were determined by the absorbance in a spectrophotometer at 532 nm.

2.6.2 Protein carbonylation

The quantification of carbonyl groups was measured as a parameter for oxidative damage to proteins, based on the reaction with dinitrophenylhidrazine (DNPH), as previously described (Levine *et al*, 1990). Proteins were precipitated by the addition of 20% TCA and re-solubilized in DNPH. Then, the absorbance was read in a spectrophotometer at 370 nm.

2.6.3 Total protein thiol content

The samples were analyzed for their proteins' thiol content, which was used as an estimation of the oxidative alterations in proteins. As previously described by Ellman (1959), an aliquot of the sample was diluted in SDS 0.1%. Then, 0.01 M 5,5dithiobis-2-nitrobenzoic acid (DTNB) in ethanol was added. The intense yellow color was developed and read in a spectrophotometer at 412 nm after 60 min.

2.6.4 Total reactive antioxidant potential

The total reactive antioxidant potential (TRAP) was an index of the non-enzymatic antioxidant capacity. As previously described by Lissi *et al* (1992), this assay is based on the peroxyl radical (generated by AAPH solution, 2,2azobis[2-amidinopropane], with luminol) quenching by sample compounds. The reading is done by chemiluminescence emission. The results were transformed in percentual and the area under curve (AUC) as described by Dresch *et al* (2009) with GraphPad Software (San Diego, CA, USA –version 5.00).

2.6.4 Antioxidant enzymes activities

The activities of two important antioxidant enzymes were analyzed: catalase (CAT) and superoxide dismutase (SOD). Catalase (EC 1.11.1.6; CAT) activity was assayed by measuring the rate of decrease in hydrogen peroxide (H_2O_2) absorbance in a spectrophotometer at 240 nm and superoxide dismutase (EC 1.15.1.1, SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm (Aebi, 1984; Misra and Fridovich, 1972). To better understand the effect of vitamin A supplementation on these free radical-detoxifying enzymes, we applied a ratio between SOD and CAT activities (SOD/CAT), two enzymes that work in sequence to reduce the superoxide anion into water.

2.6.5 Glutathione-S-Transferase activity

Glutathione S-transferase (GST, E.C. 2.5.1.18) activity was determined spectrophotometrically at 340 nm by measuring the formation of the conjugate of GSH (glutathione) and CDNB (chloro-dinitro benzene) as previously described (Habig and Jakoby, 1981). Enzyme activity was determined by adding to a buffer, GSH 20 mM, and an amount of sample. The reaction started by the addition of CDNB 20 mM was carried out at 30°C, and monitored spectrophotometrically for 3 min. Corrections of the spontaneous reaction were made by measuring and subtracting the rate in the absence of enzyme.

2.7 Statistical analysis

Body weights, body weight gains, gestation length, numbers of implants, pups delivered, delivery index and viability indices of pups were analyzed by the one-way analysis of variance (ANOVA) to determine if any statistical differences existed among the groups. If the analysis of variance presented a significant result, Dunnett's test was

performed to detect any significant differences between the treated groups and their corresponding controls. The litter was used as a unit for statistical evaluation of the data of body weights and viability index of pups. The sex ratios and completion rates of physical development of pups were analyzed by Fisher's exact probability test.

Biochemical data were analyzed using the one-way ANOVA for the dams and the two-way ANOVA for the litters, with ''exposure to retinyl palmitate or saline control'' and ''gender'' as factors. Once significant main effects were seen, specific differences between groups were analyzed using post hoc Duncan's test or Bonferroni posttest. For biochemical analysis the litter was used as a unit for statistical evaluation with distinction between males and females. All analyses were performed using the GraphPad Prism (GraphPad Software Inc, San Diego, CA, USA, version 5.0) software. The results are expressed as means ± standard deviation (S.D.) unless otherwise indicated; p values were considered significant when $p \leq 0.05$.

3. RESULTS

3.1 Reproductive, maternal and litter data

No treatment-related clinical symptoms of toxicity were found in mothers throughout the treatment period. Two animals at 12500 I.U./kg/day group and one at 25000 I.U./kg/day showed soft feces and bradypnea immediately after delivery. One of the mothers at 12500 I.U./kg/day was euthanized on lactation day 4 because it became moribund. Their pups died due to deterioration of maternal condition. The examination of the moribund female and her litter showed no treatment-related abnormality. No gross malformations were observed in pups at PND0. Incidences of gross lesions at necropsy were not found in dams or pups of the retinyl palmitate-treated groups.

Body weight gain in gestation or lactation, gestation length, delivery index, the number of pups delivered and the number of implants in retinyl palmitate-treated groups showed no treatment-related changes (Table 1). During breastfeeding, the pups exhibited no treatment-related clinical symptoms, no viability index changes and no treatment-related reduction in body weights. Also, the sex ratio of litters was comparable among all groups. Lastly, incisor eruption, pinna detachment and eye opening completion ratios of the pups showed no treatment-related differences (Table 2).

3.2 Maternal biochemical data

Biochemical data of the dams shows increased levels of oxidative damage markers in the reproductive tissues (Table 3). In the retinyl palmitate-treated dams, TBARS levels and protein carbonylation levels were higher in uteri and ovaries related to the control group. In uteri, lipid peroxidation was increased by vitamin A at all doses ($F_{[3,20]}$ = 53.49, $p<0.0001$), while protein carbonylation was found increased with 2500 and 12500 I.U./kg/day ($F_{[3,20]}$ = 6.806, $p=0.0024$). In the ovaries, TBARS levels were increased at 12500 I.U./kg/day and 25000 I.U./kg/day ($F_{[3,20]}$ = 30.09, $p<0.0001$), with increased carbonylation levels at the same doses ($F_{[3,20]}$ = 34.38, $p<0.0001$). Indeed, plasma showed a significantly decrease in total thiol content levels ($F_{[3,20]}$ = 16.25, $p<0.0001$) and SOD activity ($F_{[3,20]}$ = 23.89, $p<0.0001$) in retinyl palmitate-treated rats at 25000 I.U./kg/day. The liver and kidney showed significant alterations in the GST activity. The liver had an increase in enzymatic activity of GST at 2500, 12500 and 25000 I.U./kg/day ($F_{[3,20]}$ = 10.59, $p=0.0002$) and the kidney showed a decrease in activity at 25000 I.U./kg/day ($F_{[3,20]}$ = 3.558, $p=0.0327$). On other hand, in the heart there was no significant difference at all.

3.2 Litter biochemical data

Pups' plasma showed significant changes in TBARS levels and total reactive antioxidant potential between retinyl palmitate-treated litters and the control litters (Table 4). TBARS levels in plasma increased in male pups while they decreased in female pups of retinyl palmitate treated dams at all retinyl palmitate doses tested (according to two-way ANOVA the interaction between exposure to retinyl palmitate and gender affect the result, $F_{[3,40]} = 193.2$, $p<0.0001$, with exposure affecting the result, $F_{[3,40]} = 190.5$, $p<0.0001$, and also gender, $F_{[1,40]} = 20.74$, $p<0.0001$). Also, the total reactive antioxidant potential in plasma decreased in males at 12500 and 25000 I.U./kg/day and in females at all tested doses (according to two-way ANOVA the interaction between exposure to retinyl palmitate and gender affect the result, $F_{[3,40]} = 34.21$, $p<0.0001$, with gender also affecting the result, $F_{[1,40]} = 138.4$, $p<0.0001$). In the liver, the total reactive antioxidant potential also changes in retinyl palmitate treated offspring (Table 5). The total reactive antioxidant potential in the liver decreased in males at 25000 I.U./kg/day and in females at all tested doses (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,40]} = 11.58$, $p<0.0001$). In the kidney, we observed changes in the enzymatic activities of CAT and SOD (Table 6). CAT activity increased (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,40]} = 16.65$, $p<0.0001$) and SOD activity decreased (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,40]} = 8.656$, $p=0.0002$) in retinyl palmitate-treated litters at 25000 I.U./kg/day. The SOD/CAT ratio also decreased at 25000 I.U./kg/day (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,40]} = 24.6$, $p<0.0001$) . Moreover, GST activity also decreased in retinyl palmitate treated males in all doses and in females at 12500 and 25000 I.U./kg/day (according to two-way ANOVA the exposure to retinyl palmitate affect the result, $F_{[3,40]} = 37.43$, $p<0.0001$). Lastly, there was no significant

difference in any parameter in the heart of the offspring (Table 7). According to the above data presented, the vitamin A effects do not appear to be dose-dependent in both dams and their litters.

4. DISCUSSION

Vitamin A has several biological roles and is required in adequate amounts for healthy reproduction and development (Clagett-Dame and DeLuca, 2002; Ross et al, 2000; Underwood, 1994). However, there is growing evidence that people are taking higher than recommended amounts of vitamin A through increased consumption of rich vitamin A food, vitamin A supplementation and/or prescription drugs (Penniston and Tanumihardjo, 2006; Rothman *et al*, 1995; Moise *et al*, 2007). This excessive vitamin A intake may pose a serious threat for safe reproduction and development. Teratogenic effects have been reported for many natural and synthetic retinoids in humans, but the evidences of such effects for retinyl palmitate supplementation in humans are limited (Miller *et al*, 1998).

For this reason, there is no consensus about the safety of retinyl palmitate supplementation during gestation for humans among several researchers, nutritional experts and international organizations (Dolk *et al*, 1999; IVACG, 1998; Miller *et al*, 1998; Mills *et al*, 1997; Ross *et al*, 2000). Some authors state that is safe to supplement fertile women with 10000 I.U./daily (200 I.U./kg/day) at any time during gestation, independent of their vitamin A status (Dolk *et al*, 1999; IVACG, 1998). However, others suggest higher levels of safety (Ross *et al*, 2000). Moreover, human data regarding the effects of retinyl palmitate supplementation during gestation and while breastfeeding are mostly observational, and epidemiological studies based in morphological endpoints.

In this work, we supplemented rats during gestation and while breastfeeding with retinyl palmitate in doses of 2500, 12500 and 25000 I.U./kg/day. These doses were used in order to evaluate the effects of equivalent doses to those stated as safe for humans during gestation and while breastfeeding upon dams and their offspring. Equivalent doses may be obtained throughout the application of an uncertainty factor of 10-fold for species differences and of 10-fold for interspecies difference (U. S. Environmental Protection Agency, 1991).

In these doses and conditions no treatment-related clinical signs of maternal and offspring toxicity and no malformations were found in our study. In addition, several reproductive and developmental toxicity endpoints showed no treatment-related effects. Only a slight delay in eye completion rate was found in the 2500 I.U./kg/day group (Table 2); however, this was judged to be unrelated to the treatment since no such delay occurred in the higher dose groups. Thus, the retinyl palmitate doses that do not show toxicity in humans also show no toxicity in equivalent doses in rats.

Apparently, we may suppose that maternal serum homeostatic mechanisms were efficient in maintaining stability of the fetal acquisition of vitamin A over the range of maternal supplementation. These were expected results for doses far below the dose of 163000 I.U./kg/day, the lowest teratogenic dose (LOAEL) of retinyl palmitate in rats established in segment II developmental toxicity testing (Ritchie *et al*, 1998). However, biochemical changes induced by prenatal insults that lead to physiological deficits of organ function may not always be accompanied by detectable anatomical abnormalities (Lau and Rogers, 2004). Hence, in recent decades, considerable attention had been drawn to functional teratology, an extension of the investigation beyond morphological examinations to include the evaluations of functional integrity of organ systems.

In this work we have proposed an evaluation of the functional integrity in organ systems of mothers and their offspring by redox evaluation of several enzymatic and non-enzymatic parameters. The redox profile was important, because ROS are generated in cells by several pathways and there has been much speculation regarding the role of free radicals during development (Allen and Balin, 1989; Hitchler and Domann, 2007). According to the free radical theory of development, it is the balance in production and removal of ROS/RNS that influences development (Hitchler and Domann, 2007).

The uteri and ovaries were analyzed due to the high metabolic activity of the reproductive tissues and the increased maternal metabolism in gestation (Agarwal *et al*, 2006; Fujii *et al*, 2005). Under the free radical-rich environment produced by reproductive metabolism, vitamin A excess results in a pro-oxidant effect with increased oxidative-damage-markers in uteri and ovaries of retinyl palmitate-treated rats. Indeed, the maintenance of a high redox potential is a prerequisite for maintaining the reproductive systems in a healthy state. There is growing literature on the effects of ROS in female reproduction related to the involvement in the pathophsiology of preeclampsia, hydatidiform mole, free radical-induced birth defects and others such as abortion (Agarwal *et al*, 2003; Agarwal and Allamaneni, 2004; Llurba *et al*, 2004).

In addition, oxidative stress markers have been reported at plasmatic levels in many of these pathological conditions in gestation (Gupta *et al*, 2009; Orhan *et al*, 2003; Rajdl *et al*, 2005; Sajewicz *et al*, 2006). In this work, the decrease found in total reduced thiol content of the maternal plasma could be considered a very early product of protein oxidation. Moreover, lipoperoxidation increased in males and decreased in females of retinyl palmitate-treated litters. In this way, maternal and offspring plasma seems to, in

part, lose its extracellular antioxidant power and may be under an increased free radical environment.

Furthermore, the decreased EC-SOD activity in maternal plasma could be also a response to oxidative stress (Nozik-Grayck *et al*, 2005). Extracellular superoxide dismutase is a key antioxidant enzyme implicated in the regulation of ROS-mediated tissue damage. This decrease suggests an increased production of superoxide commonly reported in many pathophysiological conditions such as diabetes and sepsis (Adachi *et al*, 2004; Faraci and Didion, 2004; Fattman *et al*, 2003; Fukai *et al*, 2002). Since the reaction of superoxide and nitric oxide can interfere with nitric oxide signaling and produce peroxynitrite, EC-SOD is also likely to play an important role in mediating nitric oxide-induced signaling events (Fattman *et al*, 2003; Nozik-Grayck *et al*, 2005).

Here, we also evaluated the redox parameters of maternal and offspring livers, kidneys and hearts, because they have been reported as potential targets for retinoid teratogenic effects. In addition, they are metabolicly very active organs where oxidative stress plays an important role in the pathogenesis of several diseases (Romero *et al*, 1998; Korge *et al*, 2008). The liver is also the major storage site for vitamin A, and more than likely has a central role in the maintenance of retinoid homeostasis (Penniston and Tanumihardjo, 2006; Si-Tayeb *et al*, 2010). Moreover, the existence of stellate cells in kidneys, also suggests that they may be fully capable of and adapted to storing vitamin A as retinyl esters, contributing to the regulation of vitamin A homeostasis (Penniston and Tanumihardjo, 2006).

Our results show that maternal livers have increased GST activity while maternal and offspring kidneys have decreased GST activity in retinyl palmitate-treated animals. The level of GST activity is considered to be an important factor that protects organs against

the deleterious effect of toxicants, and there is indication of oxidative activation of this enzyme (Aniya *et al*, 1993). In addition, GST can also detoxify endogenous electrophiles, which are usually the consequence of free-radical damage and may be an important participant in the mechanism for the repair of free-radical damage (Ketterer and Meyer, 1989; Wu *et al*, 2004). In this way, alterations in GST activity are likely to alter the redox state and the antioxidant defenses of the tissue (Sahu and Gray, 1996). Lastly, GST is also an endogenous switch for the control of signaling cascade pathways, and alterations in GST activity may alter the balance of regulation of kinase pathways (Townsend *et al*, 2003).

On the other hand, total reduction antioxidant potential decreased in the livers of retinyl palmitate-treated offspring. The total reactive antioxidant potential is representative of the non-enzymatic capability of the tissue to prevent oxidative damage. A wide range of molecules, including uric acid, glutathione, vitamin E and vitamin C are active free-radical scavengers (Halliwell, 1996). Reduced total antioxidant potential may suggest that offsprings' livers are under oxidative stress or have deficient antioxidant defenses. Both hypotheses are dangerous for health development, because these may disrupt the hepatic development and differentiation that are under the control of signaling events highly dynamic in nature (Si-Tayeb *et al*, 2010).

Retinyl palmitate supplementation also decrease SOD activity in offspring kidneys and increase CAT activity in male offsprings, but the SOD/CAT ratio decreased in both genders. This modulation is very similar to that of other nephrotoxic compounds intake, such as ethanol or nicotine (Cid *et al*, 2003; Cooper, 2006). In addition, many studies showed that superoxide dismutase activities of teratogenically sensitive tissues were significantly lower than those of insensitive tissues (Fantel and Person, 2002). Other

works have previously reported that the mechanisms underlying renal homeostasis are fragile and can easily be disturbed by drugs, either administered to the mother during gestation, or to the neonate (Boubred et al, 2006; Guignard et al, 1995).

Finally, retinyl palmitate supplementation has no modulation upon the redox parameters analyzed in maternal and offspring hearts. These results differ from our previous results with male rats supplemented at therapeutic doses, where retinyl palmitate induces oxidative stress in the heart (Da Rocha, 2010). Our previous findings demonstrated that vitamin A supplementation potentially induces dysfunctions in the redox and bioenergetics states of different tissues, in healthy adult male Wistar rats in therapeutical doses of 2500, 4500, and 9000 IU/kg/day (de Oliveira et al., 2009; Pasquali et al., 2009). Now, in the present work, we tested vitamin A supplementation effects in a totally distinct rat model. Indeed, we supplemented pregnant and lactant Wistar rats in doses of 2500, 12500, and 25000 IU/kg/day, and investigated the effects upon their offspring. Indeed, recent findings suggest that males may be more susceptible than females to stress induced neurobehavioral changes and free radicals may exert a regulatory influence in such gender dependent responses to stress (Chakraborti et al., 2007). In addition, several other examples can be given for the apparent teratogenic insensitivity of the developing heart and remodeling has not been as clearly demonstrated in the heart as in other tissues (Fantel and Person, 2002; Langley-Evans, 2009).

5. CONCLUSION

Vitamin A deficiency is an enormous problem, especially in developing nations, and has received much attention in terms of public health initiatives. However, excessive vitamin A intake may be a growing, but underappreciated problem. The increasing

availability and interest in fortified foods and supplements in developed nations has resulted in a large percentage of the population with preformed vitamin A intakes higher than recommended. In this work we demonstrated for the first time that excessive vitamin A intake, as retinyl palmitate supplementation at doses far below the LOAEL in rats, has subclinical toxicity for during gestation and lactation in mother rats, with adverse effects on many tissues and organ system of the developing offspring. However, the full extent of the redox modulations showed in this study is not well known and further research are needed to ascertain its effects on overall health and well-being.

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Table 1 Reproductive data

	Retinyl Palmitate (IU/kg/day)			
	0 (control)	2500	12500	25000
No. of dams	6	7	7	6
Gestation weight gain (%)	16.8±4.1	15.5±3.1	17.2±3.4	18.1±3.8
Lactation weight gain (%)	12.4±2.6	15.4±6.1	14.6±6.2	15.4±3.1
Gestation length (days)	21	21	21	21
No. of implantations	9.5±1.7	10.5±3.3	10.7±1.5	8.6±2.6
Delivery index (%)	97.2±5.6	88.2±17.3	70.8±25.5	83.3±18.9
No. of pups delivered	9.3±1.9	9±2.8	7.5±2.5	7±2.5
Sex ratio of pups	0.464	0.404	0.538	0.541
Viability index (%)				
Day 0	95.3±5.6	95.9±6.9	95.8±10.2	96.2±8.9
Day 7	89.2±9.2	91.8±9.3	69.3±40.6	98.2±4.1
Day 14	97.7±4.6	98.3±4.1	95±10	98±4.5
Day 21	95±10	98.6±3.4	97.2±5.6	97.8±5
Pup weight (g)				
Day 0	5.9±0.4	6.0±0.4	5.7±0.8	6.4±0.5
Day 7	11.5±1.7	11.4±1.5	10.6±0.6	12.5±1.5
Day 14	17±3.5	20.5±2.8	18.4±0.1	22.3±3.9
Day 21	30.2±4.6	32.5±3.3	30.9±4.1	34.9±4.8

Gestation weight gain (%) = [(weight on PND0 - weight on GD0)/weight on GD0)] x 100.
Lactation weight gain (%) = [(weight on PND21 - weight on PND0)/weight on PND0)] x 100.
Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
Sex ratio of pups = no. of male pups/total no. of pups.
Viability index on postnatal day 0 (%) = (no. of live pups delivered/total no. of pups delivered)×100.
Viability index on postnatal day 7 (%) = (no. of live pups on postnatal day 7/no. of live pups delivered)×100.
Viability index on postnatal day 14 (%) = (no. of live pups on postnatal day 14/no. of live pups on postnatal day 7 after delivery)×100.
Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live pups on postnatal day 14 after delivery)×100.

Table 2 Physical development of the pups

	Retinyl Palmitate (IU/kg/day)			
	0 (control)	2500	12500	25000
No. of litters examined	6	7	6	6
Completion rate of pinna detachment (%), PND 3	92.9±10.1	92.5±9.6	95±7.1	96.3±6.4
Completion rate of incisor eruption (%), PND 11	90±14.2	90.8±10.7	88.9±15.7	72.2±25.5
Completion rate of eye opening (%), PND 15	82.9±4.1	67.2±5.1	72.5±22.6	75±8.4
Body length (mm)	65.2±5.5	67.2±5.1	68.7±7.7	68.9±5.7
Tail length (mm)	60.5±5.2	63.2±7.4	63.2±3.5	67.1±3.7
Head length (mm)	31.1±3.4	33.1±3.2	31.4±3.6	31.3±1.9

Completion ratio (%) = (number of animals showing positive response/number of animals examined)×100.

Table 3 Biochemical data of the dams.

	Retinyl Palmitate (IU/kg/day)			
	0 (control)	2500	12500	25000
No of dams examined	6	7	6	6
Uterus				
TBARS level (nmol MDA/mg protein)	0.1301±0.02	0.5319±0.07c	0.4002±0.09c	0.4310±0.07c
Carbonyl level (nmol carbonyl/mg protein)	1.4492±0.29	2.4961±0.57b	2.1577±0.49a	1.6950±0.60
Total thiol content (mmol SH/mg protein)	30.19±10.3	29.28±9.2	30.69±8.3	38.51±5.9
TRAP (under curve area)	199276±32853	217860±42323	279640±34515	273250±72347
CAT activity (U CAT/mg protein)	7.48±2.5	7.87±3.4	4.99±2.3	5.36±2.3
SOD activity (U SOS/mg protein)	33.72±6.1	26.28±8.8	24.55±7.5	28.66±8.1
SOD/CAT ratio (arbitrary units)	4.23±1.3	2.81±0.52	4.12±0.98	3.71±1.4
Ovaries				
TBARS level (nmol MDA/mg protein)	0.1333±0.02	0.1524±0.06	0.3304±0.03c	0.2905±0.07c
Carbonyl level (nmol carbonyl/mg protein)	2.0119±0.47	2.2887±0.42	2.9287±0.76a	4.7632±0.83c
Total thiol content (mmol SH/mg protein)	38.37±9.3	31.80±13.5	33.46±9.2	37.57±7.9
TRAP (under curve area)	490112±84408	504094±129932	563496±69720	526106±63875
CAT activity (U CAT/mg protein)	5.62±0.7	6.01±1.6	4.39±0.8	4.74±1.1
SOD activity (U SOD/mg protein)	27.46±5.5	31.27±2.8	30.24±8.1	25.54±4.5
SOD/CAT ratio (arbitrary units)	4.44±0.6	5.87±0.5	6.18±1.8	5.02±0.9
Blood/Plasma				
TBARS level (pmol MDA/mg protein)	23.668±11.8	23.365±11.8	23.315±2.5	22.593±6.2
Carbonyl level (pmol carbonyl/mg protein)	23.66±13	31.75±12	33.02±10	36.64±10
Total thiol content (mmol SH/mg protein)	2.71±0.1	2.72±0.1	2.73±0.1	2.34±0.2c
TRAP (under curve area)	382155±24737	409688±23833	375007±23861	372558±10702
CAT activity (U CAT/mg protein)	10.59±3.9	12.68±2.6	12.34±1.4	7.12±1.3
SOD activity (U SOD/mg protein)	2.93±0.1	3.57±0.5	2.96±0.8	1.76±0.2c
Liver				
TBARS level (nmol MDA/mg protein)	7.2731±1.4	7.5144±1.1	6.0411±1.1	6.2573±1.3
Carbonyl level (nmol carbonyl/mg protein)	1.5162±0.3	1.6246±0.5	1.7511±0.7	2.2004±0.2
Total thiol content (mmol SH/mg protein)	52.16±5.2	52.61±2.8	54.51±5.2	56.62±4.9
TRAP (under curve area)	364314±18525	337766±61921	328347±27197	328578±34686
CAT activity (U CAT/mg protein)	42.66±7.8	44.62±9.5	46.79±4.7	45.09±6.5
SOD activity (U SOD/mg protein)	36.76±9.4	30.32±3.5	36.09±9.5	33.12±3.5
SOD/CAT ratio (arbitrary units)	0.8335±0.23	0.7197±0.22	0.8007±0.09	0.6935±0.06
GST activity (U GST/mg protein)	0.2171±0.03	0.2386±0.02b	0.2545±0.02c	0.2403±0.01c
Kidney				
TBARS level (nmol MDA/mg protein)	5.3478±1.8	3.2208±1.6	4.3846±1.5	3.5538±0.8
Carbonyl level (nmol carbonyl/mg protein)	1.7047±0.2	1.7376±0.5	1.3328±0.3	1.3662±0.3
Total thiol content (mmol SH/mg protein)	44.96±5.9	45.53±6.2	47.34±5.3	46.83±4.6
TRAP (under curve area)	176693±27822	224337±32776	178748±21794	177020±32891
CAT activity (U CAT/mg protein)	31.87±4.1	37.48±3.1	36.43±3.4	35.94±3.6
SOD activity (U SOD/mg protein)	18.16±2.5	15.81±6.5	15.52±4.2	14.41±5.4
SOD/CAT ratio (arbitrary units)	0.5764±0.11	0.4272±0.18	0.4691±0.11	0.4099±0.19
GST activity (U GST/mg protein)	0.0508±0.007	0.0521±0.005	0.0499±0.006	0.0361±0.001a
Heart				
TBARS level (nmol MDA/mg protein)	2.0379±0.2	2.2703±0.5	2.1881±0.6	2.0930±0.6
Carbonyl level (nmol carbonyl/mg protein)	2.0249±0.3	1.9941±0.2	1.6428±0.4	1.5438±0.4
Total thiol content (mmol SH/mg protein)	35.01±5.8	36.91±8.5	43.95±7.4	43.60±7.4
TRAP (under curve area)	488485±48057	401583±22557	407789±60394	420904±49751
CAT activity (U CAT/mg protein)	6.42±0.5	6.43±1.6	6.51±1.3	6.94±1.4
SOD activity (U SOD/mg protein)	5.25±1.2	6.75±2.7	6.31±2.3	9.27±3.8
SOD/CAT ratio (arbitrary units)	0.8247±0.22	1.1726±0.26	0.9646±0.25	1.3228±0.5

Significantly different from the control: a = $p<0.05$; b = $p<0.01$; and c = $p<0.001$

Table 4 Biochemical data of the pups (blood/plasma)

	Retinyl Palmitate (IU/kg/day)			
	0 (control)	2500	12500	25000
No of litters examined	6	7	6	6
TBARS level (pmol MDA/mg protein)				
Male	21.096±1.2	23.241±0.6c	19.566±1.3b	28.555±0.8c
Female	28.583±0.5	22.065±1.1c	16.744±0.8c	20.938±1.3c
Carbonyl level (pmol carbonyl/mg protein)				
Male	15.74±2.9	19.93±3.8	14.25±1.4	15.48±3.2
Female	20.55±6.5	18.04±6.1	10.85±1.9	19.86±3.6
Total thiol content (mmol SH/mg protein)				
Male	2.01±0.4	1.84±0.4	1.77±0.1	1.94±0.2
Female	1.86±0.4	1.65±0.4	1.65±0.1	1.74±0.1
TRAP (under curve area)				
Male	350360±28654	370389±23135	404614±17893c	416724±28670c
Female	366172±8452	326933±39961b	288283±29781c	282166±23506c
CAT activity (U CAT/mg protein)				
Male	12.95±2.2	15.06±1.5	13.22±1.9	15.48±2.1
Female	13.22±2.6	15.77±2.6	15.02±2.2	13.29±1.9
EC-SOD activity (U SOD/mg protein)				
Male	2.84±0.9	2.68±1.4	2.25±0.7	1.85±0.1
Female	3.19±0.3	2.71±0.7	2.25±0.9	2.07±0.1

TBARS level, Carbonyl level, Total thiol content, TRAP and SOD activity in plasma. CAT activity in total blood.
Significantly different from the control: a = $p<0.05$; b = $p<0.01$; and c = $p<0.001$

Table 5 Biochemical data of the pups (liver)

	Retinyl Palmitate (IU/kg/day)			
	0 (control)	2500	12500	25000
No of litters examined	6	7	6	6
TBARS level (nmol MDA/mg protein)				
Male	2.9062±0.6	2.4011±0.7	2.9990±1.1	3.1212±1.0
Female	2.4704±0.2	3.0714±0.6	3.0672±0.7	3.2458±0.4
Carbonyl level (nmol carbonyl/mg protein)				
Male	1.6525±0.6	1.8729±0.5	1.6791±0.6	1.5356±0.5
Female	2.0595±0.6	1.4181±0.6	1.7206±0.5	1.3167±0.3
Total thiol content (mmol SH/mg protein)				
Male	51.79±2.2	52.01±3.1	52.99±4.5	54.38±5.1
Female	50.49±1.1	49.69±3.1	55.23±8.4	51.29±2.1
TRAP (under curve area)				
Male	326144±6405	370747±51482	356083±12877	389619±8484b
Female	291085±40213	352831±58704b	346917±44996a	391925±41482c
CAT activity (U CAT/mg protein)				
Male	23.27±1.4	24.29±5.4	22.35±4.8	24.79±4.1
Female	26.94±2.3	26.48±2.7	24.89±4.5	26.49±4.9
SOD activity (U SOD/mg protein)				
Male	27.97±5.4	21.56±4.1	30.12±6.5	28.62±9.4
Female	26.94±5.9	21.03±5.9	28.78±7.5	23.76±8.4
SOD/CAT ratio (arbitrary units)				
Male	1.2354±0.21	1.1552±0.32	1.3505±0.51	1.1447±0.49
Female	1.0898±0.11	1.1581±0.59	1.1677±0.41	0.9195±0.34
GST activity (U GST/mg protein)				
Male	0.1453±0.01	0.1453±0.02	0.1327±0.01	0.1494±0.01
Female	0.1456±0.01	0.1412±0.01	0.1356±0.02	0.1418±0.01

Significantly different from the control: a = $p<0.05$; b = $p<0.01$; and c = $p<0.001$

Table 6 Biochemical data of the pups (kidney)

	Retinyl Palmitate (IU/kg/day)			
	0 (control)	2500	12500	25000
No of litters examined	6	7	6	6
TBARS level (nmol MDA/mg protein)				
Male	2.1954±0.5	1.9847±0.7	2.3623±0.6	1.6698±0.7
Female	2.5506±0.4	2.0539±0.7	2.1071±0.7	1.7394±0.6
Carbonyl level (nmol carbonyl/mg protein)				
Male	1.2042±0.4	1.5181±0.5	1.4044±0.4	1.1499±0.3
Female	1.2641±0.3	0.9873±0.3	1.3848±0.3	1.3303±0.4
Total thiol content (mmol SH/mg protein)				
Male	46.63±7.4	47.39±8.1	42.87±8.1	42.78±5.7
Female	47.93±5.9	46.48±8.2	43.79±8.1	47.09±5.2
TRAP (under curve area)				
Male	220272±46134	193216±69935	225821±64152	210229±64987
Female	203044±47442	202785±69910	218950±65827	200731±39730
CAT activity (U CAT/mg protein)				
Male	32.45±5.7	36.61±4.1	37.29±2.5	40.61±3.7b
Female	33.69±5.7	32.99±5.9	38.85±4.9	47.61±4.5c
SOD activity (U SOD/mg protein)				
Male	20.92±1.6	19.15±2.8	17.89±1.6	15.56±3.4c
Female	20.44±1.8	18.28±2.4	19.39±1.1	16.21±3.9b
SOD/CAT ratio (arbitrary units)				
Male	0.6215±0.13	0.5299±0.13	0.4994±0.06a	0.3162±0.05c
Female	0.6005±0.07	0.6012±0.11	0.5121±0.08	0.3438±0.11c
GST activity (U GST/mg protein)				
Male	0.6476±0.001	0.5788±0.009a	0.4962±0.006c	0.4315±0.005c
Female	0.6237±0.002	0.6001±0.008	0.5305±0.007b	0.4307±0.003c

Significantly different from the control: a = $p<0.05$; b = $p<0.01$; and c = $p<0.001$

Table 7 Biochemical data of the pups (heart)

	Retinyl Palmitate (IU/kg/day)			
	0 (control)	2500	12500	25000
No of litters examined	6	7	6	6
TBARS level (nmol MDA/mg protein)				
Male	4.9237±0.8	5.1469±0.9	5.1349±0.9	5.6788±0.5
Female	5.0149±0.7	5.0038±1.6	5.0562±0.9	5.3839±0.6
Carbonyl level (nmol carbonyl/mg protein)				
Male	1.4433±0.4	1.4375±0.4	1.5159±0.5	1.4821±0.2
Female	1.2851±0.4	1.4677±0.2	1.2237±0.3	1.4396±0.3
Total thiol content (mmol SH/mg protein)				
Male	33.76±1.4	34.41±2.1	34.31±1.4	33.51±4.1
Female	35.36±1.9	34.70±2.5	34.87±2.1	37.28±1.5
TRAP (under curve area)				
Male	557079±47788	542698±58730	549476±60007	558962±60525
Female	536050±45177	535168±69222	564148±55616	559235±50820
CAT activity (U CAT/mg protein)				
Male	9.07±0.8	8.82±1.3	8.69±2.5	8.21±1.1
Female	8.43±1.2	9.55±1.3	8.66±1.3	10.17±1.1
SOD activity (U SOD/mg protein)				
Male	8.49±1.5	9.71±2.9	7.57±2.1	6.24±1.9
Female	7.79±1.9	4.64±2.6	6.12±2.4	3.93±2.2
SOD/CAT ratio (arbitrary units)				
Male	1.0979±0.37	1.1579±0.36	0.9557±0.32	0.6733±0.33
Female	1.1536±0.34	0.8237±0.12	1.1886±0.21	1.1755±0.36

5. DISCUSSÃO

A vitamina A exerce uma ampla variedade de funções biológicas, no desenvolvimento do SNC quanto em diversas funções relacionadas ao SNC durante a vida adulta. Entretanto, a vitamina A é necessária em quantidades adequadas para uma reprodução e desenvolvimento saudável, pois tanto o excesso quanto a deficiência podem ser teratogênicos em humanos (Ross et al, 2000; Clagett-Dame e DeLuca, 2002). Os riscos da exposição ao excesso de vitamina A durante a gestação e lactação em humanos tornam-se importantes a partir das informações recentes na literatura apontando para um consumo de vitamina A acima do RDA em algumas populações, principalmente em países desenvolvidos e industrializados. Estas evidências parecem bastante consistentes no que diz respeito ao aumento do consumo de alimentos ricos em vitamina A, de alimentos fortificados e de drogas terapêuticas (Rothman et al, 1995; Inomata et al., 2005; Penniston e Tanumihardjo, 2006).

Atualmente, efeitos teratogênicos têm sido documentados para inúmeros retinóides naturais e sintéticos em humanos, mas as evidências destes efeitos para a suplementação com palmitato de retinol continuam limitadas (Moise et al., 2007; WHO, 2009). Assim, hoje em dia não existe um consenso entre as diversas instituições internacionais preocupadas com a segurança da suplementação com palmitato de retinol durante o período reprodutivo em humanos. Entretanto, a grande maioria das instituições internacionais considera improvável a existência de riscos associados à suplementação de até 10.000 UI/kg/dia com vitamina A pré-formada durante qualquer período da gestação, independente do status de vitamina A materno (IVACG, 1998; Dolk et al, 1999; Ross et al, 2000; WHO, 2009). Outros consideram improvável risco associado até mesmo a doses ainda mais elevadas, como 25000 UI/kg/dia (Mills et al., 1997; Miller et al., 1998; Ritchie et al., 1998).

Neste trabalho foram investigados os efeitos da suplementação com palmitato de retinol para ratas durante a gestação e lactação utilizando-se doses de 2.500, 12.500 e 25.000 UI/kg/dia de uma preparação comercial hidrossolúvel (Arovit®). Estas doses foram obtidas a partir da aplicação de um fator de incerteza, considerado bastante genérico, mas muito utilizado em estudos toxicológicos, que considera as diferenças interespecíficas e intraespecíficas (U. S. Environmental Protection Agency, 1991). Portanto, as doses utilizadas neste trabalho possuem uma equivalência aproximada com as doses consideradas supostamente seguras para suplementação de mulheres gestantes e lactantes, independente do seu status nutricional.

Como era o esperado, dentro da faixa de doses estabelecidas neste estudo, nenhum animal manifestou qualquer sintoma clínico de intoxicação, como vômitos, pilo ereção, tremores, fraqueza muscular ou diarréia, por exemplo. Também não detectamos qualquer tipo de anomalia morfológica relacionada ao tratamento nos filhotes cujas mães foram suplementadas com palmitato de retinol. Novamente, estes resultados eram esperados, visto que as doses estabelecidas neste trabalho estão muito abaixo da menor dose teratogênica (LOAEL) para o palmitato de retinol em ratos que é de 163.000 UI/kg/dia (Ritchie et al, 1998). Além disso, a suplementação não se mostrou capaz de afetar diversos parâmetros de toxicidade reprodutiva e desenvolvimental, reforçando a ausência de efeitos tóxicos associados ao tratamento quando levamos em consideração apenas parâmetros morfológicos e clássicos aplicados em estudos toxicológicos.

Entretanto, hoje sabemos que um insulto pré-natal, como as alterações nutricionais, por exemplo, pode induzir alterações bioquímicas que são capazes de levar a déficits fisiológicos que nem sempre serão acompanhados de alterações morfológicas observáveis (Lau e Rogers, 2004). Assim, recentemente passou-se a dar mais atenção para a teratologia funcional e molecular, a qual busca avaliar a integridade funcional dos

órgãos, além dos exames de caráter morfológico. Entretanto, este tipo de investigação em humanos é difícil ou mesmo impossível, devido principalmente a questões éticas. Assim, os dados a nível fisiológico e molecular das doses de palmitato de retinol consideradas seguras durante a gestação e lactação em humanos são praticamente impossíveis de ser gerados, valorizando ainda mais a produção de dados experimentais em diferentes modelos animais.

Neste trabalho, nós pela primeira vez demonstramos que a suplementação com palmitato de retinol, em doses de 2.500, 12.500 e 25.000 UI/kg/dia, durante a gestação e a amamentação em ratos, é capaz de alterar parâmetros de estresse oxidativo em diversos tecidos das mães e dos filhotes, especialmente do SNC. Sabe-se que a passagem da vitamina A pela BH provavelmente ocorra naturalmente, pois existe uma grande quantidade de RBP nas células deste tecido (MacDonald et al., 1990). Assim, a vitamina A após cruzar a BH é rapidamente distribuída entre as diferentes regiões cerebrais em quantidades bastante similares, pois todas as regiões cerebrais apresentam o aparato molecular necessário para transportar e metabolizar o retinol em retinóides considerados biologicamente mais ativos (McCaferry e Dräger, 1994; Lane e Bailey, 2005; McCaffery et al., 2006). Portanto, neste modelo é provável que todas as estruturas cerebrais analisadas, o hipocampo e o estriado, tenham recebido quantidades aumentadas de vitamina A perante o tratamento aplicado.

Uma das conseqüências da suplementação com vitamina A foi a modulação das defesas antioxidantes enzimáticas tanto no estriado quanto no hipocampo de mães e filhotes, indicando que espécies reativas de oxigênio podem estar sendo produzidas em excesso. Encontramos um aumento na atividade da SOD no estriado das mães, no hipocampo dos filhotes e no estriado dos filhotes machos, o que pode ser um indicativo do aumento na produção de $\bullet O_2^-$, pois ele é o principal ativador alostérico da SOD

(Halliwell e Gutteridge, 1999). Além disso, encontramos uma diminuição na atividade da CAT nestes mesmos tecidos que também pode ser justificada pelo aumento de $\bullet O_2^-$, pois este radical pode também inativar alostericamente a enzima CAT, diminuindo sua atividade (Kono e Fridovich, 1982; Shimizu et al., 1984). Na verdade, a própria vitamina A é conhecida por aumentar a produção de $\bullet O_2^-$, como previamente demonstrado em outros estudos (Murata e Kawanishi, 2000; Klamt et al., 2005).

Em conjunto, estas modulações nas defesas antioxidantes enzimáticas resultaram em um aumento da razão SOD/CAT em todas as regiões do SNC que foram analisadas neste trabalho, tanto nas mães quanto nos filhotes que receberam suplementação com vitamina A. Entre as conseqüências deste aumento na razão SOD/CAT está o aumento na disponibilidade de H_2O_2, já que a SOD metaboliza $\bullet O_2^-$ a H_2O_2, entretanto a CAT estará convertendo H_2O_2 a H_2O em taxas menores. Além disso, o H_2O_2 pode, via reação de Fenton, tornar-se uma perigosa fonte de produção de radical hidroxil (•OH), que é considerada a mais poderosa molécula pró-oxidante, capaz de induzir um estado pró-oxidante em todos os tecidos do SNC analisados (Halliwell, 2006). Portanto, aumento na razão SOD/CAT pode rapidamente culminar em aumento no dano a biomoléculas.

O SNC, em particular, é considerado especialmente sensível ao estresse oxidativo devido, principalmente, ao seu alto conteúdo de lipídios peroxidáveis e suas defesas antioxidantes relativamente baixas (Halliwell e Gutteridge, 1999). Neste estudo, a suplementação com vitamina A claramente induziu um aumento na peroxidação lipídica e carbonilação proteica tanto no estriado quanto no hipocampo materno, assim como uma diminuição no conteúdo total de tióis reduzidos no hipocampo. Entretanto, doses mais baixas de suplementação foram capazes apenas de induzir um aumento no dano a proteínas, sugerindo uma determinada vulnerabilidade especialmente das

proteínas estriatais ao insulto oxidativo induzido por este modelo. No estriado e hipocampo dos filhotes, a suplementação com palmitato de retinol também aumentou os níveis de lipoperoxidação e carbonilação de proteínas; entretanto, encontramos redução no conteúdo total de tióis reduzidos apenas no estriado dos filhotes de sexo masculino.

Um aumento tanto nos níveis de peroxidação lipídica quanto nos níveis de carbonilação proteica, assim como uma redução no conteúdo total de tióis reduzidos, são conhecidos por facilitar a formação de ligações cruzadas inter e intra-moleculares (Goetz e Gerlach, 2004). Estas alterações oxidativas acabam favorecendo modificações conformacionais que podem levar ao aumento da hidrofobocidade e a formação de agregados protéicos que são capazes de induzir uma disfunção celular generalizada (Mattson e Magnus, 2006). Além disso, um aumento no dano oxidativo a proteínas pode resultar em um aumento do conteúdo de ferro livre, favorecendo também a manutenção do estado pró-oxidativo, conforme descrito anteriormente (Keyer e Imlay, 1996).

Adicionalmente, encontramos aumento na atividade de GST no estriado das mães e filhotes suplementados com palmitato de retinol. Esta modulação na atividade da GST pode também ser resultado de um insulto oxidativo gerado pela suplementação com vitamina A, pois se sabe que esta enzima pode ser ativada via oxidação (Aniya et al, 1993). Entre as muitas funções descritas, sabemos que a GST também é responsável pela detoxificação de muitos eletrólitos endógenos, que são usualmente gerados como conseqüência do dano oxidativo, e compõem os mecanismos de reparo ao dano provocado por espécies reativas (Ketterer e Meyer, 1989; Wu et al, 2004). Assim, não podemos descartar que a ação do sistema de detoxificação via GST tenha sido estimulado em virtude do insulto oxidativo gerado pela suplementação com vitamina A. Além disso, o sistema de detoxificação via GST, que necessita de glutationa, talvez seja também responsável, pelo menos em parte, pela redução no TRAP que foi detectado

nestes mesmos tecidos, pois uma grande variedade de moléculas compõem as defesas antioxidantes não-enzimáticas, incluindo a glutationa (Fang et al., 2002).

Neste trabalho, nós também demonstramos que a suplementação com vitamina A, nestas mesmas doses, durante a gestação e lactação, foi capaz de induzir alterações comportamentais tanto nas mães quanto nos filhotes. Estes resultados não chegam a ser completamente surpreendentes, visto que previamente já havíamos demonstrado que a suplementação com vitamina A em doses terapêuticas é capaz de induzir ansiedade em ratos adultos, assim como redução na atividade exploratória (De Oliveira et al., 2007b). Adicionalmente, outros autores já haviam demonstrado também que a vitamina A, principalmente na forma de palmitato de retinol, é capaz de induzir alterações comportamentais em humanos, como irritabilidade, fadiga e ansiedade (Myhre et al., 2003). Entretanto, salientamos que aqui neste trabalho nós demonstramos algo totalmente diferente; pela primeira vez, demonstramos que um insulto nutricional, provocado pela suplementação com vitamina A durante o período perinatal, foi capaz de afetar não apenas o comportamento das mães, mas também dos seus filhotes.

Em ratos, a base molecular do aprendizado olfatório em filhotes envolve uma cadeia complexa de eventos cujo desenvolvimento pode ser monitorado através de testes simples, como o teste de Homing (Nakamura et al., 1987; Sullivan et al., 1989; Rangel e Leon, 1995; Langdon et al., 1997). Esta capacidade de discriminação e preferência pelo odor da mãe é inclusive considerada uma das etapas críticas para a sobrevivência e desenvolvimento em todos os mamíferos. Portanto, é essencial que todos os filhotes aprendam rapidamente a identificar, localizar, alcançar e preferir o odor materno naturalmente existente no ninho (Sullivan et al., 1989; Leon, 1992; McLean et al., 1999; Roth e Sullivan, 2005). Entretanto, existem algumas evidências bastante consistentes de que o tratamento com determinados retinóides, como o ácido

retinóico 13-cis são capazes de diminuir a capacidade de aprendizado em animais devido à indução de uma perturbação na progressão normal do ciclo celular na região hipocampal, além de induzir aumento nos níveis de morte celular nesta mesma região (Crandall et al., 2004; McCaffery et al., 2006).

Neste trabalho, a suplementação com vitamina A, na forma de palmitato de retinol, via intragástrica, durante a gestação e lactação em ratas, alterou o desempenho dos filhotes das mães suplementadas no teste de Homing. O tratamento com vitamina A aumentou a porcentagem de tempo gasto pelos animais na área de homing quando o teste foi realizado no dia pós-natal 5 (DPN5), mas diminuiu esta mesma porcentagem quando o teste foi realizado no DPN10 nas fêmeas. Provavelmente, a maior proporção de células imaturas sensíveis no DPN5 torne os animais especialmente suscetíveis ao insulto da vitamina A, pois foi notável a diferença entre estas duas fases distintas do desenvolvimento (Ikonomidou e Kaindl, 2010). Além disso, a capacidade de reconhecimento e preferência pelo odor materno parece ser mais resistente ao insulto da suplementação com vitamina A nos machos do que nas fêmeas, pois durante o DPN10 foram detectadas alterações no teste de Homing apenas para as fêmeas. Uma possibilidade que talvez nos ajude a compreender esta diferença talvez resida no maior comprometimento maternal usualmente demonstrado aos filhotes machos por parte das ratas, mas é possível que estas diferenças possam ser atribuídas também a diferenças nos hormônios sexuais (Melniczek e Ward, 1994; Moore et al., 1997).

A suplementação com vitamina A também alterou parâmetros comportamentais dos filhotes no teste de Campo Aberto, onde foi possível detectar uma redução no número de *rearings*, no número de *groomings* e número de visitas ao centro do aparato, assim como no número de cruzamentos nos machos. Adicionalmente, o tratamento aumentou o número de *freezings* em filhotes de ambos os sexos. Entretanto, não foram

detectadas alterações comportamentais apenas nos filhotes, mas também nas mães que receberam a suplementação; a dose de 25.000 UI/kg/dia reduziu a atividade locomotora e na dose de 12.500 UI/kg/dia reduziu o número de *groomings* e aumentou o número de *freezings*. Portanto, nossos resultados sugerem que a suplementação com vitamina A foi capaz de diminuir a atividade exploratória e locomotora dos filhotes, além de reduzir a atividade locomotora das mães. Adicionalmente, alguns destes parâmetros analisados formam indícios de um possível aumento em comportamentos tipo-ansiedade, que pode representar um passo inicial para a depressão em diversos modelos experimentais, assim como também é observado em humanos (Burroughs e French, 2007).

Portanto, demonstramos que a suplementação com palmitato de retinol, em doses de 2.500, 12.500 e 25.000 UI/kg/dia, durante a gestação e a amamentação em ratos, é capaz de alterar parâmetros de estresse oxidativo em determinadas regiões do SNC das mães e dos filhotes, assim como é capaz de alterar parâmetros comportamentais em dois testes distintos, o teste de Homing e o teste de Campo Aberto. Adicionalmente, sabemos que a relação entre estresse oxidativo e alterações comportamentais tem sido extensivamente demonstrada em diversos outros modelos experimentais. Cassol-Jr e colaboradores (2010) demonstraram, por exemplo, que o estresse oxidativo exerce um papel importante no desenvolvimento de déficits cognitivos associados à sepse. Outro trabalho demonstrou recentemente que terapias antioxidantes, com n-acetilcisteína e desferroxamina, como aditivos a cloroquina, previnem as seqüelas cognitivas associadas à malária cerebral (Reis et al., 2010). Além disso, o estresse oxidativo também foi demonstrado como sendo importante em um modelo animal de mania em ratos, assim como em déficits cognitivos após a isquemia, anoxia, envenenamento por monóxido de carbono, injúria traumática cerebral e doença de Alzheimer (Dal-Pizzol et al., 2010; Steckert et al., 2010).

Estudos recentes, incluindo os do nosso Laboratório, também apontam para o envolvimento direto do estresse oxidativo com comportamentos do tipo-ansiedade e déficit locomotor/exploratório em roedores (Gingrich, 2005; Hovatta et al., 2005; Bouayed et al., 2007; de Oliveira et al., 2007; Souza et al., 2007; Masood et al., 2008; Salim et al., 2010). Entretanto, neste trabalho a relação entre estresse oxidativo e as alterações comportamentais detectadas em mães e filhotes suplementados com vitamina A permanece por ser melhor elucidado. Uma alternativa seria o co-tratamento com antioxidantes clássicos, como as vitaminas E e C. Além disso, nós também sabemos que estresse oxidativo não é o único fator causador de distúrbios comportamentais. 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.

Além disso, a suplementação com palmitato de retinol nestas mesmas doses também apresentou efeitos sobre o ambiente redox de outros tecidos das mães e dos seus filhotes. Nas mães, encontramos aumento nos níveis de lipoperoxidação e carbonilação proteica nos tecidos reprodutivos (útero e ovários), diminuição no conteúdo de tióis reduzidos e atividade de SOD extracelular no plasma, assim como aumento na atividade de GST no fígado e no rim. Nos filhotes, encontramos uma modulação dos níveis de lipoperoxidação e do TRAP no plasma influenciada pelo gênero, enquanto no fígado o TRAP aumentou em ambos os gêneros. Além disso, outras alterações órgão-específicas foram encontradas nas defesas antioxidantes enzimáticas (SOD, CAT e razão SOD/CAT), mas no coração dos filhotes não detectamos nenhuma alteração nos parâmetros redox investigados. Portanto, apesar dos efeitos sobre o SNC serem mais acentuados, a suplementação com palmitato de retinol também resultou em um insulto sobre outros tecidos das mães e dos filhotes.

Neste trabalho, em nenhum momento foram realizadas quantificações dos níveis de qualquer retinóides plasmático ou tecidual porque sabemos que as moléculas originadas deste tipo de tratamento podem ser bastante numerosas, incluindo muitas moléculas instáveis demais para que possam ser detectadas por metodologias convencionais (Napoli, 1999). Adicionalmente, existem alguns trabalhos que demonstram que os níveis séricos de retinóides podem não ser parâmetros adequados para evidenciar uma possível toxicidade. Inclusive, alguns trabalhos demonstram que, em situações de hipervitaminose A, a concentração de retinóides não necessariamente atinge valores acima de variações dentro de faixas normais (Ellis et al., 1986; Croquet et al., 2000; Mills e Tanumihardjo, 2006). Além disso, ainda que pudesse ser detectada uma concentração elevada de uma determinada espécie de retinóide, isso não significaria necessariamente que pudéssemos inferir que esta fosse a única espécie diretamente responsável por todas as modulações encontradas neste trabalho.

Por fim, sabe-se que um aumento na produção de espécies reativas ou de radicais livres pode influenciar de diversas maneiras o destino celular. Um pulso de produção de espécies reativas pode, entre muitos eventos celulares, disparar o processo apoptótico dependente de NFkB. Este mesmo pulso pode também induzir o desligamento do citocromo C a partir da cardiolipina e, conseqüente, liberar este fator pró-apoptótico para o citosol, estimulando a ativação da pró-caspase-9 via apoptossomo (Hengartner, 2000; Zanzami e Kroemer, 2001; Fink e Cookson, 2005). Entretanto, a manutenção deste pulso pode ter efeitos deletérios sobre o destino celular, como a inibição da apoptose, por exemplo, pois as caspases, enzimas efetoras da apoptose, são sensíveis à oxidação (Halliwell e Gutteridge, 1999).

Conseqüentemente, se a caspase estiver com sua atividade inibida, a célula perde sua capacidade de manter o processo apoptótico, tão importante principalmente durante

o desenvolvimento. Além disso, é provável que se tenha um aumento na taxa de morte celular por necrose, o que pode levar um tecido a sofrer um severo processo inflamatório. Situações como esta são frequentemente descritas em inúmeros processos neurodegenerativos, onde existe um aumento considerável na taxa de morte celular via necrose (Berg et al., 2004; Halliwell, 2006).

Portanto, a partir dos dados apresentados neste trabalho, recomendamos cautela quanto ao consumo de vitamina A durante a gestação e lactação, principalmente em relação ao uso de suplementos vitamínicos hidrossolúveis, pois esta suplementação pode facilitar, ou mesmo manter, um processo pró-oxidante. Por sua vez, esse estado pró-oxidante pode ter conseqüências severas sobre o desenvolvimento e saúde do embrião, do feto, do neonato e da mãe, pois o estresse oxidativo pode perturbar diversos fenômenos biológicos, incluindo a sinalização neuronal e a neurotransmissão, que podem levar a inúmeros déficits comportamentais. De fato, a suplementação com vitamina A induziu alterações comportamentais em dois testes, mas a relação entre estas alterações comportamentais e o estresse oxidativo permanece por ser elucidada. Adicionalmente, a exposição ao estresse de diversos tipos durante as etapas iniciais da vida podem induzir uma vulnerabilidade a diversos distúrbios do humor durante a vida adulta (Sanchez et al., 2001). Neste sentido, sabe-se que a origem de muitas doenças na vida adulta como a depressão, a ansiedade e as desordens de controle de impulsos podem ser encontradas na infância (Kaffman e Meaney, 2007; Swain et al., 2007).

6. CONCLUSÕES

A partir dos resultados obtidos nesta dissertação de Mestrado, podemos concluir que:

1) A suplementação com vitamina A, em doses com equivalência aproximada com as doses consideradas por muitas instituições internacionais como seguras durante o período reprodutivo é capaz de induzir aumento nos marcadores de estresse oxidativo nos tecidos analisados, em especial no estriado e no hipocampo, tanto das mães quanto dos filhotes;

2) O tratamento foi capaz de modular a atividade das enzimas antioxidantes de forma tecido-específica, com alterações na atividade da SOD, da CAT e na relação SOD/CAT. Nas estruturas cerebrais, o tratamento induziu o aumento na relação SOD/CAT devido a modulações na atividade da SOD e da atividade da CAT. Essa mudança na relação SOD/CAT pode indicar um aumento na produção de $\bullet O_2^-$ que é metabolizado a H_2O_2 pela SOD. Por sua vez, o H_2O_2 parece não ser metabolizada 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, o mais deletério dos radicais livres;

3) A suplementação nestas mesmas doses com palmitato de retinol foi capaz de induzir alterações comportamentais no teste de Homing em dois períodos distintos do desenvolvimento, no DPN5 e no DPN10, assim como alterações comportamentais no teste de Campo Aberto tanto nas mães quanto

nos filhotes. Todos estes resultados podem indicar que a suplementação com vitamina A interfere no processo de aprendizado olfatório dos filhotes durante o desenvolvimento. Além disso, podem indicar que a suplementação prejudica sua atividade locomotor-exploratória e induz comportamento do tipo ansiedade nos filhotes, além de prejudicar a atividade locomotora e induzir comportamentos do tipo ansiedade nas mães.

4) Em resumo, mostramos que a suplementação com vitamina A, na forma hidrossolúvel (palmitato de retinol – Arovit®), foi capaz de induzir modulações sobre o ambiente redox de forma tecido-específica, assim como um estado pró-oxidante no hipocampo e estriado de ratos suplementados durante a gestação e lactação. É importante salientar que estes efeitos não foram acompanhados de sintomas clínicos de intoxicação, nem por mal-formações congênitas nos filhotes, assim como não houve alterações em parâmetros reprodutivos clássicos em estudos toxicológicos. Ainda, mostramos que esta suplementação induziu diminuição nas capacidades locomotora e exploratória dos animais, além de induzir comportamento tipo-ansiedade.

7. PERSPECTIVAS

Os resultados obtidos nesta dissertação de Mestrado abrem um leque de perspectivas bastante interessantes no que diz respeito principalmente às conseqüências das modulações encontradas na vida adulta destes animais. Além disso, existe um grande número de opções no que diz respeito ao refinamento dos dados comportamentais apresentados neste trabalho, assim como investigar se os efeitos encontrados estão diretamente relacionados ao estresse oxidativo, ou se ambos são fenômenos distintos resultantes da suplementação com vitamina A. Dentre as inúmeras perspectivas de continuação com este trabalho, temos:

1) Investigar o outros parâmetros redox que permitam uma melhor compreensão sobre o ambiente redox nas estruturas analisadas neste trabalho, como avaliação de estresse nitrosativo pela quantificação de 3-nitrotirosina e a avaliação da taxa de produção de radical ânion superóxido mitocondrial, além de avaliar também o funcionamento da cadeia transportadora de elétrons;

2) Investigar a relação entre as alterações comportamentais e o estresse oxidativo resultantes da suplementação com vitamina A através da co-suplementação com moléculas de poder antioxidante reconhecido, como a vitamina C e vitamina E. Além disso, existe um grande número de opções em relação ao refinamento das alterações comportamentais encontradas neste trabalho, através de técnicas específicas que permitam a análise da capacidade de memória e aprendizado nos animais tratados com vitamina A;

3) Investigar outros mecanismos que poderiam estar envolvidos nas alterações comportamentais encontradas neste trabalho, como a quantificação de morte celular via intrínseca e extrínseca, além de 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.

4) Investigar as conseqüências e impactos das modulações moleculares e comportamentais encontradas neste trabalho sobre a qualidade de vida e saúde destes animais na vida adulta, como a resistência ou suscetibilidade destes animais aos déficits cognitivos persistentes em um modelo de septicemia induzida por ligação e perfuração cecal. Outros modelos, como aqueles que estudam doenças neurodegenerativas, onde o estresse oxidativo está claramente envolvido, também poderiam ser testados.

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9. ANEXOS

9.1 Anexo I

“Long-term vitamin A supplementation at therapeutic doses induces mitochondrial electrons transfer chain (METC) impairment and increased mitochondrial membrane-enriched fraction (MMEF) 3-nitrotyrosine on rat heart.”

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Long-term vitamin A supplementation at therapeutic doses induces mitochondrial electrons transfer chain (METC) impairment and increased mitochondrial membrane-enriched fraction (MMEF) 3-nitrotyrosine on rat heart

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Abstract

The aim of the present study was to compare electrons flux and oxidative/nitrosative stress parameters on the heart among rats supplemented with vitamin A and one not supplemented long-term. Vitamin A has important roles for the cardiovascular system as well as antioxidant properties. However, pro-oxidant properties have been reported. Male adult rats were treated with four different doses of retinyl palmitate (1000–9000 IU/Kg/day) or saline (control) for 28 days and the heart was removed for analysis. Electrons flux and oxidative/nitrosative stress parameters were evaluated and statistics were conducted with Anova one-way followed by Dunnet's *post hoc* and significance level of $p \leq 0.05$. The supplementation induced increase on lipids/proteins oxidation and mitochondrial 3-nitrotyrosine content, an imbalance on enzymatic activity and a decrease on respiratory chain complexes activities. The results suggest that vitamin A induces oxidative/nitrosative stress and mitochondrial impairment on a cardiac level.

Keywords: *Oxidative stress, myocardio, nitrosative stress, respiratory chain, retinol*

Introduction

Vitamin A has many important physiological functions during the development and adult life by regulating cell processes, as proliferation and differentiation, on the central nervous system (CNS), reproduction, vision, cardiovascular system and others. Besides the cellular control capacity, vitamin A has antioxidant properties, principally on lipophilic environments, due its liposolubility. Vitamin A is the isoprenoid retinol, which can be found as pro-vitamin A, on animal sources (mainly on liver meats, as retinyl palmitate) and as pre-vitamin A on vegetal sources (mainly as carotenes, which are precursors of retinol) [1].

However, our group has demonstrated that it can be a mistake categorize vitamin A as an antioxidant substance, since it also has pro-oxidant properties. It was observed that a few modifications on retinol concentration lead to oxidative damage on biomolecules (lipids, proteins and deoxyribonucleic acid), antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) imbalance, mitochondrial impairment and modulation of cell proliferation/death pathways

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through nuclear factor kappa B (NFκB) in a Sertoli cells culture model [2–5]. Thereafter, using an *in vivo* model, we found oxidative and nitrosative stress in several structures of the central nervous system, as well as behaviour disorders and bioenergetic impairment [6–10]. Recently, we gave a good contribution for the understanding of retinoids pro-oxidants effects, suggesting a cytosolic source of superoxide radical ($\cdot O_2^-$) by xhantine oxidase, since retinol competes with xanthyne for this enzyme and the given reaction release ($\cdot O_2^-$) [11].

Other groups have also demonstrated negative results about vitamin A usage, which give support to our findings. Interestingly, a study that treated smokers with vitamin A was discontinued because the intervention raised the lung cancer incidence among the participants [12]. Indeed, many other dysfunctions are attributed to the elevated vitamin A intake, such as behavioural disturbance and hepatic fibrosis [13,14].

Therefore, it is a worry if vitamin A usage is really safe, since it is used for many treatments, such as psoriasis, cancer, cystic fibrosis and others [15–18]. Additionally, vitamin A is suggested as a candidate for treatment of myocardial hypertrophy and remodelling, because retinoic acid inhibits angiotensin II effects on neonatal rat cardiac myocytes [19]. Moreover, it was demonstrated that there is a mobilization of retinol from its physiological resources to the myocardium after infarction, what also indicates this molecule as a possible therapeutic agent [20,21].

Regarding the large vitamin A usage and its possible complications, our aim in the present study was to compare cardiac electrons flux and oxidative/nitrosative stress parameters among rats treated with saline solution and ones treated with different doses of vitamin A long-term.

Materials and methods

Animals

Adult male rats (90 days old) were used and were maintained on a 12 h light–dark cycle with water and food *ad libitum*. All experimental procedures were performed in accordance to the National Institute of Health Guide for Care and Use of Laboratory Animals. Retinyl palmitate (Arovit®), water soluble form, was purchased from Bayer (São Paulo, Brazil). All others reagents, when not specified, were purchased from Sigma Chemicals (St. Louis, MO).

Experimental design and treatment

Initially, the animals were randomly divided into the supplementation groups: Control (vehicle: saline solution 0.9%) and four different vitamin A doses (1000, 2500, 4500 and 9000), expressed as International Units (IU) per body mass Kilogram (Kg) per day (IU/Kg/day) and thereafter were treated for 28 days with retinyl palmitate. The retinyl palmitate solution was prepared daily using saline solution (NaCl 0.9%) as a vehicle and was administrated orally, by intra-gastric gavage, in a total 0.8 mL volume, always in the dark cycle beginning.

Samples preparation

After the respective treatments, the rats were killed by decapitation and the heart was carefully removed and then cleaned with iced saline solution to remove blood excess contamination. For general analysis, the organ was homogenized in phosphate buffer for samples (PBS) pH 7.4, so the homogenate was centrifuged at 700 × g to remove debris and the resulting supernatant was used as the mother solution. To obtain the cardiac mitochondrial membrane-enriched fraction (MMEF), in order to assess the mitochondrial electron transfer chain (METC) complexes activities, the tissue was homogenized in a buffer containing sucrose 250 mM, EDTA 2 mM, Tris 10 mM pH7.4 and heparin 50 IU/mL. The samples were then centrifuged at 1000 × g and the supernatants were collected. Thereafter the samples were frozen and thawed three times. The protein content of general analysis and MMEF was measured by the Lowry et al. [22] method in order to correct the results.

Redox status and damage

The thiobarbituric acid reactive species (TBARS) test were evaluated as an index of lipids oxidation. The TBARS consists of an acid-heating reaction of the lipid peroxidation end product, malondialdehyde, with thiobarbituric acid (TBA). The TBARS were determined at 532 ηm and the results were expressed as ηmol/mg protein [23]. For protein oxidation analysis the carbonyl content was measured, which is based on the reaction of dinitrophenylhydrazine with protein carbonyl groups. The results are expressed as ηmol/mg protein [24]. The total sulphydryl (SH) content, present in proteins as well as in glutathione, was measured at 412 ηm by its reaction with 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB) and the results were expressed as ηmol/mg protein [25].

Non-enzymatic antioxidant potential

The total reactive antioxidant potential (TRAP) has been used as an index of the non-enzymatic antioxidant capacity, based on the peroxyl radical (generated by AAPH solution, 2,2'-azobis[2-amidinopropane], with luminol) quenching by sample compounds. The reading is done by chemiluminescence emission. Briefly, we prepared AAPH solution, added luminol (AAPH + luminol, radical generating system) and then we waited for the

system to stabilize for 2 h to do the first reading. After the sample addition, we analysed the readings at the lumineter counter for 96-well microplates for nearly 30 min [26]. The results were transformed in percentual and the area under curve (AUC) was calculated by software (GraphPad Software Inc.®, San Diego, CA; version 5.00) as described [27]. For the TRAP, it is important to note how much lower the AUC is and higher the antioxidant potential is, playing an inversely proportional relation. The total antioxidant reactivity (TAR) was also analysed and it is based on the same technical principles of TRAP. The TAR results were calculated as the ratio of light in absence of samples (I^0)/light intensity right after sample addition (I) [28]. For the TAR, the values play a directly proportional relation to the antioxidant capacity. Although TAR and TRAP evaluations are obtained in the same experiment, they represent a different observations, since the TAR is more related to the antioxidant quality (reactivity, the scavenging capacity in a short-term period) and the TRAP is more related to the antioxidants amount [28].

Enzymes activity

Enzymes activities were also assessed. The superoxide dismutase (SOD) catalyses superoxide anion radical ($\cdot O_2^-$) dismutation to generate hydrogen peroxide (H_2O_2) and its activity measurement is based on the principle that adrenaline undergo auto-oxidation in $\cdot O_2^-$ presence, so at 480 ηm we determine the SOD activity by the exogenous adrenaline auto-oxidation inhibition in sample presence [29]. The catalase (CAT) catalyses the H_2O_2 conversion to water (H_2O) and to determine its activity we added H_2O_2 and analysed the capacity of the sample to decrease the H_2O_2 amount at 240 ηm [30]. Glutathione S-Transferase (GST) activity was measured in a reaction mixture containing 1-chloro-2,4-dinitronemzene (CDNB) and glutathione as substrate (GSH) and was calculated by the slope of the initial linear portion of the absorbance time curve at 340 ηm [31].

Indirect enzyme-linked imunosorbent assay (ELISA) to 3-nitrotyrosine

Indirect assay was performed to measure 3-nitrotyrosine contents by using a polyclonal antibody to nitrotyrosine (Calbiochem®), diluted 1:5000 in PBS pH 7.4 with albumin 5%. Briefly, microtiter plate (96-well flat bottom) was coated for 24 h (at ~8°C) with the samples, thereafter the plates were then washed four times with wash buffer (PBS with Tween-20 0.05%), the antibodies were added to the plate and an incubation of 2 h (at room temperature) was performed. After incubation, four more washings were conducted and a second incubation for 1 h (at room temperature) with anti-rabbit antibody peroxidase conjugated (diluted 1:1000) was carried out. Again, four more washings were conducted and the substrates (hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine, 1:1, v/v) were added. The readings were done at 450 ηm, in a plate spectrophotometer. The results were expressed as changes in percentage among the groups.

Mitochondrial electrons transfer chain (METC)

The electrons transference from the complex I to complex III (complexI-CoQ-III activity) was determined by following the increase in absorbance, due to reduction of cytochrome C, at 550–580 ηm as reference range, in a reaction started by nicotinamide adenine dinucleotide (NADH) [32]. The electrons transference from the complex II to complex III (complexII-CoQ-III activity) was also determined by following the increase in absorbance, due to reduction of cytochrome C, at 550–580 ηm as reference, but its reaction is started by succinate [33]. Complex II (succinate-2,6dichloroindophenol-oxidoreductase) activity was measured by following the decrease in the absorbance, due to reduction of 2,6-dichloroindophenol (DCIP), at 600–700 ηm as a reference range, in a

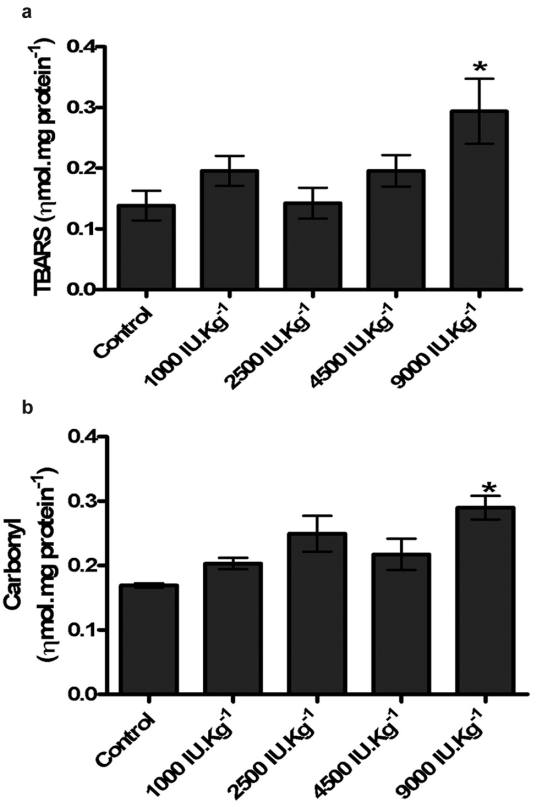

Figure 1. (A) TBARS levels ($n=7$ for each group) and (B) Carbonyl content ($n=7$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

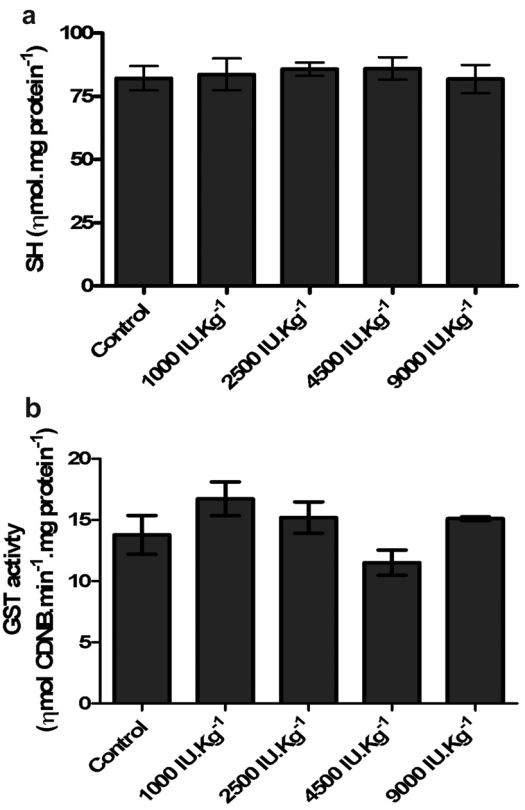

Figure 2. (A) SH content ($n=7$ for each group) and (B) GST activity ($n=5$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

reaction started by succinate [33]. Succinate dehydrogenase (SDH) was measured after adding phenazine methasulphate to the same mixture reaction used for complex II activity measurement and is also analysed by following the decrease in the absorbance, due to reduction of 2,6-dichloroindophenol (DCIP), at 600–700 ηm as a reference range, in a reaction started by succinate [33].

Statistical analysis

All data are presented as mean ± standard error of mean (SEM), Anova one way was used, followed by Dunnet's *post hoc*, to determine the differences among groups and the significance level considered was $p \leq 0.05$. The statistical analysis and the graph making were conducted with GraphPad Software Inc.® (San Diego, CA; version 5.00).

Results

As biomolecules oxidative damage parameter, TBARS levels were assessed, for lipid peroxidation and carbonyl levels for protein oxidation. An increase in these parameters was observed at the highest vitamin A dose (Figure 1). Additionally, the SH oxidation status was assessed, since this group is present in proteins as well as in glutathione molecules and plays an important role as a redox parameter. As the function of GSH oxidation status is involved not only with redox mechanisms, indeed it is also related to detoxifying actions, we decided to evaluate the GST activity. However, no changes were observed for both SH and GST (Figure 2).

The non-enzymatic antioxidant properties were evaluated by TRAP (antioxidant capacity more related to antioxidants amount) and TAR (antioxidant capacity more related to antioxidant quality), but only for TAR were differences observed, which happened at the three higher doses (Figure 3). Antioxidants enzymes activities were also analysed and no modifications were observed for SOD activity, while CAT activity decreased at the three higher doses, which yielded an enzymatic imbalance that can be seen by the increased SOD/CAT ratio (Figure 3). [AQ4]

To assess the possible RNS effects, in response to retinol treatment, the 3-nitrotyrosine levels in the total heart homogenate as well as in the MMEF were analysed. No differences were observed on total tissue,

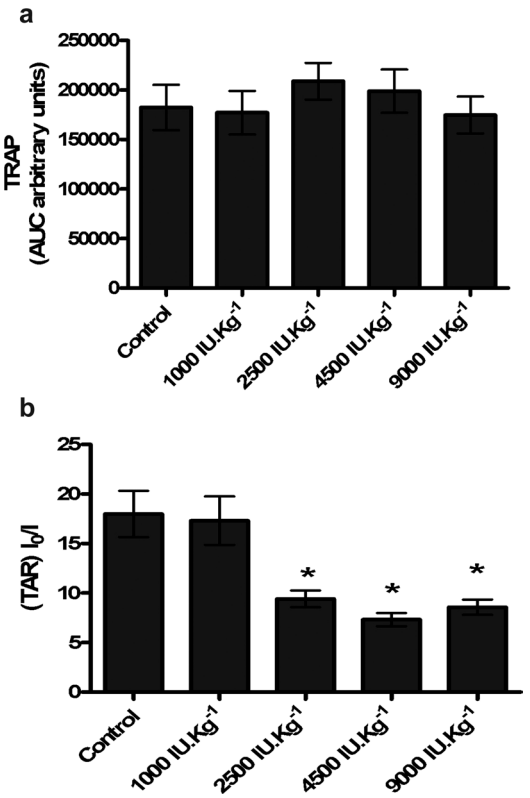

Figure 3. (A) TRAP ($n=7$ for each group) and (B) TAR ($n=7$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

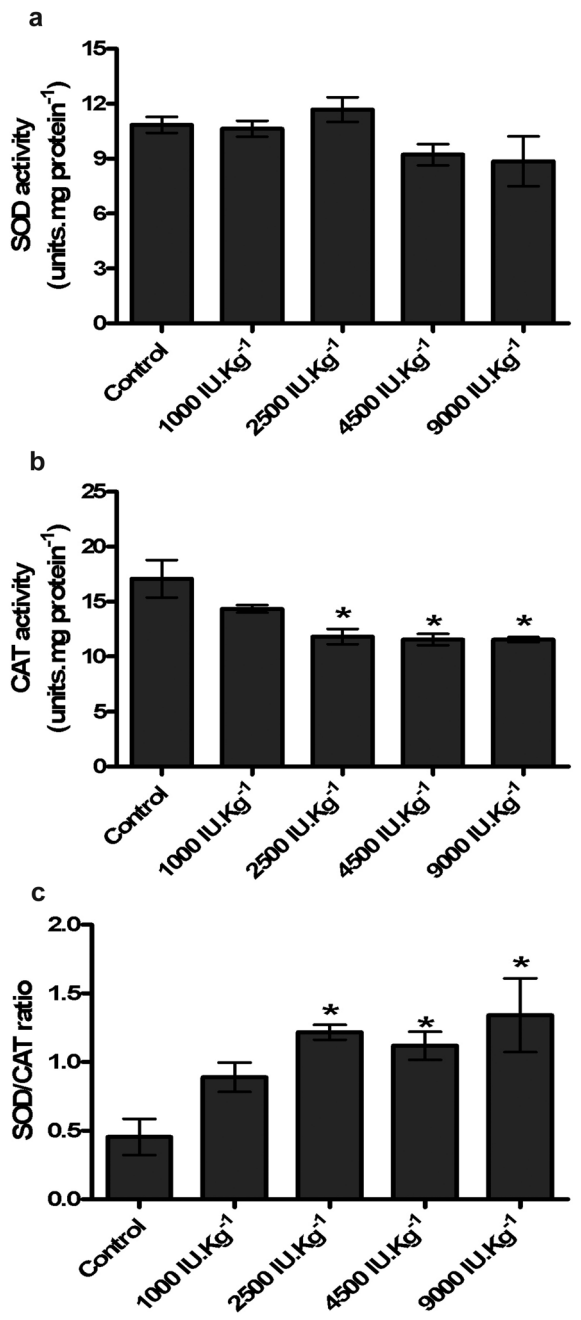

Figure 4. (A) SOD activity ($n=7$ for each group), (B) CAT activity ($n=7$ for each group) and (C) SOD/CAT ratio ($n=7$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

but an increase was found at the two higher doses for MMEF (Figure 5). Additionally, we analysed the respiratory chain complexes activities and detected a decrease in electrons transfer from complex I to III only at the highest dose, while at the three higher doses the electrons transfer was decreased on complexes II–III and the activities of complex II and SDH were also diminished (Figure 6).

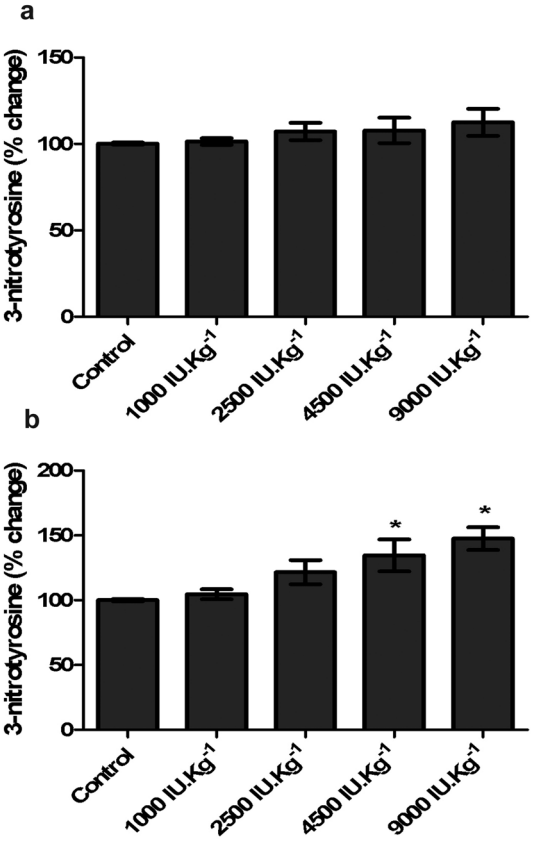

Figure 5. (A) Total 3-nitrotyrosine ($n=5$ for each group) and (B) MMEF nitrotyrosine ($n=5$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

Discussion

The present work evaluated the effects of a long-term retinyl palmitate supplementation on rat heart, regarding oxidative/nitroasative stress and electrons flux parameters. Despite the retinol being largely used for treatment of several diseases and conditions, negative effects are also attributed to this molecule, which become very important studies that help us to better understand the possible toxic effects of vitamin A.

We noted that the highest dose of the treatment was able to increase the lipid and protein oxidation, observed by the increased levels of TBARS and carbonyl. These results are in accordance with previous studies developed by our group [8,34,35]. Regarding the non-enzymatic capacity, we observed that the supplementation diminished the sample antioxidants reactivity without changing the amount of sample antioxidants, which led us to believe that retinol could interfere with glutathione (GSH) metabolism, the main redox buffer of the intracellular mean. However, no modifications were found for SH content or GST activity, which indicates that GSH status is not modified [36].

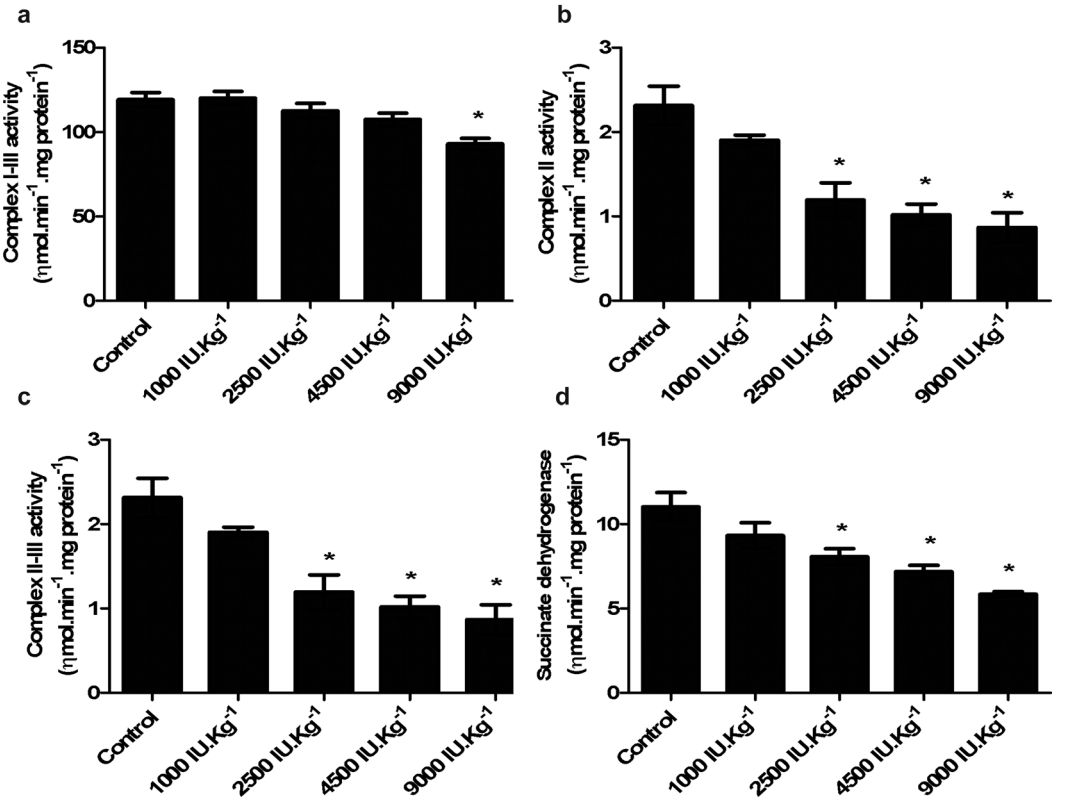

Figure 6. (A) Complex I–III activity ($n=5$ for each group), (B) Complex II–III activity ($n=5$ for each group), (C) Complex II activity ($n=5$ for each group) and (D) SDH activity ($n=5$ for each group). Data are expressed as mean ± standard error of mean.*Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

In agreement to prior studies developed by our research group, in the present one, an imbalance on SOD and CAT activities was detected, as a result of the decreased CAT activity together to an unchanged SOD activity. Previously, we had also found changes on enzymatic system [34]. The SOD enzyme is responsible for $\cdot O_2^-$ dismutation to generate H_2O_2, which is removed by some peroxidase enzymes (as CAT). If an imbalance occurs, as that yielded by decreased SOD/CAT ratio, the H_2O_2 accumulates and in transition metals it's presence can generate the most potent free radical, the ·OH (hydroxyl), which is highly reactive and produces damage on several other molecules [37]. Otherwise, it can be one of the mechanisms responsible for the oxidative damages, which were observed in the present work.

In function of several studies are relating cardiovascular dysfunctions to the imbalance on reactive nitrogen species (RNS), for instance peroxynitrite ($ONOO^-$), in a condition known as nitrosative stress, we have decided to evaluate the content of 3-nitrotyrosine [38]. By assessing the total tissue, we could not observe any modification in this marker, but when it was analysed the MMEF an increase of 3-nitrotyrosine was detected. The 3-nitrotyrosine is produced by the attack that protein tyrosil residues undergo in front of $ONOO^-$, which is formed by the reaction between $\cdot O_2^-$ and the radical nitric oxide (·NO) [39]. Prior studies have also demonstrated nitrosative stress induced by retinol treatment [6,7].

Additionally, as the respiratory chain is an important source of $\cdot O_2^-$, substrate for $ONOO^-$ formation, we decided to measure the activity of the mitochondrial complexes responsible for electrons flux [37]. Our findings, which demonstrated inhibition on respiratory chain complexes (mainly at complex II level), are in agreement with our previous studies, which also demonstrated decreased complexes activities [7,8,40]. In addition, it is suggested that the respiratory chain electrons transference inhibition can yield $\cdot O_2^-$ [37,41].

Taken together, our results suggest that the pro-oxidants effects of vitamin A are related to an impairment on mitochondrial level, since the respiratory chain is inhibited and there is an increase on MMEF 3-nitrotyrosine. However, we have here a possible cycle, in which we do not know where it begins. As a first hypothesis, we believe that retinol can directly inhibit the respiratory chain complexes, because it was previously demonstrated that their decreased activities are associated to increased $\cdot O_2^-$ production. The $\cdot O_2^-$, generated by complexes inhibition, could then activate the nitric oxide shynthase (NOS), increasing ·NO production. So, these two

molecules ($\cdot O_2^-$ and $\cdot NO$) can react, forming $ONOO^-$ [42–44]. The second hypothesis is that the cycle beginning would be the direct activation of NOS by retinol and the resulting $\cdot NO$ could inhibit the respiratory chain complexes activities [41,45–48]. However, more studies are needed to confirm one of these theories.

In conclusion, our results suggest that vitamin A supplementation at therapeutic doses generates oxidative damage and it is associated to RNS metabolism disturbance and inhibition of respiratory chain, but the mechanisms involved need to be better clarified.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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9.2 Anexo II

“Vitamin A supplementation to pregnant and breastfeeding female rats induces oxidative stress in the neonatal lung.”

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Vitamin A supplementation to pregnant and breastfeeding female rats induces oxidative stress in the neonatal lung

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ABSTRACT

Vitamin A is an essential micronutrient that regulates many biological processes through modulation of retinoic acid receptor-responsive genes. Vitamin A acts as a systemic antioxidant, participating in the modulation of diverse redox mechanisms involved in physiological and pathological processes. Different studies, however, observed that vitamin A and other retinoids may induce pro-oxidant/deleterious actions under certain conditions, leading to impairment of brain and lung function. Here, we studied the effect of vitamin A treatment at oral doses of 100 IU/kg, 200 IU/kg, and 300 IU/kg to female rats (*Rattus norvegicus*) during pregnancy and lactation on oxidative parameters of lungs from the offspring vitamin A supplementation induced increases in lipoperoxidation, protein carbonyl, activities of the antioxidant enzymes superoxide dismutase and catalase (200 IU/kg, and 300 IU/kg), and decreased sulphydryl protein (500 IU/kg) content in the neonatal lung.

1. Introduction

Vitamin A (retinol, retinoic acid) plays a key role in many essential biological processes. It is essential for embryonic development and the regulation of proliferation and differentiation of many cell types [1]. The discovery of nuclear receptors for retinoic acid and other retinoids has provided a conceptual basis to explain how these compounds preside over a large network of gene activation processes [2]. The physiological actions of vitamin A are generally described as being mediated by nuclear receptors from steroid/thyroid superfamily.

Studies have described a protective role of vitamin A in several diseases, which was related to its ability to scavenge toxic forms of oxygen and other free radicals mainly in rats and mice [3]. Some patients with acute myeloid leukemia respond to very large doses of all-*trans*-retinyl palmitate of the order 50,000 IU/kg/day. The role of vitamin A is also particularly important for embryogenesis [4]. Hypovitaminosis A may lead to failure of embryo segmentation and growth, ceasing vascularization and impairing development in rats and guinea pigs (*Cavia porcellus*) [5]. Hypervitaminosis A, on the other hand, is teratogenic in many model systems [6]. Human susceptibility to the teratogenic effects of vitamin A have been associated to even a single supplement of 500,000 IU in the first 2 months or at 25,000 IU daily throughout pregnancy. In addition, excess of vitamin A can disrupt membranes and is hepatotoxic in adults [7].

Our previous results demonstrated that vitamin A supplementation (retinol at 7 μM) induces oxidative damage in biomolecules [8–10], upregulation of antioxidant enzymes [11,12], preneoplasic transformation [13], and activation of phosphorylation signaling pathways in cultured Sertoli cells [14–16]. Thus, vitamin A may promote oxidative stress and modulate redox-dependent processes in tissues. Oxidative stress may follow from the generation of reactive oxygen species (ROS)/reactive nitrogen species (RNS) through increased production or decreased antioxidant capacity, or when such factors are combined [17]. Oxidative stress may contribute to the pathogenesis of diffuse lung diseases (DLD) [18].

Data regarding effects of vitamin A supplementation in human lung development are limited. Given that vitamin A supplementation causes oxidative stress in rat lungs at doses from 1000 IU/kg/day to 9000 IU/kg/day, as we previously demonstrate [19,20], the aim of this study was to investigate the effects of vitamin A supplementation at lower dosages of 100 IU/kg, 200 IU/kg, and 500 IU/kg on parameters of oxidative stress in neonatal rat lung from dams treated with vitamin A supplementation during pregnancy and lactation.

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2. Materials and methods

2.1. Animals

Nulliparous female Wistar rats (*Rattus novergicus*) at 90 days of age and 200–250 g in weight were obtained from an in-house breeding colony. All animals used in this study were healthy and free of pathogens. Dams were caged in groups of five animals with free access to standard commercial food (CR1 lab chow, Nuvilab, Curitiba, Brazil) and water. They were maintained on a 12-h light–dark cycle (7:00–19:00 h) at 23 ± 1 °C. The females were selected at their sexual receptive phase of the estrous cycle by vaginal smear and were placed individually with a single mature male of proven fertility from the same colony, overnight. Successful mating was regarded as the presence of vaginal plug and/or viable sperm at vaginal smear, and this was designed as day 0 of gestation. All housing, handling and euthanasia 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). Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

2.2. Treatment

Pregnant dams were randomly selected for daily treatment throughout gestation and lactation (21 days of gestation and 21 days of lactation). Vitamin A was given at night in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. The number of females in each group varied between 5 and 12 animals and the number of pups per litter ranged from 7 to 12. All newborns were analyzed regardless of gender.

Dams were treated with vehicle (0.15 M saline; control group), 100 IU/kg, 200 IU/kg and 500 IU/kg of retinol palmitate (Arovit®; a water-soluble form of vitamin) orally, via a metallic gastric tube (gavage) in a maximum volume of 0.6 ml. Adequate measures were taken to minimize pain or discomfort. The recommended daily intake for humans is 5000 IU/day (1.5 mg/day of retinol) of vitamin A. The doses used in this study are doses of supplementation that can be easily achieved in humans through the ingestion of foods fortified with vitamin A. Furthermore, these dosages are considered safe for humans [6] and this intake of foods fortified with vitamins is observed mainly in developed countries or developing countries.

2.3. Drugs and reagents

Arovit® (retinol palmitate, a commercial water-soluble form of vitamin A) was purchased from Roche, São Paulo, SP, Brazil. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A administration solutions were prepared daily, protected from light exposure and temperature.

2.4. Lung extraction and samples preparation

The animals were euthanized by decapitation 24 h after the last treatment. Lungs were quickly dissected out on ice and stored at −80 °C for subsequent biochemical analyses. Samples were homogenized in 50 mM phosphate buffer (pH 7.0) and the lysate was clarified by centrifugation (700 × *g*, 5 min) to generate the crude supernatant fraction used in all biochemical assays described. Results were normalized by the protein content using bovine albumin as standard [21].

2.5. Thiobarbituric acid reactive species (TBARS) assay

As an index of lipid peroxidation, we measured the formation of TBARS during an acid-heating reaction, which is widely adopted for measurement of lipid redox state [22]. 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.

2.6. Measurement of protein carbonyls

Oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) as previously described [23]. Briefly, proteins were precipitated by the addition of 20% TCA, resolubilized in DNPH, and the absorbance read in a spectrophotometer at 370 nm. Results are expressed as nmol carbonyl/mg protein.

2.7. Measurement of protein thiol content

Protein thiol content was analyzed to estimate oxidative alterations in proteins. Briefly, an aliquot was diluted in SDS 0.1% and 0.01 M 5,50-dithionitrobis 2-nitrobenzoic acid (DTNB) in ethanol were added and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min [24]. Results are expressed as mmol SH/mg protein.

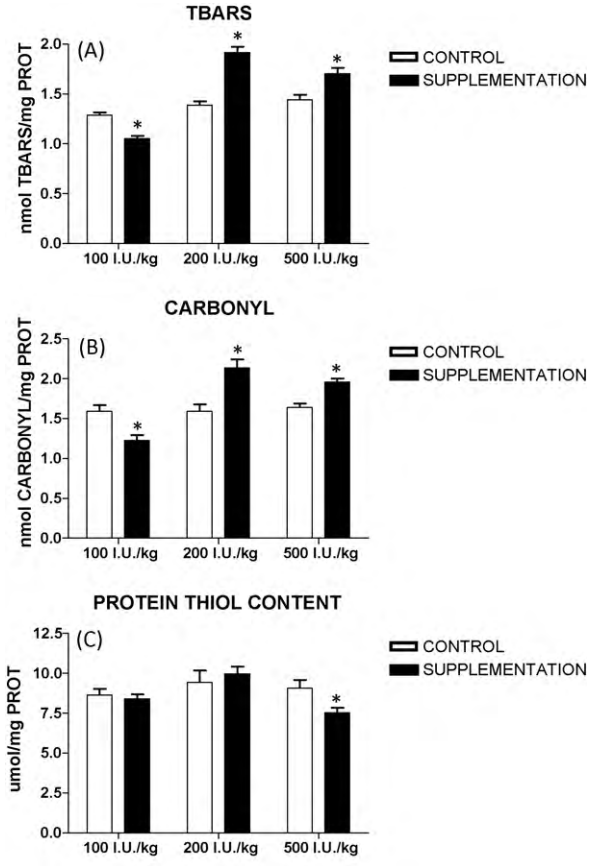

Fig. 1. Effects of vitamin A supplementation on lipid peroxidation (A), protein carbonylation (B), protein thiol content (C) in the offspring lungs. Data are mean ± SEM ($n = 5$–12 L). *$p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

2.8. Antioxidant enzyme activities estimations

Catalase (EC 1.11.1.6; CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm [25], and the results are expressed as units of CAT/mg protein. Superoxide dismutase (EC 1.15.1.1, SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described [26], and the results are expressed as U SOD/mg protein. A ratio between SOD and CAT activities (SOD/CAT) was applied to better understand the effect of vitamin A supplementation upon these two free radical-detoxifying enzymes that work in sequence converting superoxide anion to water. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

2.9. Statistical analysis

Biochemical results are expressed as means ± standard error of the mean (SEM); *p* values were considered significant when $p < 0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test whenever necessary (GraphPad Prism 4 – Software). We used the average of each litter for statistical analysis.

3. Results

We first analyzed the effect of vitamin A supplementation on lipoperoxidation in the neonatal lung as determined by TBARS levels. Vitamin A supplementation at 100 IU/kg decreased TBARS levels (Fig. 1A); however, at 200 IU/kg and 500 IU/kg, vitamin A supplementation induced an increase in the levels of TBARS, indicating an increase in lung lipoperoxidation (Fig. 1A). Treatment induced a similar effect on lung protein carbonylation. At 100 IU/kg, vitamin A administration induced a decrease in the levels of protein carbonyls, whereas at 200 IU/kg and 500 IU/kg carbonylation levels

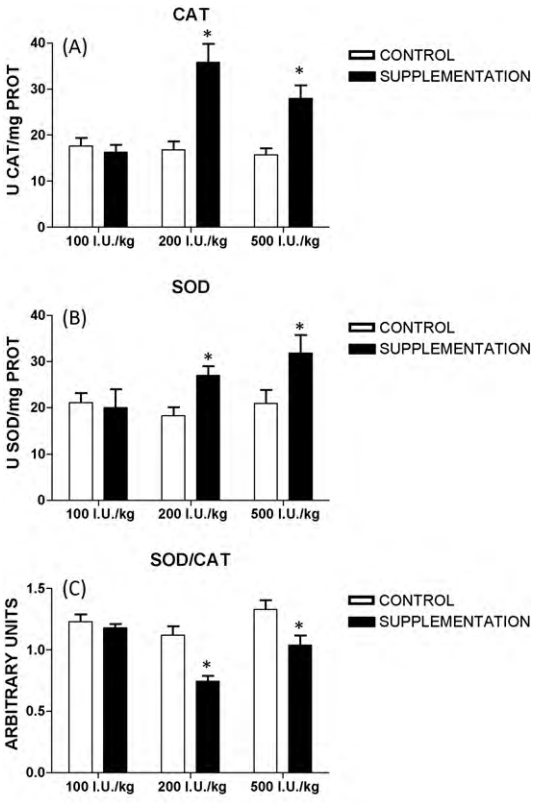

Fig. 2. Effects of vitamin A supplementation on SOD (A), CAT (B), activity in the offspring lungs. (C) The lung SOD/CAT ratio. Data are mean ± SEM (n = 5–12 L). *$p < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

were found to be increased (Fig. 1B). Finally, protein thiol content was observed to be decreased by vitamin A supplementation only at 500 IU/kg (Fig. 1C).

We next analyzed the enzymatic activity of CAT, which is involved on hydrogen peroxide (H_2O_2) detoxification, and SOD, which metabolizes superoxide radicals. CAT activity was enhanced in newborns that receiving vitamin A supplementation at doses of 200 IU/kg and 500 IU/kg (Fig. 2A). These same doses induced a stimulation on SOD activity as well (Fig. 2B). Plotting a ratio between the activities of SOD and CAT showed that, besides increasing the activities of SOD and CAT, vitamin A supplementation at 200 IU/kg and 500 IU/kg caused in imbalance in SOD/CAT ratio, suggesting that vitamin A exerts differential effects on the regulation of SOD and CAT activities, which probably contributes to the pro-oxidant effects observed here.

4. Discussion

Vitamin A is known to influence the maturation and differentiation of the lungs during development by affecting different physiological functions of lung cells through modulation of retinoic acid-responsive genes. Type II alveolar cells are especially prepared to synthesize and secrete lung surfactant protein, and retinoic acid was observed to modulate the expression of such protein in fetal lungs [27]. Deficient vitamin A intake was also observed to induce an emphysematous condition in lungs, reducing the content of lung elastin and decreasing type II pneumocyte synthesis of surfactant [28]. Vitamin A is known to preserve and maintain the integrity of the lung epithelium [29]; retinoids have been shown to enhance alveolar septation in neonatal rats and mice [30], and in some cases to improve lung repair after injury in adults [31].

Vitamin A supplementation to human populations considered at elevated risk to lung cancer was nevertheless observed to increase the incidence and mortality of such neoplasia [32], indicating the complexity of vitamin in lung function. Different studies examined the modulation of oxidative parameters in lungs by vitamin A. Diffuse lung disease (DLD) pathogenesis may partly involve oxidative stress driven by an imbalance between oxidant production and antioxidant defenses, since pro-oxidant conditions may promote DLD [33]. Although different studies have explored the potential pro- or antioxidant effects of vitamin A on lung function in animal models, few focused on potential harmful effects on neonates. Considering the role of vitamin A on lung development, the effect of vitamin A supplementation on oxidative stress-related parameters in neonate lung needs to be better understood.

Here, we observed that maternal vitamin A supplementation induced a pro-oxidative state in neonatal lungs that was vitamin A-dose dependent. Vitamin A supplementation increased lipid peroxidation and protein carbonylation levels, and decreased protein thiol content. Furthermore, we observed that vitamin A supplementation to mothers induced a increase on CAT and SOD activities of rat offspring lungs. It is known that vitamin A auto-oxidation *in vitro* increases superoxide anion production in cell cultures [34,35]. Previous studies showed that vitamin A leads to impairment on electron transfer system, thus increasing the rate of superoxide production. This effect was reported either in isolated mitochondria incubated with retinol or in mitochondria isolated from vitamin A supplemented rats [36,37]. Here, we observed that vitamin A supplementation caused an increase in SOD activity, suggesting that superoxide production is enhanced. Taken together, these results indicate a possible exacerbation in superoxide production in this experimental model of supplementation. Increased superoxide production may stimulate SOD activity due to the substrate allosteric activation [26]. In this regard, impairment between the concerted SOD and CAT detoxifying activities may take place, as can be observed in the imbalanced SOD/CAT ratio.

There are many possible deleterious consequences from imbalanced SOD/CAT activities. Increased SOD activity may result in an enhanced formation of H_2O_2 because its production from superoxide dismutation by SOD is also enhanced. An excess of H_2O_2 facilitates the production of the highly reactive and toxic hydroxyl radical ($OH^{\bullet}$) through a reaction with iron or copper (Fenton chemistry). Van Helden et al. [36] showed that β-carotene and its metabolites (vitamin A, retinal, and retinoic acid) were able to increase $OH^{\bullet}$ formation in a system containing iron and H_2O_2 through the Fenton reaction. *In vitro* experiments show that either β-carotene or retinol is able to increase cellular iron uptake, thus increasing iron availability in the cytosol to react with H_2O_2 [38,39]. In addition, H_2O_2 is relatively stable and able to diffuse into the nucleus, where it can react with DNA-associated transition metals to form $OH^{\bullet}$. Oxidatively damaged DNA is known to be pro-mutagenic and is therefore an important marker for carcinogenesis. Previously, we showed that retinol increases iron uptake and induces damage and mutagenesis in the DNA of Sertoli cells through iron-dependent Fenton chemistry [38]. Other authors also observed that vitamin A was able to induce DNA damage [35].

The increase in CAT activity may be associated with the increase of SOD activity and, in turn H_2O_2 formation. Some studies related a conformational change of CAT to (compound II) during exposure to its own substrate H_2O_2 decreases CAT proteasomic degradation and increases its enzymatic activity [40,41]. An imbalance in the SOD/CAT ratio is thought to facilitate oxidative-dependent alterations in the cellular environment. Thus, impaired SOD/CAT is likely to culminate in increased oxidative damage to biomolecules. The basal or physiological SOD/CAT ratio varies according to the tissues, depending on specific-tissue factors such as production of superoxide and H_2O_2 in the electron transport chain, peroxisomes,

and other intrinsic oxidative processes as well. Many studies have shown that an imbalance between SOD and CAT activities is responsible for increasing oxidative stress in biological systems [8,10,42], and this is consistent with our results with maternal vitamin A supplementation in neonatal lungs.

We found that the level of lipid peroxidation increased with vitamin A supplementation. Lipid peroxidation is one of the major sources of free radical-mediated injury that directly damages membranes and generates a number of toxic secondary products. This may involve the interaction of oxygen-derived free radicals with polyunsaturated fatty acids and finally results in a variety of highly reactive electrophilic aldehydes that are capable of easily attaching covalently to proteins by forming adducts with cysteine, lysine, or histidine residues [43]. A large portion of biological properties and functions involving protein structure, enzyme catalysis, and redox signaling pathways depends on the redox properties of the thiol group present both in protein and in low-molecular-weight molecules. Numerous cell signaling proteins are regulated by alterations of the protein thiol groups [44]. This signaling involves cell proliferation, differentiation, and death. Most protein damage is irreparable and may lead to a wide range of downstream functional consequences, such as protein dimerization or aggregation; unfolding or conformational changes to expose more hydrophobic residues to an aqueous environment; loss of structural or functional activity; alterations in cellular handling/turnover; effects on gene regulation and expression; modulation of cell signaling, induction of apoptosis and necrosis [45]. Therefore, an increase of protein carbonylation and excessive decrease in the protein thiol content may facilitate the formation of protein aggregates, as a result of protein cross-links, and this is very likely to culminate in a widespread cellular dysfunction.

Additionally, increased oxidative damage to proteins might result in increased free iron, because of its release from damaged ferritin and other iron-containing proteins, favoring the maintenance of the pro-oxidative state [46,47]. We observed that vitamin A supplementation increases protein carbonylation of manner dose dependent. In addition, protein thiol levels were decreased with vitamin A supplementation at dose of 500 IU/kg of vitamin A. These effects are also observed in pathological conditions that lead to DLD or lung cancer [48].

In summary, we for the first time show that maternal vitamin A supplementation during pregnancy and lactation increases oxidative stress parameters of neonatal lungs in the rat. Furthermore, these pro-oxidant effects here observed may be involved in the onset of lung diseases associated to redox dysfunctions and free radical-induced damage to biomolecules, such as lung cancer, lung fibrosis, and asthma, and more precautions should be taken when vitamin supplements are prescribed, mainly when administered to children and pregnancy, for therapeutic or preventive interventions.

Conflicts of Interest

None.

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9.3 Anexo III

“Vitamin A supplementation for different periods alters rat vascular redox parameters.”

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ORIGINAL PAPER

Vitamin A supplementation for different periods alters rat vascular redox parameters

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Abstract Vitamin A plays physiological and antioxidants properties and is associated with protective effects on arterial level. However, deleterious effects have been reported, including those observed by our group, which has demonstrated pro-oxidant properties in other systems. Therefore, it is needed to better understand the redox effects of retinoids on arterial system. Thus, our aim was to compare vascular redox parameters among animals supplemented or not with vitamin A. Eighty-five adult male rats were treated with different retinyl palmitate doses (1,000–9,000 IU kg^{-1} day^{-1}) or saline for 3 (25 rats, n=5 for each group), 7 (25 rats, n=5 for each group), and 28 (35 rats, n=7 for each group)days periods. Aorta artery was surgically removed, cleaned to remove the blood, and homogenized. It was evaluated thiobarbituric reactive species (TBARS), total reduced sulfhydryl (SH), and activities of superoxide dismutase (SOD) and catalase (CAT). Statistics were conducted by one-way ANOVA with Dunnet's post hoc and significant value of $p \leq 0.05$. About TBARS, we observed no modifications after 3 days, but a decrease after 7 days in all doses and after 28 days in three higher doses. The two higher doses yielded an increase on SH only after 3 days. SOD activity decreased in three higher doses after 3 days and in all doses after 28 days, but no modifications after 7 days, while CAT activity increased in all doses after 3 days, decreased in all doses after 7 days, and did not change after 28 days. In conclusion, vitamin A induces antioxidant status on vascular level.

Keywords Antioxidants · Aorta artery · Oxidative stress · Reactive oxygen species · Retinol

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Introduction

Vitamin A is the given name to retinol and any molecule that presents retinol activity, being found as pro-vitamin in animal sources (retinyl palmitate and

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retinyl acetate) and as pre-vitamin on vegetable sources (carotenoids). The retinol plays important actions on physiological systems by regulating cell maturation, differentiation, and proliferation, since development period until adult life. Its importance for eyes, reproduction, central nervous system (CNS), and others has been well demonstrated. Moreover, antioxidants properties are also associated to retinol and its related molecules [32]. Additionally, significant roles have been attributed to retinoids at vascular level. In spontaneously hypertensive rats, the chronic treatment (3 months) with retinoic acid prevented medial thickening of intramyocardial and intrarenal arteries as well as the ventricular fibrosis [24]. Furthermore, it was demonstrated that retinoic acid inhibits angiotensin II actions on vascular smooth muscle cells [20].

The administration of retinol or carotenoids has been largely used for treatment and prevention for several cases, such as psoriasis, cystic fibrosis, cancer, and others [26, 29, 31, 34]. However, contrary effects are observed after to retinol administration. Long-term vitamin A supplementation induces hepatic toxicity and can yield in some cases cognitive and behavior disturbances, for instance anxiety, depression, and irritability [27]. It has been reported that foods fortification with vitamin A is also associated to toxicological effects [22]. Interestingly, retinoids and its metabolites are nowadays not indicated to smokers, since the risk for lung cancer development in this population was demonstrated [28].

Our research group has been developing several experimental models to better understand the contradictory effects of retinoids. Performing an in vitro model, we recently demonstrated that retinol molecule competes with xanthine molecule for xanthine oxidase enzyme, and the catalyzed reaction generates superoxide radical, which lead us to believe that it can be one of the cytosolic mechanisms responsible for vitamin A pro-oxidant effects [35]. Additionally, we previously described ex vivo pro-oxidant properties of retinol, as result of a little modification on its concentration, from 5 to 7 μM, on Sertoli cells culture. Our studies were able to demonstrate increased oxidation in lipids, proteins, and deoxyribonucleic acid, as well as impairment on antioxidant enzymatic activity and mitochondrial dysfunction [4–6, 15, 18, 21]. Furthermore, in vivo models have been developed, and redox imbalance was found in response to vitamin A supplementation at therapeutical doses. Increased oxidative stress status was observed in cerebellum, hippocampus, and *substantia nigra*, associated with anxiety behavior and decreased exploratory and locomotory activity [3, 7–9].

Therefore, putting the physiological importance of vitamin A in vascular level, its use for many treatments and its known antioxidant properties against its toxic and pro-oxidant effects, we aimed in the present study to compare vascular redox parameters among animals supplemented or not with vitamin A.

Materials and methods

Animals and materials

Eighty-five adult male rats (90 days old) were used in this work and were maintained on a 12 h light–dark cycle with water and food ad libitum. All experiment procedures were performed in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals. Retinyl palmitate (Arovit®), water soluble form, was purchased from Bayer, São Paulo, São Paulo, Brazil. All other reagents were purchased from Sigma Chemicals (St. Louis, MO, USA).

Experimental design and drug administration

Three different treatment periods were performed, two acute (3 and 7 days) and one chronic (28 days). Initially, the 85 animals were randomly divided into the three treatments: 25 rats for the treatment of 3 days (n=5 for each group), 25 rats for the treatment of 7 days (n=5 for each group) and 35 rats for the treatment of 28 days (n=7 for each group). The supplementation groups were the following: control (vehicle: saline solution 0.9%) and four different vitamin A doses (1,000, 2,500, 4,500, and 9,000), expressed as International Units (IU) for body mass kilogram (kg) for day ($IU kg^{-1} day^{-1}$). Retinyl palmitate solution was prepared daily using saline solution (NaCl 0.9%) as vehicle, and it was administrated via oral, by intragastric gavage, in a total of 0.8 mL volume, always in the dark cycle beginning.

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Sample preparation

After the respective treatments, the rats were killed by decapitation, and thoracic aorta artery was surgically removed and cleaned with iced saline solution to remove blood. The vessel was homogenized in phosphate buffer for samples pH 7.4, the homogenate was centrifuged at 700×*g* to remove debris, and the supernatant was used as a mother solution. The protein content from aorta artery homogenates was quantified by Lowry method in order to correct the results of the evaluated redox parameters (all expressed per milligram protein) [23].

Redox parameters

The thiobarbituric acid reactive species (TBARS) test as an index of lipids oxidation was evaluated. The TBARS consists of an acid-heating reaction of the lipid peroxidation end product, malondialdehyde, with thiobarbituric acid (TBA). The TBARS were determined at 532 nm, and the results were expressed as $\mathrm{nmol\,mg\,protein^{-1}}$ [13]. The total sulfhydryl (SH) content, present in proteins as well as glutathione, was quantified at 412 nm by its reaction with 5,5′-dithio-bis 2-nitrobenzoic acid, and the results were expressed as $\mathrm{nmol\,mg\,protein^{-1}}$ [14].

Antioxidant enzymes activity was also assessed. The superoxide dismutase (SOD) catalyzes superoxide anion radical (O_2^-) dismutation to generate hydrogen peroxide (H_2O_2), and its activity measurement is based on the principle that adrenaline undergo auto-oxidation in O_2^- presence; so at 480 nm, we determine the SOD activity by the exogenous adrenaline auto-oxidation inhibition in sample presence [2]. The catalase (CAT) catalyzes the H_2O_2 conversion to water (H_2O), and to determine its activity, we added H_2O_2 and analyzed the capacity of sample to decrease the H_2O_2 amount at 240 nm [1].

Statistical analysis

All data are presented as mean±standard error of mean, and one-way ANOVA followed by Dunnet's post hoc was used to determine the differences among groups and the significance level considered was $p \leq 0.05$. The statistical analysis and the graphs making were conducted with *GraphPad Software Inc.®*, *San Diego*, *CA*, *USA—version 5.00*.

Results

For measure oxidative damage, we assessed TBARS, which has been used as an index of lipoperoxidation, and it was observed in the present work that there is a decrease on this parameter in all doses after 7 days treatment period and after 28 days treatment period in the three higher doses, but no modifications after 3 days treatment period (Fig. 1). Regarding the sulfhydryl oxidation, which is present in proteins as well as glutathione and has been used as a marker of redox status, we could observe an increase on its reduced form after 3 days treatment period in two

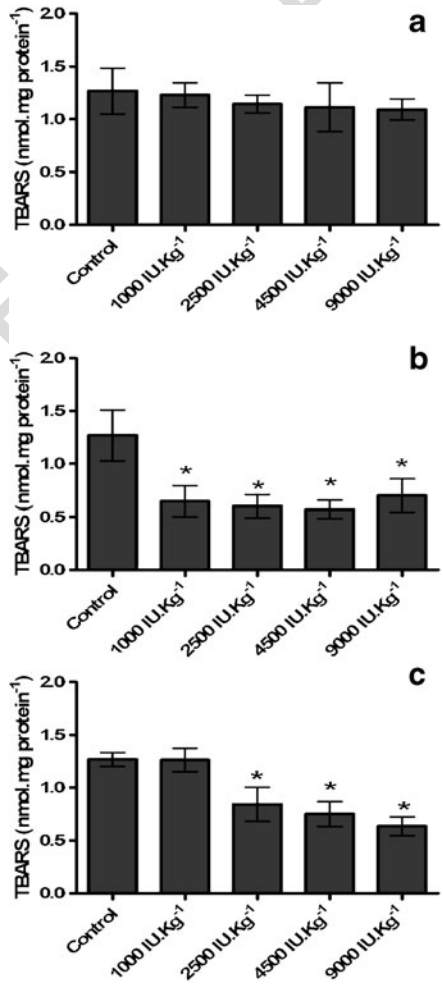

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Fig. 1 Effects of different doses of vitamin A after **a** 3, **b** 7, and **c** 28 days on TBARS. Data are expressed as mean± standard error of mean. *Single asterisk* indicates different of control. Differences were determined by one-way ANOVA followed by Dunnet's post hoc, and the accepted significance level was $p \leq 0.05$

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higher doses. No changes were seen on SH status after 7 and 28 days treatment period (Fig. 2).

Additionally, the activities of two important antioxidant enzymes, SOD (responsible for O_2^- dismutation) and CAT (responsible for H_2O_2 conversion to water), were evaluated. After 3 days treatment period, a decrease on SOD activity in three higher doses was observed; after 7 days treatment period, its activity did not change; and after 28 days treatment period, SOD activity decreased in all doses (Fig. 3), while on CAT activity, we detected an increase after 3 days treatment period in all doses, a decrease after 7 days treatment period in all doses, and no modifications after 28 days treatment period (Fig. 4).

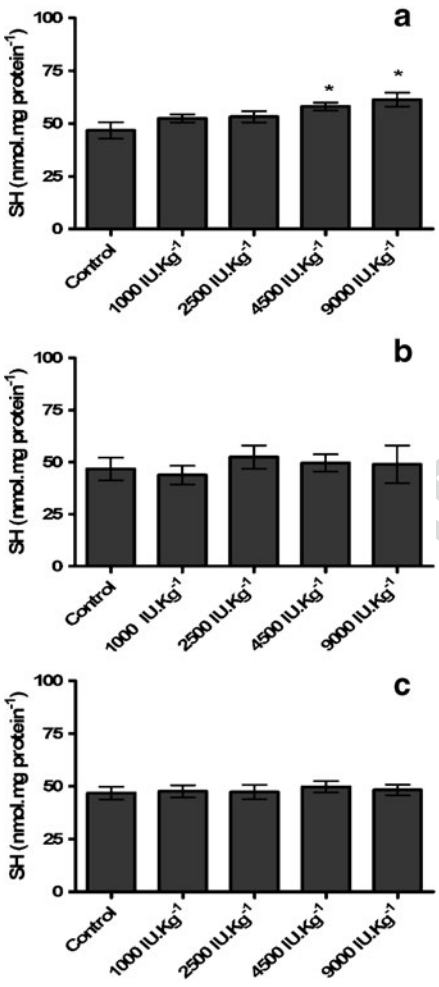

Fig. 2 Effects of different doses of vitamin A after **a** 3, **b** 7, and **c** 28 days on SH. Data are expressed as mean±standard error of mean. *Single asterisk* indicates different of control. Differences were determined by one-way ANOVA followed by Dunnet's post hoc, and the accepted significance level was $p \leq 0.05$

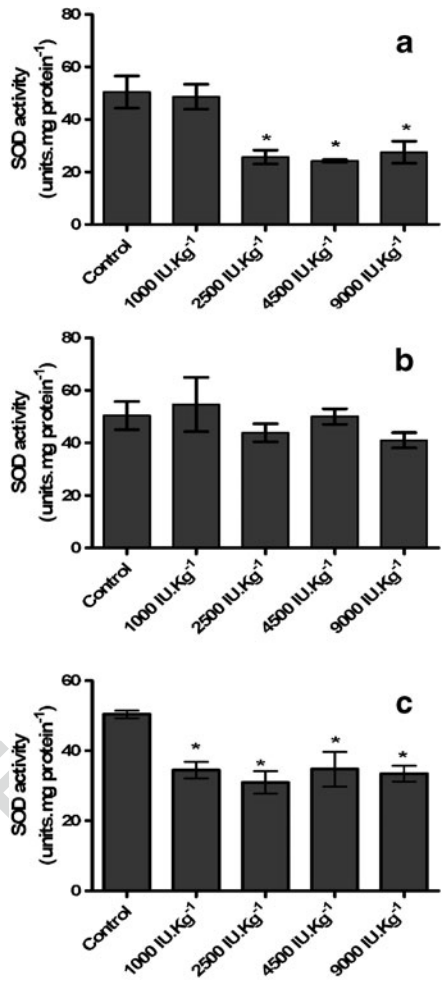

Fig. 3 Effects of different doses of vitamin A after **a** 3, **b** 7, and **c** 28 days on SOD activity. Data are expressed as mean± standard error of mean. *Single asterisk* indicates different of control. Differences were determined by one-way ANOVA followed by Dunnet's post hoc, and the accepted significance level was $p \leq 0.05$

Discussion

In the present work, we have evaluated redox parameters on vascular level in response to vitamin A supplementation, since important roles are attributed to this vitamin at arterial level but growing literature has also shown negative effects of the use of this compound. Additionally, a few studies have regarded the redox effects of vitamin A at arterial

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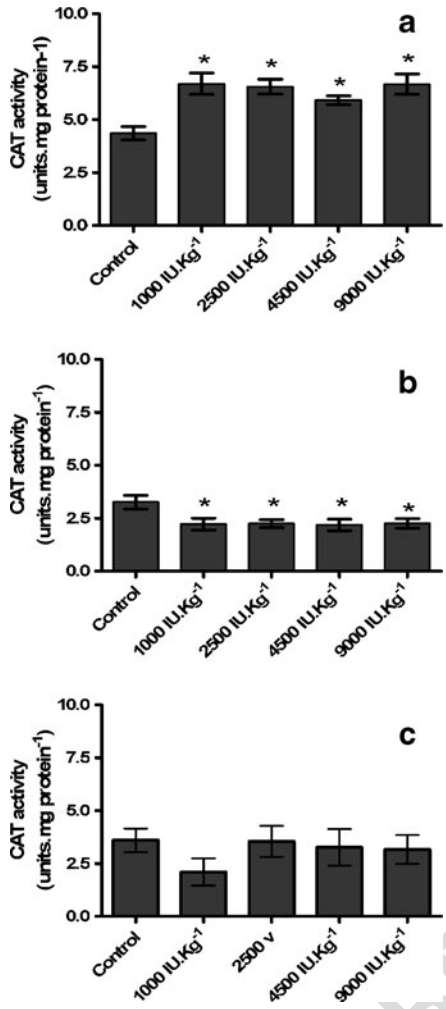

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Fig. 4 Effects of different doses of vitamin A after **a** 3, **b** 7, and **c** 28 days on CAT. Data are expressed as mean±standard error of mean. *Single asterisk* indicates different of control. Differences were determined by one-way ANOVA followed by Dunnet's post hoc, and the accepted significance level was $p \leq 0.05$

Q5 system, probably due the difficulty in handling the aorta and its low tissue yield.

On lipoperoxidation terms, we could observe a decrease on TBARS levels in response to the retinyl palmitate treatment, acutely (7 days) and chronically, what is not in agreement to previous results of our group for other tissues. In the liver of animals treated with retinyl palmitate, it was detected an increase on TBARS levels in one treated for acute (3 days) and chronic period [11, 12]. When evaluated in the hypothalamus, TBARS levels are also increased in a Q6 chronic treatment period [10]. However, it is needed to regard that these studies analyzed different tissues, which can be a strong point for these distinct results, in function of diverse physiological roles. Indeed, when we compare our data with another study that also evaluated TBARS in aorta in response to vitamin A deficiency, our results make more sense. Gatica et al. performed their study with a vitamin A-deficient diet model in rats for 3 months, and it resulted in increased TBARS levels at aorta and serum, as well as, serum retinol concentrations in sub-clinical levels [16]. Thus, our TBARS results indicate that vitamin A acts as an antioxidant agent at aorta artery, by diminishing the oxidative damage induced by reactive species. Nevertheless, there is still a doubt if the lower lipoperoxidation could be considered a good adaptation, since it is well-known that reactive species are involved with several physiological roles, including cell-signaling processes [19, 33].

Besides pro-oxidants effects of vitamin A deficiency demonstrated by the Gatica et al., they found increased nitrite levels associated with increased nitric oxide synthase expression as result of the retinol deficiency. However, their results about reactive nitrogen species need to be complemented to better clarify if it is indicating an improvement on nitric oxide availability or is yielding increased nitration by peroxynitrite [30]. Furthermore, by using the same retinol deficiency model for 3 months, Gatica et al. observed changes on lipid metabolism, such as diminished serum triacylglycerol (TAG) cholesterol levels and, in opposite, increased TAG and cholesterol levels on aorta [17].

In addition to lipoperoxidation, the SH content give us another important idea about redox status, since it is present in proteins and glutathione molecules and, for this reason, is considered as the main intracellular redox buffer. Interestingly, SH is increased just after 3 days treatment period, the unique period that TBARS had no changes, and did not play modifications after 7 or 28 days treatment period, the same one in which TBARS is diminished [25]. The results presented here, in part, are in accordance to previous works, which found no modifications on SH content after 7 or 28 days treatment period, with identical doses to those tested here, but a difference is notated after 3 days, since in the present study was observed an increase while in the prior a decrease was found [11, 12]. Despite the opposite results after 3 days, a coincidence is played, it is exactly in this period that occur the changes,

independently if it is to up or down. This coincidence point could be happening in function of the SH redox buffer role [25]. Obviously our results must be carefully analyzed; however, it is possible that it represents a time-related adaptation mechanism, where in a first moment the system responds to the new situation increasing its defenses, for instance SH groups, and as consequence the damage to macromolecules decreases.

For SOD activity, our results are not in agreement to other works, since we observed decreased activity after 3 and 28 days treatment period (no modifications after 7 days) while there was a reported increased SOD activity in liver (acutely and chronically) and hypothalamus (chronic period) [10–12]. However, one more time we have to consider the organ analyzed and the fact that a very few studies have done oxidative stress parameters analysis in vascular level. Analyzing the study with diminished vitamin A intake, it can be seen lower SOD activity on aorta in response to retinol deficiency [16]. However, there is an experimental difference here, since in the Gatica's study the cytosolic SOD activity was assessed, while in the present work we evaluated the total SOD activity. Moreover, the SOD activity observed in our study is in accordance with TBARS and SH results because we believe that in this situation the retinol is acting as an antioxidant molecule, so less radical is offered, and SOD activity do not need be increased. Our results about CAT activity are in accordance with our previous works, which an increase on hepatic activity after 3 days treatment period, and no changes after 28 days treatment period were demonstrated; no modifications of hypothalamus CAT activity after 28 days treatment period were also observed as well. The only exception is the CAT activity after 7 days treatment period, which did not change in the liver in the previous work, but increased in aorta in the present work [10–12]. In response to retinol deficiency, CAT activity has played a diminished activity [16]. The differences on enzymatic activity results, mainly regarding CAT activity, can be attributed to the fact that in the present work we did not assess other peroxidases (enzymes group responsible for hydrogen peroxide removal, like the CAT), such as glutathione peroxidase, and thereby we do not know how is the balance of these enzymes. Another important consideration is if the observed modifications on enzymatic activities are related to changes on their immunocontent or not, since it has been demonstrated that retinol can interfere on nuclear factor kappa B activation, and this transcription factor is be related to antioxidant enzymes expression [36].

In conclusion, vitamin A plays an antioxidant effect on vascular level in our experimental model, what is the opposite observed in other in vivo works produced by our group, where were observed pro-oxidant effects on CNS, liver, and lungs. However, two points must be regarded, in one hand the different role of the studied organic system and in another hand the real impact of this antioxidant adaptation, since it is a biochemical concept and cell function and physiological (vasorelaxation) parameters are needed to suggest if it is a positive or a negative adaptation. Another important consideration for future works will be to investigate the possible different effects among the cell types that form the vascular wall, for instance endothelial and smooth muscular cells.

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9.4 Anexo IV

“Increased blood oxidative stress in experimental menopause rat model: the effects of vitamin A low-dose supplementation upon antioxidant status in bilateral ovariectomized rats”

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ORIGINAL ARTICLE

Increased blood oxidative stress in experimental menopause rat model: the effects of vitamin A low-dose supplementation upon antioxidant status in bilateral ovariectomized rats

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ABSTRACT

Menopause has been reported to be associated with increased oxidative stress and metabolic disorders among women worldwide. Disarrangements in the redox state similar to those observed in women during the decline of ovarian hormonal activity can be obtained experimentally through rat bilateral ovariectomy. The search for alternative treatments to improve life quality in postmenopausal woman is really important. The aim of this study was to evaluate biochemical and oxidative stress parameters that distinguish sham-operated female rats from Wistar rats bilaterally ovariectomized (OVX). Additionally, we have also investigated the effects of retinol palmitate (a vitamin A supplement) low-dose supplementation (500 or 1500 IU/kg/day, during 30 days) upon blood and plasma antioxidant status in OVX rats. Ovariectomy caused an increase in body weight gain, pronounced uterine atrophy, decreased plasma triglycerides and increased total cholesterol levels, and reduced acid uric content. Moreover, we found increased blood peroxidase activities (catalase and glutathione peroxidase), decreased plasma non-enzymatic antioxidant defenses total reactive antioxidant potential and total antioxidant reactivity, decreased protein and non-protein SH levels, accompanied by increased protein oxidative damage (carbonyl). In addition, vitamin A low-dose supplementation was capable to ameliorate antioxidant status in OVX rats, restoring both enzymatic and non-enzymatic defenses, promoting reduction in plasma SH content, and decreasing protein oxidative damage levels. This is the first work in the literature showing that vitamin A at low dose may be beneficial in the treatment of menopause symptoms. Further studies will be made to better understand the effects of vitamin A supplementation in menopause rat model.

INTRODUCTION

Over the last hundred years, the average human lifespan in developed countries has greatly increased and more people are living long lives. Therefore, an increased number of women experienced menopause, a period that extends for almost one-third of their lives. Menopause is an unavoidable process that usually affects women between the ages of 40 and 60 years and signals the end of the fertile phase of a woman's life. After 12 months of permanent loss of menstruation, a woman is considered to be in menopause [1]. Menopausal

women are going to experience a wide variety of physiological changes (the climacteric or climacterium) principally associated with the cessation in sexual hormone secretion. In fact, female sexual hormones have important known benefic biologic functions, such as, controlling depressive episodes [2], reducing risk factor for coronary artery and general cardiovascular diseases [3,4], and also acting as endogenous antioxidants [5–7].

According to the scenario described here, it is not difficult to visualize the metabolic complexity presented by menopausal women, showing important alterations in signaling cascades and metabolic pathways. Common menopausal symptoms include mood and cognition disturbances, vasomotor symptoms (i.e. hot flushes), vaginal and uterine atrophy, and sleep disruption [8]. The literature provides evidence of oxidative stress affecting the entire reproductive lifespan of a woman, even menopause. More recently, many studies suggested the involvement of free radicals and oxidative stress in aging and some age-related processes that often accompany menopause [1,9]. Increased production of reactive oxygen species (ROS) is considered to be one of the major causes of several age-related diseases. These species are continuously generated in physiological conditions and effectively controlled/eliminated by intracellular and extracellular antioxidant systems. Oxidative stress has been defined as an unbalance between increased ROS production and inadequate antioxidant activity [10].

In the recent years, diverse authors suggest the use of different treatments for women in menopause instead of the traditional hormonal replacement. First, not all postmenopausal women with menopausal symptoms are considered likely candidates to receive hormonal therapy; second, to avoid the increased risk to develop breast cancer, stroke, and/or cardiovascular complications associated with the hormonal replacement [11]. Alternative treatments include non-hormone drugs, herbal remedies, vitamins, minerals, antioxidant supplementation, and alternative therapies [12–15]. A balanced diet with adequate amounts of vitamins, minerals, and other nutrients, plays an important role in the prevention and treatment of cardiovascular disease, osteoporosis, obesity, diabetes, cancer, depression, and other menopause-related diseases [16]. Furthermore, in some cases postmenopausal woman appear to be healthier when taking specific supplementations, such as vitamins D and E, or minerals. [17,18]. Despite this, both basic science and clinical studies are still needed to elucidate the mechanisms and true effects of these treatments, which make it difficult to provide evidence-based recommendations [19,20].

Animal models serving in research may have an existing, inbred or induced disease or injury that is similar to a human condition, for the purpose of better understanding the disease/condition or to try a new treatment, therapy, or strategy [21–23]. Menopause experimental models are widely used for research purposes, and a variety of different methodologies are presented in the literature. The two most common ways to induce menopause-like symptoms in experimental animals are by surgical procedures (ovariectomy, with dramatic cessation in hormonal secretion) and by chemical induction (with progressive ovarian function loss) [24,25]. It has also been shown that each model develops different biochemical characteristics in rodents. In this way, it is possible to decide on one or other model according to specific research applications.

The best characterized and reported surgical procedure to induce experimental menopause in rats and mice is the bilateral ovariectomy. This procedure makes possible in a short period of time the acquisition of female rats without ovarian hormones secretion. In addition, ovariectomized (OVX) rats show higher risk to present osteoporoses symptoms [26], cardiac hypertrophy [27], important cardiovascular dysfunctions [28], uterine atrophy [29], increased tail skin temperature [30], decreased plasma vitamin A, C, and E concentrations [31], and an imbalance between free radical production and antioxidant defenses levels, with increased oxidative stress and consequently an acceleration of aging process in different tissues [9,26,32].

To date, rat models of menopause are largely based on surgical or chemical induction, and none of them represent the progressive failure of ovarian function that occurs in natural menopause [1,33]. The transition to menopause can occur over a 10- to 15-year period. Early in perimenopause, menstrual cycles are typically more frequent and characterized by more extreme fluctuations in oestrogen levels. Later in the menopausal transition, cycles become unpredictable and decrease in number, exposing women to progressively longer periods of oestrogen withdrawal. One year of oestrogen absence marks the initiation of the menopause period called early menopause [33]. Although widely used, OVX rats are problematic with regard to reproducing the effects of natural menopause transition. Ovariectomy produces a rapid, dramatic cessation of ovarian function, rather than

the gradual decline that occurs in perimenopause. In addition, the administration of chemicals like 4-vinylcyclohexene diepoxide has been described recently to induce ovarian function loss [24]. But this is a pharmacological rather than physiological rodent model. With the use of any drug-induced physiological changes, care will need to be taken to assure that the drug has no secondary effects on the target tissue and others.

Several works have been focused on the effects of vitamin A in menopausal woman, others focused on the oxidative stress profile established during the transition to menopause. But, to our knowledge, this paper is the first work linking menopause, vitamin A treatment and blood-related oxidative stress parameters. In this work, we evaluated biochemical and oxidative stress parameters that distinguish sham-operated female rats from Wistar rats bilaterally OVX. Additionally, we have also investigated the effects of retinol palmitate (a vitamin A supplement commercially available at drug stores) low-dose supplementation (500 or 1500 IU/kg/day, during 30 days) upon blood and plasma antioxidant status in OVX rats.

MATERIAL AND METHODS

All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication number 80–23 revised 1996) and were carried out according to the determinations of the Brazilian College of Animal Experimentation, COBEA.

Animals and reagents

Thirty-two female Wistar rats (200–250 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. Animals were supplied with commercial pellet food (Nuvilab® CR-1 type – Curitiba, PR, Brazil) and water ad libitum.

Arovit® (retinol palmitate, a commercial water-soluble form of vitamin A) was purchased from Roche (Rio de Janeiro, RJ, Brazil). Ketamine hydrochloride was purchased from Virbac Ltda (Jurubatuba, SP, Brazil) and xylazine hydrochloride from Vetbrands Ltda (Goiânia, GO, Brazil). All other chemicals used in the study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Surgical procedures

Rats were allowed 2 weeks to acclimatize to the surroundings before beginning any experimentation. Thirty-two 60-day-old female rats were randomly divided into either sham-operated group ($n = 8$) or OVX group ($n = 24$). All animal dissections were conducted by surgical procedures with aseptic technique. Rats were anesthetized by an intraperitoneal injection (ketamine, 100 mg/kg; plus xylazine 15 mg/kg, respectively). The ventral part of the abdominal region was shaved and then cleaned with ethanol. One small incision (1 cm) was made through the skin and the muscle wall on the center of peritoneal area. Ovaries were then located, a braided silk sterile suture (Shalon LTDA; São Luis de Montes Belos, GO, Brazil) was performed around the area of the uterine horns (closely to oviducts and to the cervical junction), and the ovaries were removed. The wound was closed in two layers, i.e. muscle and skin using sterile sutures. Sham animals were also anesthetized, the skin and muscle layers were opened, the uterus and ovaries were gently manipulated but not excised, and the wound was closed in two layers. After surgery, rats were housed individually for some hours to allow recovery and then re-grouped in their home cages. Sixty days after surgical procedures, we started the treatment.

The reproductive cycle of female rats is called the estrous cycle, and the mean cycle length is 4–5 days [34]. Two months into the rat reproductive life represents 12–15 estrous cycles. In women, this number of ovulation episodes represents approximately one reproductive life year. After 1 year of permanent loss of menstruation, a woman is considered to be in menopause. From that, the menopause experimental model used here is similar to a human early menopause period. Also, some related symptoms between human menopause and the experimental protocol used here were reported previously.

Treatment

Two months after surgical procedures, animals were treated once a day for 30 days. All treatments were carried out at night (i.e. when the animals are more active and take a greater amount of food) to ensure maximum vitamin A absorption, because this vitamin is better absorbed during or after a meal. Sham-operated and one OVX group were treated with vehicle (physiological saline – NaCl 0.9%, $n = 8$ each). Two other OVX groups ($n = 8$ each) were treated with retinol palmitate (vitamin A) 500 and 1500 IU/kg/day (OVX + 500 and OVX + 1500, respectively). Vitamin A

treatment was prepared daily while protecting vitamin A from light. Treatment was performed orally via a metallic gastric tube (gavage) in a maximum volume of 0.4 ml. Adequate measures were taken to minimize pain or discomfort. During treatment, the animals were weighted weekly and weight gain analyzed.

Samples acquisition

The animals were killed 90 days after surgical procedures (with treatment in the last 30 days). Rats were decapitated 18 h after last vitamin A administration with researchers blinded to group. Blood samples were collected for analysis, and the plasma was separated immediately. The uterus was cut above the cervical junction, visible fat removed, and the cleaned uterus was weighed. Whole blood was rapidly collected (and plasma separated) by one researcher, and another analyzed and weighed uteri. Blood and plasma samples were stored at −80 °C for posterior analyses. On the day of the experiment, a sample aliquot was used. Blood samples were frozen (−80 °C) and thawed (25 °C) two times and centrifuged (600 ***g***, 5 min). Supernatants were used for all biochemical assays described herein. Plasma samples were thawed (25 °C), mixed (vortex), and directly used for posterior assays. Plasma TBARS (thiobarbituric acid reactive species) measurements were normalized by the lipid content in the samples. Aminotransferase activities and redox parameters were normalized by the protein content using bovine albumin as a standard [35].

Plasma lipid profile and biochemical parameters

The plasma was separated to determine the levels of total cholesterol, high-density lipoprotein (HDL), and triglycerides. The plasma lipid profile was determined with commercial kits. Levels of triglycerides and total cholesterol were determined with commercial kits produced by Human do Brasil S/A (Itabira, MG, Brazil). Quantitation of HDL was determined with commercial kit produced by in vitro Diagnostica S/A (Barbacena, MG, Brazil). The concentrations of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) were assessed by using the Friedewald equation [36]. Plasmatic uric acid levels, aspartate aminotransferase (AST, E.C. 2.6.1.1) and alanine aminotransferase (ALT, E.C. 2.6.1.2) plasma activities were quantified with commercial kit produced by in vitro Diagnostica S/A. Iron concentrations in plasma were determined with commercial kit produced by Wiener Laboratórios S.A.I.C (Rosario, Argentina). Blood glucose levels were measured with Accu-Chek® (Roche Diagnostics GmbH, Mannheim, Germany) Active, blood glucose monitor, strips and lancing device produced by Roche Diagnostics GmbH, just before the killing. Animals were not fasted for the blood glucose measurement.

Antioxidant enzyme activities quantitation

Blood superoxide dismutase (SOD, EC 1.15.1.1) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described [37]. The same protocol was used to assess the extracellular form of CuZn-SOD (EC-SOD) activity in plasma samples. Results are expressed as Units SOD/mg protein. Blood catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm [38]. CAT activity is expressed as Units CAT/mg protein. Blood glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by measuring the rate of NADPH oxidation in a spectrophotometer at 340 nm, as previously described [39]. GPx activity was expressed as Units (nmol NADPH oxidized/min)/mg protein.

Non-enzymatic antioxidant defenses measurement

We used the total reactive antioxidant potential (TRAP) test as an index of the non-enzymatic antioxidant capacity on plasma, based on the peroxyl radical (generated by AAPH solution, 2,20-azobis[2-amidinopropane], with luminol) quenching by sample compounds [40]. The reading is taken by chemiluminescence emission. Briefly, we prepared AAPH solution and added luminol (system); thereafter, we waited 2 h for the system to stabilize to do the first reading. After the addition of the sample, we analyzed the readings for nearly 60 min. The results were transformed in percentage, and the area under curve (AUC) was calculated by software GraphPad (San Diego, CA, USA) as described [41]. When the sample was more reduced AUC (in relation to the system AUC), more antioxidant is the sample. The total antioxidant reactivity (TAR) was also analyzed in the plasma and it is based on the same technical principles of TRAP, but TAR is more related to the quality of samples antioxidants. The TAR results were calculated as the ratio of light in the absence of samples (Io)/light intensity right after sample addition (I) [42]. A higher value means a higher antioxidant potential.

Measurement of total and non-protein thiol content

An assay that serves to analyze oxidative alterations in proteins was used to measure the level of reduced thiol

content (SH) in samples [43]. Briefly, for total SH content measurement, a 100-µg sample aliquot (blood or plasma) was diluted in PBS 10 and 10 mM 5,5′-dithionitrobis 2-nitrobenzoic acid and read in a spectrophotometer at 412 nm after 60 min incubation at 25 °C. For non-protein total SH content, a 1-mg sample aliquot was reacted with trichloroacetic acid (10% v/v), centrifuged (10 000 ***g***, 10 min), and the supernatants were used to measure the level of SH. All results are expressed as umol SH/mg protein.

Oxidative damage parameters

As an index to plasma lipoperoxidation, we used the thiobarbituric acid reactive species (TBARS) test, which is widely adopted as a method for measurement of lipid redox state, as previously described [44]. The TBARS consists of an acid-heating reaction of the lipid peroxidation end product, malondialdehyde (MDA), with thiobarbituric acid (TBA, 4,6-Dihydroxypyrimidine-2-thiol). The TBARS was determined at 532 nm and was expressed as nmol/mg lipid. The oxidative damage to plasma proteins was measured by the quantitation of carbonyl groups, as previously described [45]. Briefly, this method is based on the reaction of dinitrophenylhydrazine with protein carbonyl groups, and the absorbance read in a spectrophotometer at 370 nm. Results were expressed as nmol carbonyl/mg protein.

Statistical analyses

Results were expressed as mean ± SEM. All analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) software (version 15.0), and GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) software (version 4.02). The influence of the surgery, the effect of vitamin A in OVX rats, and the possible vitamin A action restoring sham values were analyzed. In this way, we performed three different statistical analyses. First, differences between sham-operated and OVX saline-treated groups (Sham and OVX) were determined by the *t* test analysis. Second, differences among OVX experimental groups (OVX, OVX + 500, and OVX + 1500) were determined by one-way ANOVA followed by the post hoc Tukey's test. Finally, to better see whether Sham and OVX vitamin A-treated groups differed significantly, a one-way ANOVA was performed followed by the post hoc Tukey's test. Differences were considered statistically significant at $P \leq 0.05$. Statistical results were presented in the result section, as follows: first, the *P*, *t* and df values, of Sham vs. OVX; second, the ANOVA *P* and *F* values, and the Tukey's test *P* value for the selected OVX groups comparison; and finally, if necessary, the ANOVA *P* and *F* values, and the Tukey's test *P* value for the selected groups comparison. To better observe differences resulting from the surgery or from the treatment, we decided to use three different statistical tests. Also, a single test would probably mask important differences, for instance between sham-operated and OVX groups.

RESULTS

Body weight gain and uterine tissue weight

For weight gain analyses (*Figure 1a*), we monitored rat weights during the 30-day vitamin A treatment. In this period, weight gain (g) was significantly higher in the OVX saline-treated group (OVX = 13.50 ± 1.476) when compared to the Sham group (Sham = 7.500 ± 1.225) ($P = 0.0074$, $t = 3.128$, df = 14). Body weight gain in OVX rats was not significantly altered by vitamin A treatment (OVX + 500 = 7.875 ± 1.093, OVX + 1500 = 8.625 ± 1.194) ($P = 0.2722$, $F = 1.385$).

After killing, we collected and weighted the uterine tissue (*Figure 1b*). Sham rats presented different uterine morphology according to the estrous cycle phase upon the day of decapitation. Seven sham female rats presented uterine morphology related to non-proestrus phases, estrus, and diestrus (0.32–0.51 g, low fluid

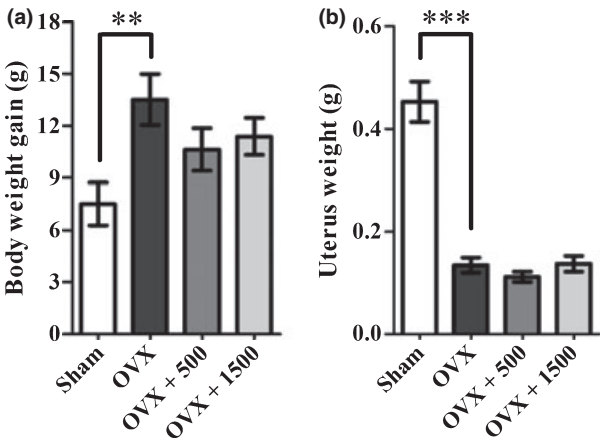

Figure 1 Effects of bilateral ovariectomy and vitamin A supplementation on body weight gain and uterus weight. The body weight gain (a) and the uterus weight (b) were analyzed after the end of the treatment. Data are mean ± SEM ($n = 8$ per group). Statistical difference between sham and ovariectomized (OVX) groups, $^{**}P < 0.01$, $^{***}P < 0.001$ (*t*-test). Statistically different from OVX group.

content and thin tissue), and one sham rat presented a characteristic proestrus uterus morphology (0.65 g, high fluid content and thick tissue). All OVX rats (with or without vitamin A treatment) presented a significant reduction in the uterine tissue weight (OVX = 0.134 ± 0.015, OVX + 500 = 0.112 ± 0.010, OVX + 1500 = 0.137 ± 0.016) when compared to the sham group (Sham = 0.452 ± 0.040) ($P < 0.001$, $t = 7.524$, df = 14; and $P = 0.3668$, $F = 1.052$). OVX uterine tissues were highly atrophied at the end of the study (90 days after ovariectomy procedure), showing the absence of ovarian hormones secretion, and also ovulation, during this period. OVX uterine weight was not altered by vitamin A treatment ($P = 0.3668$, $F = 1.052$) as well as visual morphology.

Plasma lipid profile and biochemical parameters

At the end of the experimental period, OVX groups (with or without vitamin A treatment) showed alterations in the plasma lipid content when compared to sham group (*Table I*). OVX saline-treated triglycerides levels were found to be reduced ($P < 0.001$, $t = 4.204$, df = 14), and total cholesterol content increased ($P = 0.0014$, $t = 3.966$, df = 14) when compared to sham-operated group. Also, LDL and VLDL estimations showed significant alterations when compared to sham group ($P = 0.0067$, $t = 3.177$, df = 14, and $P < 0.001$, $t = 4.204$, df = 14). Vitamin A treatment during 30 days was not capable of restoring the normal lipid profile in OVX rats (triglycerides $P = 0.1412$, $F = 2.152$, total cholesterol $P = 0.7432$, $F = 0.3010$, and HDL $P = 0.9919$, $F = 0.0081$).

In blood/plasma biochemical parameters tested (*Table II*), we found reduced plasmatic uric acid values in OVX saline-treated group when compared to the sham group ($P = 0.004$, $t = 3.441$, df = 14), and vitamin A was not capable of changing these levels ($P = 0.5057$, $F = 0.7044$). The other biochemical parameters did not differ significantly among all analyzed groups.

Blood antioxidant enzyme activities

Blood CAT activity showed an increase in the OVX saline-treated group (OVX = 19.12 ± 0.941) when compared with the sham group (Sham = 15.28 ± 1.204) ($P = 0.025$, $t = 2.510$, df = 14) (*Figure 2c*). CAT activity in OVX groups treated with vitamin A 500 and 1500 IU/kg/day (OVX + 500 = 16.21 ± 0.571, OVX + 1500 = 14.77 ± 0.800) differ significantly from OVX saline-treated group ($P = 0.0027$, $F = 7.936$, $P < 0.05$ OVX vs. OVX + 500 and $P < 0.01$ OVX vs. OVX + 1500); however, only the higher dose restored the CAT activity close to sham values. OVX saline-treated group also showed an increase in blood GPx activity (OVX = 15.37 ± 0.595) when compared with the sham group (Sham = 10.45 ± 0.520) ($P < 0.001$, $t = 6.228$, df = 14) (*Figure 2d*). Both vitamin A treatment doses were able to promote restoration in GPx activity near to the sham values (OVX + 500 = 12.93 ± 0.545, OVX + 1500 = 11.26 ± 0.522) differing significantly from OVX saline-treated group ($P < 0.001$, $F = 13.93$, $P < 0.05$ OVX vs. OVX + 500 and $P < 0.001$ OVX vs. OVX + 1500). The two SOD measures (total SOD activity in blood and EC-SOD in plasma) showed no differences among all four analyzed groups ($P = 0.9291$, $F = 0.1496$; and $P = 0.2065$, $F = 1.622$, respectively) (*Figure 2a* and *b*). We observed an increased blood CAT and GPx activities, in response to ovariectomy, but no changes in blood SOD activity, which led to SOD/CAT + GPx ratio imbalance (OVX = 0.672 ± 0.029) when compared to sham group (Sham = 1.000 ± 0.102) ($P = 0.0077$, $t = 3.107$, df = 14) (*Figure 3*). Vitamin A treatment with 1500 IU/kg/

Table I Plasma lipid profile.

	Sham	OVX	OVX + 500	OVX + 1500
Triglycerides (mg/dL)	66.89 ± 6.91	35.69 ± 2.71[a]	42.58 ± 4.67[b]	46.55 ± 3.61[b]
Total cholesterol (mg/dL)	73.80 ± 2.68	89.24 ± 2.83[a]	86.06 ± 4.41	90.39 ± 4.77[b]
HDL (mg/dL)	22.24 ± 2.25	23.27 ± 3.16	23.37 ± 1.55	22.98 ± 1.57
LDL (mg/dL)	38.18 ± 5.26	58.84 ± 3.82[a]	54.18 ± 4.91	58.10 ± 3.94[b]
VLDL (mg/dL)	13.38 ± 1.38	7.138 ± 0.54[a]	8.516 ± 0.93[b]	9.31 ± 0.72[b]

Triglycerides, total cholesterol, and HDL-cholesterol fraction were measured in plasma samples, LDL and VLDL values were indirectly obtained with the Friedwald methodology. Sham and OVX groups were treated with saline; OVX + 500 group treated with retinol palmitate 500 U/kg/day; and OVX + 1500 group were treated with retinyl palmitate 1500 U/kg/day. Animals were treated once a day for 30 days. Data are mean ± SEM ($n = 8$ per group). Statistically different from sham group, [a]$P < 0.05$ (*t*-test), [b]$P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

HDL, high-density lipoprotein; LDL, low-density lipoprotein; OVX, ovariectomized; VLDL, very-low-density lipoprotein.

Table II Blood/plasma biochemical data.

	Sham	OVX	OVX + 500	OVX + 1500
Aminotransferases				
AST activity (U/dL)	26.70 ± 1.374	29.83 ± 2.428	28.64 ± 1.083	28.75 ± 1.020
ALT activity (U/dL)	15.16 ± 0.812	15.70 ± 1.551	14.84 ± 1.051	14.05 ± 0.877
Uric acid (mg/dL)	0.477 ± 0.033	0.295 ± 0.042[a]	0.339 ± 0.037	0.368 ± 0.052
Iron (mg/dL)	0.339 ± 0.0125	0.341 ± 0.0243	0.290 ± 0.0243	0.303 ± 0.0332
Glycemia (mg/dL)	96.3 ± 7.1	99.5 ± 11.4	104.2 ± 9.2	106.9 ± 10.6

AST and ALT activities, uric acid content and iron content were measured in plasma samples. The glycemic level just before killing was measured in blood samples. Sham and OVX groups were treated with saline; OVX + 500 group was treated with retinol palmitate 500 U/kg/day; and OVX + 1500 group was treated with retinyl palmitate 1500 U/kg/day. Animals were treated once a day for 30 days. Data are mean ± SEM (n = 8 per group). Statistically different from sham group, [a]$P < 0.05$ (t-test).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; OVX, ovariectomized.

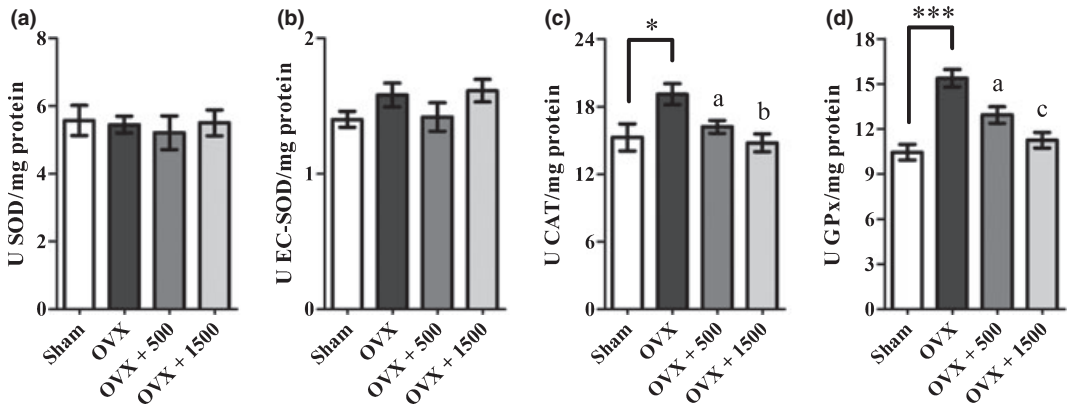

Figure 2 Effects of bilateral ovariectomy and vitamin A supplementation on blood antioxidant enzyme activities. Total superoxide dismutase (SOD) activity (a), catalase activity (c), and glutathione peroxidase activity (d) were measured in blood samples. Extracellular SOD activity (b) was measured in plasma samples. Data are mean ± SEM (n = 8 per group) and the experiments were performed in triplicate. Statistical difference between sham and ovariectomized (OVX) groups, *$P < 0.05$, ***$P < 0.001$ (t-test). Statistically different from OVX group, [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. (one-way ANOVA followed by the post hoc Tukey's test).

day in OVX rats (OVX + 1500 = 1.001 ± 0.075) was able to restore this ratio near to sham value (P = 0.0061, F = 6.564, $P < 0.01$ OVX vs. OVX + 1500).

Plasma non-enzymatic antioxidant defenses

The plasmatic non-enzymatic potential was measured (*Figure 4*); the function of these substances (like polyphenols, vitamins, and protein sulfhydryl) is the main antioxidant defense system in blood plasma. A decreased non-enzymatic potential was observed in OVX saline-treated group when compared to sham-operated group, seen by increased AUC (*Figure 4b*) in TRAP (more related to the amount of the antioxidant) (Sham = 16.79 ± 2.712, OVX = 43.53 ± 3.638) ($P < 0.001$, t = 5.893, df = 14). Furthermore, the TAR (*Figure 4c*) (more related to the quality of the antioxidant, that is, the scavenger capacity) also showed significant difference between sham and OVX saline-treated group (Sham = 52.27 ± 3.358, OVX = 21.06 ± 3.071) ($P < 0.001$, t = 6.859, df = 14).

Vitamin A treatment was able to improve plasmatic non-enzymatic antioxidant defenses in OVX rats. The treatment with 500 IU/kg/day showed an improvement in both TRAP and TAR analyses (OVX + 500 = 26.56 ± 4.418, and 41.07 ± 3.044, respectively) when compared to OVX saline-treated group ($P < 0.001$, F = 12.25, $P < 0.01$; and $P < 0.001$, F = 24.62, $P < 0.001$, respectively). Moreover, the treatment with 1500 IU/kg/day presented a higher improvement in TRAP and TAR analyses (OVX + 1500 = 18.63 ± 2.626, and 51.89 ± 3.334, respectively) when compared to OVX saline-treated group ($P < 0.001$ and $P < 0.001$, respectively).

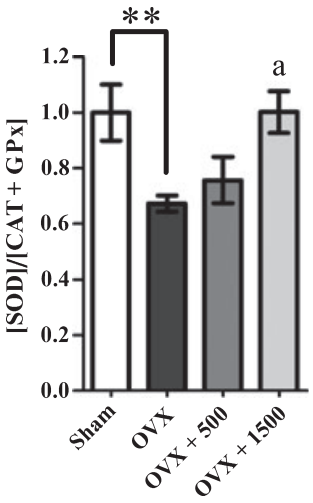

Figure 3 Effects of bilateral ovariectomy and vitamin A supplementation on blood antioxidant enzymatic defenses ratio. The ratio between superoxide dismutase and catalase plus glutathione peroxidase activities were analyzed (arbitrary units) with the blood results. Data are mean ± SEM (n = 8 per group). Statistical difference between sham and ovariectomized (OVX) groups, $^{**}P < 0.01$ (t-test). Statistically different from OVX group, $^{a}P < 0.01$. (one-way ANOVA followed by the post hoc Tukey's test).

Blood- and plasma-reduced sulfhydryl content

Blood-reduced sulfhydryl content (total SH content *Figure 5a* and non-protein SH content *Figure 5b*) and plasma-reduced sulfhydryl content (total SH content *Figure 5c* and non-protein SH content *Figure 5d*) presented decreased levels in OVX saline-treated rats (OVX = 40.25 ± 1.000, 0.396 ± 0.048, 1.070 ± 0.023, and 0.183 ± 0.013, respectively) when compared with the sham-operated group (Sham = 48.42 ± 1.815, 0.702 ± 0.103, 1.212 ± 0.028, and 0.346 ± 0.040, respectively) (P = 0.0015, t = 3.944; P = 0.0173, t = 2.697; P = 0.0016, t = 3.914; and P = 0.0016, t = 3.894, df = 14; respectively), showing an increase in thiol oxidation levels in OVX group. Vitamin A treatment at 1500 IU/kg/day in OVX rats was able to restore reduced thiol content in the plasma total SH content (OVX + 1500 = 1.228 ± 0.027) ($P < 0.001$, F = 10.95, $P < 0.001$ OVX vs. OVX + 1500) and in the plasma non-protein SH content (OVX + 1500 = 0.256 ± 0.024) (P = 0.0158, F = 5.082, $P < 0.05$ OVX vs. OVX + 1500) analyses.

Plasma oxidative damage levels

OVX saline-treated rats presented significant increase in the plasma protein carbonylation levels (OVX = 10.19 ± 0.376) when compared to the sham group (Sham = 7.767 ± 0.445) (P = 0.001, t = 4.165, df = 14) (*Figure 6b*). Vitamin A treatment with 1500 IU/kg/day in OVX rats (OVX + 1500 = 8.187 ± 0.353) was able to reduce plasma protein damage near to sham values (P = 0.0362, F = 3.901, $P < 0.05$ OVX vs. OVX + 1500). On the other hand, ovariectomy and/or vitamin A treatment did not alter plasma lipid peroxidation levels measured (P = 0.9036, t = 0.123, df = 14; and P = 0.5900, F = 0.5411) (*Figure 6a*).

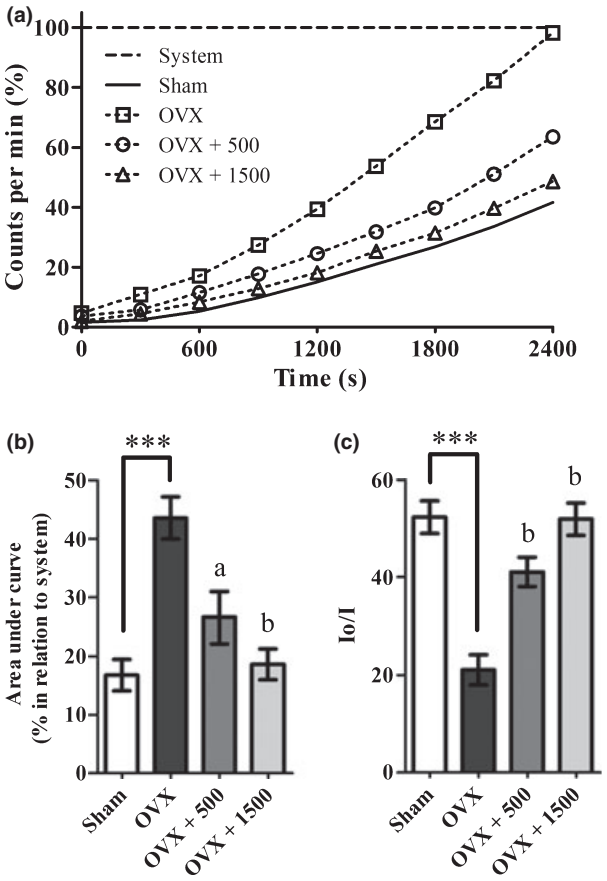

Figure 4 Effects of bilateral ovariectomy and vitamin A supplementation on plasma non-enzymatic antioxidant potential. An experiment's representative graphic (a), the area under curve of total reactive antioxidant potential (b), and the total antioxidant reactivity of plasma samples were analyzed. Data are mean ± SEM (n = 8 per group) and the experiments were performed in duplicate. Statistical difference between sham and ovariectomized (OVX) groups, $^{***}P < 0.001$ (t-test). Statistically different from OVX group, $^{a}P < 0.01$, $^{b}P < 0.001$ (one-way ANOVA followed by the post hoc Tukey's test).

DISCUSSION

Currently, there is no single experimental model that specifically represents the progressive failure of ovarian

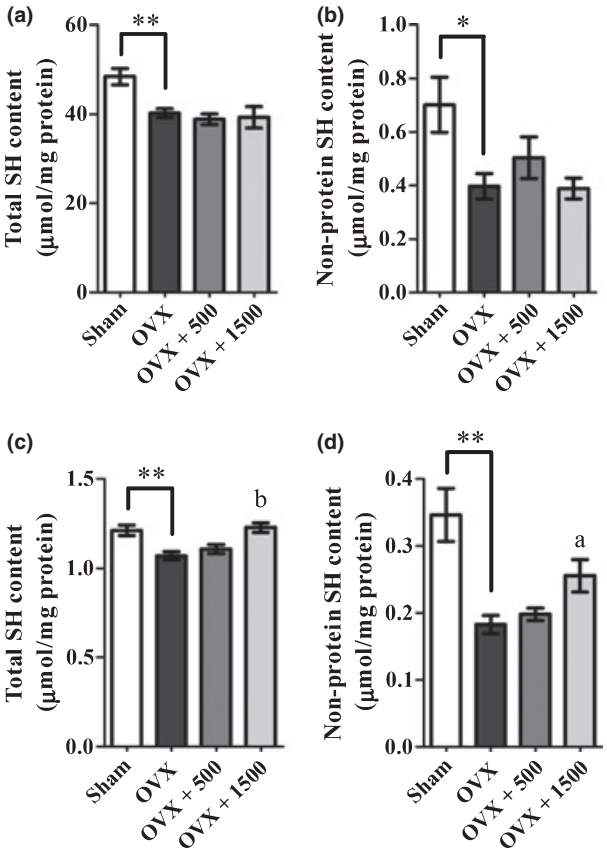

Figure 5 Effects of bilateral ovariectomy and vitamin A supplementation on blood and plasma reduced thiol content. Total reduced thiol content and non-protein thiol content on blood (a and b, respectively), and plasma (c and d, respectively) samples. Data are mean ± SEM (n = 8 per group), and the experiments were performed in triplicate. Statistical difference between sham and ovariectomized (OVX) groups, $^{*}P < 0.05$, $^{**}P < 0.01$ (t-test). Statistically different from OVX group, $^{a}P < 0.05$, $^{b}P < 0.001$ (one-way ANOVA followed by the post hoc Tukey's test).

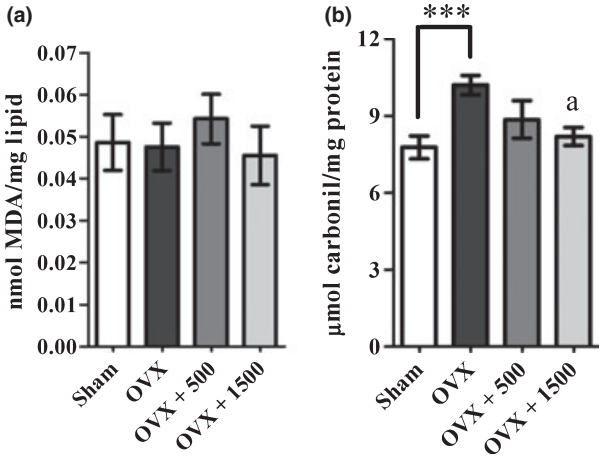

Figure 6 Effects of bilateral ovariectomy and vitamin A supplementation on plasma oxidative damage parameters. Lipid peroxidation (a) and protein carbonylation (b) were analyzed in plasma samples. Data are mean ± SEM (n = 8 per group) and the experiments were performed in duplicate. Statistical difference between sham and ovariectomized (OVX) groups, $^{***}P < 0.001$ (t-test). Statistically different from OVX group, $^{a}P < 0.05$. (one-way ANOVA followed by the post hoc Tukey's test).

function that occurs in natural menopause transition, and in the same time using the available models based on surgical or chemical induction to accurately recreate and study the erratic hormonal state that occurs during the natural menopause transition remains to be seen. With this caveat in mind, the OVX rat model remains the most popular choice as it has been proven to represent some of the most important clinical features of oestrogen deficiency-induced (or postmenopausal) bone loss, circulatory dysfunctions, and nervous system aging in the adult human [26,32,46]. Associated with the higher risk of showing menopause-related symptoms, OVX rats are able to present increased oxidative stress levels and consequently an accelerated aging process in different tissues [9,26,32]. In this scenario, oxidative stress has been proposed to explain the biologic side effects of experimental menopause. In the present work, our first objective was to evaluate the effects of bilateral ovariectomy upon biochemical and oxidative stress parameters in Wistar rats. Our results confirmed that OVX rats presented increased blood oxidative stress and considerable changes in the lipid profile, associated with an increase in body weight gain and expected uterine tissue atrophy.

The ovary of the premenopausal human female, as well as the ovary of various animal species, serves as the body's primary source of oestrogen (17β-estradiol or E2), the hormone associated with protection of the premenopausal woman from a variety of potential postmenopausal health problems, such as increased risk for a decline in cardiovascular, skeletal, and nervous system function, and for accelerated aging process. An important anti-atherosclerotic effect of oestrogen is probably its beneficial influence on lipid metabolism. Postmenopausal women usually exhibit increased levels of LDL, lipoprotein(a), and total cholesterol and decreased HDL level [47]. In the present study, OVX rats presented an increase in total cholesterol levels, but no alterations in HDL fraction, and an interesting decrease in triglycerides. According to the lipid profile performed, an increase in LDL levels of OVX rats may be suggested (*Table I*). Additionally, OVX rats presented reduced

triglycerides levels compared to sham-operated animals (*Table I*). However, the literature provides evidences that experimental menopause rodent models and menopausal woman exhibit increased plasmatic triglycerides levels [48,49]. According to another study comparing the group of patients in surgical menopause following bilateral oophorectomy with natural menopause group, that while total cholesterol and LDL levels were found higher in surgical menopause group, no statistical difference was found between the two groups in HDL, VLDL, and triglycerides levels [50]. Furthermore, the factors determining the level of triglycerides are not well defined, although diet, smoking, and physical activity are important factors [51]. The data also showed a decrease in plasmatic uric acid levels in OVX rats (*Table II*). Recently, increased uric acid levels were associated with increased risk for metabolic syndrome in both premenopausal and postmenopausal women [52]. In other hand, another recent study suggests that menopause explains a substantial portion, but not all, of the serum uric acid level age-associated increase among women [53]. In addition, we show that female rats bilaterally OVX presented increased body weight gain and extremely atrophied uterine tissue (*Figure 1*). These findings confirm the efficiency of ovariectomy surgery causes drastic cessation of sexual hormones secretion.

The increased risk of coronary heart disease in postmenopausal women is closely related with the fall in female hormones secretion. Accumulating evidence suggests that cardiovascular diseases are associated with increased oxidative stress in blood vessels. Increased ROS, such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), causes blood vessels to become thicker, produce inflammation in the vessel wall, and thus are regarded as risk factors for vascular disease. Whereas controlled ROS concentrations also act as signaling molecules in many aspects of growth factor-mediated physiological responses [54]. Oxidative stress is a particular state characterized by an overload in oxidants, which may culminate in cellular dysfunction [10,55]. In our study, OVX rats presented an increase in blood antioxidant peroxidase activities (*Figure 2c* and *d*), a decrease in plasma non-enzymatic defenses (*Figure 4*), a decrease in both protein and non-protein SH content (*Figure 5*), accompanied by increased plasma protein oxidative damage levels (*Figure 6b*).

The increase in blood peroxidase activities (CAT and GPx), without changes in blood SOD measures (*Figure 2*), suggests that other source of H_2O_2 instead of SOD presented in blood, is active. Blood vessels express the three isoforms of SOD: cytosolic (CuZn-SOD), mitochondrial SOD, and an EC-SOD [56]. A consequence of SOD activity is the formation of H_2O_2 that it is relatively stable and diffusible (including through cell membranes), compared with many other ROS. Although recognized as a key player in both oxidative damage and redox-regulated cellular processes, there are still many gaps in our knowledge of how it acts. H_2O_2 directly reacts with biologic molecules causing thiol redox transformations, cell signaling, and also oxidative damage [57,58]. In the current study, we have also shown a decrease in plasma non-enzymatic defenses, on both TRAP and TAR analyzes (*Figure 4*). Non-enzymatic antioxidants, such as vitamin C, vitamin E, selenium, zinc, taurine, glutathione (GSH), beta-carotene, and carotene, usually are obtained from dietary sources [7]. The literature reported concentrations of vitamins A, C, and E in the plasma of OVX rats are lower than in controls [31]. Thus, plasma concentrations of vitamins A, C, and E in OVX rats may be decreased as a result of their action in inhibiting free radicals insult. Also, the blood-reduced GSH pool may be depleted by the oxidative insult caused by ovariectomy. In agreement with this idea, a decrease in both protein and non-protein SH content (*Figure 5*) in OVX rats were showed, suggesting increased levels of oxidized protein and GSH. In addition, we found decreased plasmatic uric acid levels in OVX rats (*Table II*), which represents the main plasma non-protein antioxidant. The decreased non-enzymatic antioxidant potential observed in OVX saline-treated group (*Figure 4*) may be correlated with the lower uric acid levels found. Indeed, OVX vitamin A-treated groups showed higher uric acid values and increased non-enzymatic antioxidant potential. Different studies have shown a positive correlation between non-enzymatic potential and plasmatic uric acid levels [59]. Particularly in septic shock, non-enzymatic antioxidant levels are strongly influenced by uric acid levels [60].

Moreover, OVX rats presented increased plasma carbonyl levels, but no changes in lipid peroxidation (*Figure 6*). Most parts of the literature reported increased lipid peroxidation levels in OVX rats and in postmenopausal women [1,31,61,62]. On the other hand, it is well recognized that protein oxidative damage is closely involved with many menopause-related pathologies [63]. Of the many biologic targets of oxidative stress, lipids are the most involved class of biomolecules. One of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products is the MDA. This molecule can be quantified by different approaches. The two most com-

mon are by chromatography (analyzing the MDA content) and spectrophotometry (reaction of sample with TBA solution, TBARS test) [64]. The utilization of MDA analysis and/or the TBARS test in studies of lipid peroxidation require caution, discretion, and correlative data from other indices of oxidative stress. In our work, we found no significant lipid peroxidation in animals with decreased antioxidant capacity. More than likely, the measurement of TBARS levels is not an accurate/specific approach to evaluate lipid peroxidation in blood/plasma samples. More recently, the measurement of F2-isoprostanes by methods utilizing mass spectrometry is gaining force. This test is widely regarded as the best currently available biomarker of lipid peroxidation [65]. F2-isoprostanes are certainly the most specific markers of lipid peroxidation but also the most difficult to measure. In summary, according to the biochemical and oxidative stress parameters presented, we emphasize that rat ovariectomy methodology to induce human menopause-related conditions is valid and suitable for research purposes, corroborating with the previous literature [26–29,32,46].

Our second objective was to evaluate the effects of vitamin A supplementation in OVX rats upon the previously described parameters. Our results suggest that vitamin A at doses of 500 and 1500 IU/kg have potentially beneficial effects in OVX rats, improving the blood antioxidant status after 30 days of supplementation. Regarding the vitamin A dose used in this study, we chose it according to the vitamin A quantity often supplemented in the pellet food (25 200 IU/kg of food, according to the manufacturer) and the amount that each adult rat eats per day (15–25 g of pellet food). Therefore, in our own breeding colony, the dietary consumption of vitamin A in food is around to 375–625 IU per rat. In addition, several works originating within our research group demonstrated that vitamin A supplementation in higher doses (up to 2500 IU/kg/day) potentially induces dysfunctions in the redox and bioenergetics states of different tissues in healthy male rats [66–69]. It is important to emphasize that here we tested the supplementation of vitamin A in a totally distinct rat model, with clearly noticeable gender and physiological differences being considered. Since we started the treatment 2 months after surgical procedures, the results lead us to believe vitamin A treatment probably acts more to reverse some of the changes induced by OVX surgery than it does to prevent it. However, this is not a simple phenomenon. It is not possible to discard that vitamin A treatment may also be important in prevention of some menopause-related symptoms during the last 30 days of the experiment. In addition, longer periods of treatment may induce different changes in metabolism, sometimes leading to hepatic toxicity. In our study, we did not observe changes on plasmatic AST and ALT activities (hepatic function markers) among all groups (*Table II*).

Moreover, when deciding to use vitamin A as an antioxidant therapy, the possible pro-oxidative effects elicited by such treatment may be taken into account, mainly in CNS tissues because of its low capacity to tolerate reactive species [70]. Today, there is no consensus on a definitively safe supplemental dose of retinyl palmitate during menopause for humans among literature [71]. Literature reports common safety factors applied to extrapolate animal data to the human. Approximate equivalence was obtained by the application of a 10-fold factor for difference in species and another 10-fold factor to account for interspecies difference [72]. Taking this information and comparing with the doses used in our work (500 and 1500 IU/day/kg), we observed sevenfold and 21-fold ratios. Our results suggest that vitamin A low-dose treatment (doses not significantly higher than the ingested diary amount) seems to be safe for menopausal women. However, further investigations need to be made in other structures to better determine these issues. In recent studies, higher doses of vitamin A have been associated with adverse effects on bone metabolism. A moderately high vitamin A intake (three times the adult recommended dietary intake – RDA) and high plasma vitamin A levels have been associated with a low bone mineral density in menopausal woman [73]. On the other hand, serum retinyl esters are not elevated in different populations of postmenopausal women with and without osteoporosis who have taken vitamin A supplements that are either higher or not than the RDA [74,75]. However, retinyl ester concentration (percentage of total vitamin A) was marginally associated with osteoporosis and should be further investigated. Thus, at present it is unclear whether a high vitamin A intake should be considered as a risk factor for osteoporosis and other menopause-related diseases.

Hormonal replacement therapy may be prescribed if severe side effects (menopause-related symptoms) caused by low levels of sexual hormones are experienced. However, not all postmenopausal women with menopausal symptoms are considered likely candidates to receive hormonal therapy. Thus, to avoid the increased risk of developing complications and diseases associated with the hormonal replacement, many women choose

alternative treatments [11–15]. It is well known that vitamin and mineral supplementation may play an important role in the management of menopausal symptoms [16]. Despite this fact, both basic scientific and clinical studies are still needed to elucidate the mechanisms and true effects of these treatments [19,20]. Our work suggests that the blood tissue is an important site of oxidative stress induced by ovariectomy, which is important because the blood and plasma parameters are good markers for whole organism conditions [23]. In addition, low-dose vitamin A treatment was capable of ameliorating the blood antioxidant profile. In the present study, we found that vitamin A was capable of promoting restoration in CAT and GPx activities near to sham-operated levels (*Figure 2*). Furthermore, vitamin A treatment at 1500 IU/kg/day was able to restore the blood SOD/CAT + GPx ratio near to sham value (*Figure 3*). More importantly, we have shown that plasma non-enzymatic antioxidant defenses were greatly improved, in a dose-dependent manner, after vitamin A treatment (*Figure 4*). As expected, this improvement in antioxidant defenses is shown by the visualization in a reduced protein oxidative damage in plasma samples (*Figure 6b*). Additionally, plasmatic reduced SH content was increased after treatment with vitamin A 1500 IU/kg/day (*Figure 5c* and *d*). On the other hand, this phenomenon was not observed in blood SH analyses. Instead of the variety of studies regarding the use of different vitamins (B6, C, D, E and K) in the treatment of menopausal symptoms [1,17,18,76,77], the vitamin A (or retinoids in general) is not studied as much [78]. More recently, low plasma retinol was reported to strongly predict a poorer prognosis in postmenopausal breast cancer patients [79]. Another study suggested that dietary vitamin A and beta-carotene are modestly protective against ovarian cancer, particularly among smokers [80]. Moreover, retinoids have important functions in the activation of many blood-related signaling pathways, regulating epithelial cell growth, immune system, and hematopoiesis [81,82].

Many vitamins inhibit nitric oxide (NO) production by inducible NO synthase (iNOS), as supported by their known antiatherogenic and antineuroinflammatory roles [83]. For example, vitamin A inhibits iNOS gene transcription in vascular smooth muscle cells [84], and endothelial cells [85]. By reducing NO generation by iNOS, vitamin A plays an important role in preventing radical induced cytotoxicity. Also, retinol and retinoic acid (an active metabolite of vitamin A) modulate different redox-dependent signaling pathways [86]. The beneficial effects of vitamin A against oxidative events found in our study could be related to the activation of these pathways. However, the molecular mechanisms related to these effects still need to be clarified. In addition, the restoration of plasma antioxidants could also indicate that vitamin A had scavenged specific oxidant radicals, shown by decreases in both CAT and GPx activities, and accompanied by restoration in non-enzymatic antioxidant potential and plasmatic SH level. Unfortunately, it is almost impossible to indicate which vitamin A metabolite is the responsible for the observed effects, given the vast number of existing vitamin A metabolites [87]. Indeed, different vitamins directly scavenge ROS. However, among them, vitamins E and C have been recognized as two of the most important antioxidants [88]. On the other hand, several studies suggested that β-carotene and retinoids could exert antioxidant effects through a mechanism of free radical scavenging and/or detoxification [88,89]. While substantial experimental evidence has been accumulated demonstrating the potency and nature of the biologic effects of retinoids, in most cases their underlying mechanisms of action remain uncertain.

Several works have been focused on the effects of ROS in pathophysiological changes in the skeleton, cardiovascular system, and thermoregulatory control mechanisms in OVX rats. But, to our knowledge, the present paper is the first work demonstrating that female blood oxidative profile (CAT and GPx activities; TRAP and TAR measures; protein and non-protein SH levels; and protein oxidative damage parameter) is altered by bilateral ovariectomy, thus suggesting that the cessation in sexual hormones secretion, accompanied by increased oxidative stress, may play an important role in the development of menopause-related symptoms. In addition, this is the first work to show that low-dose supplementation on vitamin A was capable of ameliorating antioxidant status in OVX rats. Further investigations will be made to better determine the influence of vitamin A supplementation in oxidative profile of other tissues and organs of OVX rats. Menopause is an inevitable milestone in the reproductive life of every woman. Traditionally, in developing countries, menopause and problems thereof are accepted as normal physiological phenomena. However, with increasing life expectancy among women in developing countries, the prevalence of osteoporosis, cardiovascular disease, and postmenopausal problems in women continue to increase substantially. Most women not only care about living long lives but also about living healthy lives. In this sense, both basic scientific and clinical studies are very important to elucidate the

mechanisms associated with menopause symptoms and to potentially prospect new alternative treatment.

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