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Dissertação de Mestrado

ESTADO ENERGÉTICO E REDOX CARDIOVASCULAR DE RATOS SUPLEMENTADOS COM VITAMINA A

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Não temas Abrão. Sou para ti escudo. Tua recompensa será muito grande.

Gênesis 15:1

DEDICATÓRIA

Dedico esta dissertação para minha avó paterna, vó Rosália, por toda sua luta, seus ensinamentos e seu amor. Ela foi uma grande incentivadora de meus estudos e também a pessoa que me mostrou a palavra de Deus.

Maria Rosália da Luz *(in memorian)*, 1920 - 2008

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ÍNDICE

PARTE I

RESUMO

A vitamina A apresenta importantes papéis no sistema cardiovascular, desde a fase embrionária até a vida adulta. Além disso, propriedades antioxidantes são atribuídas a essa vitamina; Contudo, propriedades pró-oxidantes lhe são atribuídas. Portanto, nosso objetivo foi comparar parâmetros energéticos/redox (no sistema cardiovascular) entre animais submetidos a uma suplementação com vitamina A e animais não submetidos a essa suplementação. Ratos adultos Wistar machos foram tratados com 4 diferentes doses (1000-9000 $UI.Kg^{-1}.dia^{-1}$) de palmitato de retinol ou com salina (controle) ao longo de 3 diferentes períodos (3, 7 e 28 dias). Após o tratamento, a artéria aorta e o coração foram retirados para as análises. Foram avaliados parâmetros de estresse oxidativo em ambas as estruturas, e parâmetros de estresse nitrosativo e energéticos no coração dos animais tratados por 28 dias. A análise estatística foi realizada por Anova de uma via, seguida do *pos hoc* de Dunnet, com significância de $\rho \leq 0,05$. Na artéria aorta, foi observada uma diminuição na lipoperoxidação após 7 e 28 dias, bem como aumento de sulfidril em 3 dias e alterações enzimáticas em todos períodos. No coração, foi detectado um aumento de lipoperoxidação e de carbonilação após 7 e 28 dias e uma dimuição na reatividade não enzimática nos 3 períodos. Alterações enzimáticas foram vistas nos 3 períodos. No período mais longo, foi observado aumento de nitração na fração mitocondrial e diminuição da atividade dos complexos da cadeia transferidora de elétrons. Concluímos que ocorre uma adaptação oposta entre coração e artéria, onde há um aumento de estresse oxidativo no coração e uma diminuição na aorta, no entanto, uma futura análise fisiológica ainda é necessária para se sugerir um ou outro como positivo ou negativo. Além disso, o estresse cardíaco crônico está associado com um possível bloqueio energético e um estado de estresse nitrosativo mitocondrial.

ABSTRACT

Vitamin A plays important roles on cardiovascular system, since development until adult life. Besides physiological roles, antioxidants properties are also attributed to its. However, pro-oxidants properties have been associated to vitamin A. Therefore, our aim was to compare redox parameters among animals supplemented with vitamin A and one not. Adult male Wistar rats were treated with different doses (1000-9000 $IU.Kg^{-1}.day^{-1}$) of retinyl palmitate or saline (control) for 3 different periods (3, 7 and 28 days). Thereafter, the aorta artery and the heart were removed to follow analysis. Oxidative stress parameters were assessed in both organs, while nitrosative stress and energetic parameters were evaluated only at heart from animals treated for 28 days. Statistics were conducted with Anova one way, followed by Dunnet's *pos hoc*, and significance of $\rho \leq 0.05$. On arterial level it was observed a decrease in lipoperoxidation after 7 and 28 days, as well as an increase in sulfhydryl after 3 days and changed enzymatic activity for all periods. In the heart, it was detected an increased lipoperoxidation and carbonyl levels after 7 and 28 days, and a decreased non-enzimatic reactivity for all periods. Additionally, enzymatic changes were also seen. For the chronic treatment we could observe an increase in mitochondrial nitration and a decrease in respiratory chain complexes activities. Thus, we concluded that happens an opposite adaptation between heart and aorta, since there is an increased oxidative stress status in the heart, while in the aorta there is a decreased status. However, a physiological evaluation is needed to suggest what is positive or negative. Furthermore, the cardiac oxidative stress is associated to an energetic impairment and a mitochondrial nitrosative stress.

LISTA DE ABREVIATURAS E SIGLAS

CAT – Catalase

CNS – Sistema nervoso central

CRALBP – Proteína celular ligadora de retinaldeído

CRBP – Proteína celular ligadora de retinol

DNA – Ácido desóxiribonucleico

GSH – Glutationa (forma quimicamente reduzida)

GSSG – Dissulfeto de glutationa (forma quimicamente oxidada)

GST – Glutationa S-transferase

GPx – Glutationa peroxidase

EDRF – Fator de relaxamento derivado do endotélio

MAPK – Proteína cinase ativada por mitógenos

METC – Cadeia transferidora de elétrons mitocondrial

MMEF – Fração enriquecida em membrana mitocondrial

RAR – Receptor de ácido retinóico

RARE – Elemento responsivo ao ácido retinóico

RALDH – Retinal desidrogenase

RDH – Retinol desidrogenase

RBP – Proteína ligadora de retinol

RNS – Espécies reativas de nitrogênio

ROS – Espécies reativas de oxigênio

RXR – Receptor X de retinóides

SOD – Superóxido dismutase

SDH – Succinato desidrogenase

TAR – Reatividade antioxidante total

TBARS – Substâncias reativas ao ácido tiobarbitúrico

TRAP – Potencial antioxidante reativo total

INTRODUÇÃO

VITAMINA A

Vitamina A é o nome dado ao retinol ou qualquer molécula que apresente atividade de retinol. Essa vitamina pode ser obtida tanto como pró-vitamina de fontes animais (esterificado) quanto como pré-vitamina de fontes vegetais (precursores conhecidos coletivamente como carotenóides, principalmente o β-caroteno). Adicionalmente, ela pode ser obtida em suplementos, na forma de palmitato ou acetato de retinol (Sporn 1994). De fato, existem muitos tratamentos (psoríase, câncer, fibrose cística e outros) baseados na utilização de retinóides, bem como a "fortificação" de alimentos (Montrone et al. 2009; O'Neil et al. 2008; Smith et al. 1992; Van Zander and Orlow 2005). Diversas funções fisiológicas são atribuídas ao retinol, desde o desenvolvimento até a vida adulta, principalmente por sua ação na regulação de processos celulares de maturação, diferenciação e proliferação. Além disso, propriedades antioxidantes são atribuídas ao retinol e suas moléculas relacionadas (Sporn 1994).

O retinol é transportado no plasma ligado a proteínas ligadoras de retinol (RBP), enquanto intracelularmente é transportado ligado a proteínas celulares ligadoras de retinol (CRBP) (Sporn 1994). Existem dois diferentes modelos para o transporte de retinol do meio extracelular para o meio intracelular, o primeiro é o modelo de difusão, que propõe que o tempo de dissociação do retinol da RBP seria o passo limitante, enquanto o segundo sugere uma captação mediada pela interação da RBP com uma proteína receptora na membrana plasmática (Hussain et al. 2001; Ross 1993). Dentro do citoplasma, o retinol passa a ser convertido em *cis*-retinal, por catálise da enzima retinol desidrogenase (RDH). Então o *cis*-retinal pode ter dois destinos, ou se mantém ligado a uma proteína celular ligadora de

retinaldeído (CRALBP) ou é oxidado a ácido retinóico, por catálise da retinal desidrogenase (RALDH). A degradação enzimática do ácido retinóico é catalisada por enzimas da família 26 da citocromo P450 (CYP26), e ele pode ser isomerizado da forma *trans* para a forma *cis* (Noy 2000).

Classicamente, os retinóides agem celularmente de forma genômica, onde o ácido retinóico pode ativar receptores nucleares conhecidos como RAR (receptor de ácido retinóico) e RXR (receptor X de retinóides). Esses receptores atuam como dímeros, e cada um possui 3 isoformas (α, β, e γ) codificadas por genes diferentes. Após ativados, esses receptores dimerizados se ligam a elementos responsivos ao ácido retinóico (RARE), presentes na região promotora de certos genes-alvo. Dessa forma então ocorre a ação genômica do retinol, mediada pela forma biologicamente ativa, o ácido retinóico (Chambon 1994; Kastner et al. 1997; Ross 1993; Zile 2001). No entanto, outros mecanismos de ação têm sido sugeridos para o retinol, falando-se na ação não clássica do retinol, possivelmente por ativação de vias de sinalização celular, como das MAPK (proteínas cinases ativadas por mitógenos), sendo ou não mediada pela formação de espécies reativas (Gelain et al. 2006; Gelain et al. 2007).

ESPÉCIES REATIVAS E RADICAIS LIVRES

Radicais são átomos que possuem um ou mais elétrons desemparelhados em sua orbital externa, enquanto o termo radical "livre" não possui uma normatização por parte da IUPAC (União Internacional de Química Pura e Aplicada, http://www.iupac.org/), sendo um conceito mais utilizado por pesquisadores da área, que tratam como "livres" aqueles radicais altamente reativos, envolvidos em cascatas de reação que levam a formação de outros radicais mais reativos, e capazes de agir "livremente". Espécies reativas são intermediários, radicais ou não, envolvidos em vias de formação de radicais livres, como o peróxido de hidrogênio

(H_2O_2), pouco reativo e muito solúvel, mas que pode dar origem ao mais potente dos radicais livres, o radical hidroxila ($^{\cdot}OH$) (Gutteridge 2001).

Uma classe de espécies reativas que merece muita atenção são as espécies reativas de oxigênio (ROS), onde primariamente é formado o radical íon superóxido ($^{\cdot}O_2^-$), por uma redução monovalente do oxigênio (O_2). Uma das principais fontes de radical superóxido é a cadeia transferidora de elétrons, já que se algum dos seus complexos for sobrecarregado ou inibido, passa a haver uma redução parcial (monovalente) da molécula de oxigênio. Isso ocorre pelo fato de o complexo IV (citocromo C oxidase) ser o único capaz de reduzir o oxigênio de forma tetravalente, e se os complexos anteriores não conseguem carrear o elétron até esse complexo, eles próprios passam a reduzir o oxigênio, mas não conseguem fazer isso tetravalentemente. O superóxido pode então sofrer uma dismutação, catalisada pela superóxido dismutase (SOD), gerando peróxido de hidrogênio. Por ação da enzima catalase (CAT), o peróxido pode então ser convertido à água (H_2O), entretanto se isso não ocorrer, em presença de metais de transição, por reações conhecidas como Fenton e Haber-Weiss, esse peróxido dá origem ao radical hidroxila, altamente reativo e capaz de gerar danos em biomoléculas, tais como lipídios, proteínas e DNA (ácido desóxiribonucleico). Alternativamente a catalase, as células possuem outras peroxidases capazes de remover o peróxido de hidrogênio, e entre elas destaca-se a glutationa peroxidase (GPx), enzima que necessita da molécula de glutationa (GSH), sintetizada pelas células, como co-fator (Gutteridge 2001; Poderoso et al. 1996).

Além dos sistemas enzimáticos descritos acima, as células dispõem de sistemas não-enzimáticos de defesa antioxidante. Os antioxidantes não-enzimáticos podem então ser classificados em endógenos (sintetizados pelo nosso organismo) e exógenos (obtidos da dieta). Dentre os endógenos destaca-se a molécula de glutationa, um tripeptídeo que além de ser co-fator da GPx, exerce importante papel antioxidante tanto prevenindo como revertendo

dano oxidativo, através do grupamento sulfidril. A glutationa é considerada o principal tampão redox do citosol, sendo usada na pesquisa como um marcador de estado redox intracelular. No entanto, essa molécula também está relacionada com processos de detoxificação, onde a célula bombeia substâncias do meio intracelular através da formação de conjugados de glutationa. Portanto, alterações no estado de oxidação da GSH devem ser analisadas com precaução, pois essa alteração pode não estar relacionada com uma alteração redox, mas sim com uma situação de desintoxicação (Gutteridge 2001; Meyer and Hell 2005). Várias substâncias podem ser obtidas exogenamente, dentre elas os compostos fenólicos e as **vitaminas A, C e E**. Convém lembrar que a vitamina C (hidrossolúvel) está mais relacionada com "proteção" em meio hidrofílicos, enquanto a A e a E (lipossolúveis) estão mais relacionadas com "proteção" em ambientes lipofílicos (como as membranas). Talvez o termo "proteção" não seja o mais adequado, já que seria precipitado tratar essas substâncias exclusivamente como antioxidantes, já que propriedades pró-oxidantes são atribuídas a todas elas, e de nosso interesse cabe chamar atenção para a vitamina A (Dal-Pizzol et al. 2001; de Oliveira et al. 2007; Gutteridge 2001; Klamt et al. 2003).

Um termo comumente encontrado na literatura científica é "estresse oxidativo", sendo que esse se refere a um desbalanço entre sistemas anti e pró-oxidantes, o que acaba resultando em dano oxidativo a biomoléculas, e isso está relacionado a uma disfunção celular, o que pode resultar em progressão de doenças. De fato, muitas doenças e disfunções vêm sendo relacionadas a esse desbalanço (Gutteridge 2001).

ESTRESSE NITROSATIVO

Além das ROS, outra classe de espécies reativas tem recebido muita atenção recentemente, são as espécies reativas de nitrogênio (RNS). Dentre elas, duas merecem

grande destaque, são o óxido nítrico ($^{\cdot}NO$) e o peroxinitrito ($ONOO^{-}$). O óxido nítrico é formado por catálise da enzima óxido nítrico sintase (NOS), que possui 3 isoformas (endotelial, neural e induzível). Além das 3 isoformas clássicas, vem sendo discutido recentemente a isoforma mitocondrial. O óxido nítrico foi descoberto primeiramente em nível vascular, pois já se sabia da existência de um fator de relaxamento derivado do endotélio (EDRF), produzido por células endoteliais e que se difunde para as células musculares lisas para gerar o relaxamento delas. No entanto, somente depois veio a se descobrir de que o óxido nítrico era esse fator (Gutteridge 2001; Ignarro et al. 1999).

Assim como o que foi relatado acima para as vitaminas, para o óxido nítrico um balanço também é importante, pois apesar de apresentar importantes funções fisiológicas, em excesso ele também apresenta efeitos nocivos. Entre esses efeitos, dois possuem notável importância, um deles se refere a capacidade desse gás em inibir a cadeia transportadora de elétrons, e o outro ocorre pelo fato de ele ser precursor do peroxinitrito (Boveris et al. 2000; Poderoso et al. 1996; Riobo et al. 2001). O peroxinitrito, que não é um radical, é formado pela reação entre os radicais óxido nítrico e superóxido, e tem recebido grande atenção dos pesquisadores por sua capacidade em atacar resíduos tirosil de proteínas, formando 3-nitrotirosina, e inibir essas moléculas. Esse processo é conhecido como nitração, e parece estar relacionado com vários processos patológicos, principalmente no sistema cardiovascular, no qual já é sugerido como marcador plasmático de risco cardiovascular (Ferrer-Sueta and Radi 2009; Peluffo and Radi 2007). O desbalanço entre as espécies reativas de nitrogênio é conhecido como “estresse nitrosativo”; entretanto, uma pequena confusão pode ser feita quando se comparam as expressões “estresse oxidativo” e “estresse nitrosativo”, pois em virtude do termo nitrosativo estar relacionado ao nitrogênio, poderia se cometer o erro de achar que o termo oxidativo estaria relacionado ao oxigênio. Na verdade, o oxigênio é quem está relacionado à oxidação, e não o contrário, pois oxidação significa perda de perda de

elétrons (o oposto de redução, que significa ganho de elétrons). Dessa forma, o oxigênio recebeu seu nome por ser um forte agente que "gera oxidação", ou seja, ele tira elétrons de outras substâncias, ao mesmo tempo em que ele é reduzido, exatamente o que ocorre na cadeia respiratória (Gutteridge 2001).

VITAMINA A E SISTEMA CARDIOVASCULAR

Importantes papéis cardiovasculares são desempenhados pela vitamina A, além dos clássicos em termos de desenvolvimento (Hoover et al. 2008). Foi proposto que o retinol apresenta um papel diferente em nível arterial, que depende do estado desse tecido. Na artéria sadia, o retinol parece atuar como agente de proliferação de células musculares lisas, enquanto na artéria com ateroma, o retinol atuaria induzindo a morte celular (Owens et al. 2004). Além disso, interessantes aspectos são relatados em nível cardíaco, já que ocorre uma mobilização das reservas de retinol para o miocárdio após um infarto (Hoover et al. 2008; Palace et al. 1999a; Palace et al. 1999b). Outra importante ação é a capacidade do ácido retinóico em inibir alguns efeitos gerados pela angiotensina II no coração de fetos de ratos, o que acabou sugerindo ele como um possível agente terapêutico para o remodelamento cardíaco (Wang et al. 2002). A suplementação com palmitato de retinol também interfere nos níveis de triacilgliceróis e de colesterol plasmáticos, o que também tem grande relevância em termos cardiovasculares (Gatica et al. 2006).

EFEITOS ADVERSOS DA VITAMINA A

Apesar dos relatados efeitos positivos da vitamina A, dados negativos também são encontrados como resultado do consumo em excesso. Entre eles podem-se citar enjoos,

vômitos, distúrbio comportamental, fibrose hepática e risco de desenvolvimento de câncer em fumantes (Lam et al. 2006; Myhre et al. 2003; Omenn et al. 1994). Aliado a isso, nosso grupo desenvolveu uma série de trabalhos (usando modelos *in vitro*, *ex vivo* e *in vivo*) demonstrando efeitos pró-oxidantes do retinol. Foi visto que pequenas variações na concentração de retinol em células de Sertoli são capazes de induzir lipoperoxidação, oxidação de proteínas e DNA, desbalanço de enzimas antioxidantes e disfunção mitocondrial (Dal-Pizzol et al. 2001; Dal-Pizzol et al. 2000; Klamt et al. 2003). Interessantemente, alterações de parâmetros comportamentais também foram descritas em animais, aliadas a desbalanço redox no sistema nervoso central (CNS) (De Oliveira and Moreira 2008; de Oliveira et al. 2009a; de Oliveira et al. 2008; de Oliveira et al. 2007). Recentemente, nosso grupo propôs uma fonte citosólica de radical superóxido, já que o retinol compete com xantina pela enzima xantina oxidase, e essa reação gera esse radical (Zanotto-Filho et al. 2008).

JUSTIFICATIVA

Muitas intervenções utilizam a vitamina A na prevenção e no tratamento de uma série de doenças e condições especiais, assim como alguns alimentos são "fortificados" com ela, e seu uso em forma de suplementação não possui nenhum tipo de controle. Adversamente, a literatura vem relatando efeitos nocivos dos retinóides, incluindo as propriedades pró-oxidantes (o que contrasta com a idéia de que o retinol seja exclusivamente antioxidante). De forma preocupante, outros usos terapêuticos têm sido sugeridos, principalmente para doenças cardiovasculares, mesmo frente aos possíveis efeitos tóxicos. Portanto, torna-se de fundamental importância investigar o real impacto que a suplementação com vitamina A tem sobre o sistema cardiovascular.

OBJETIVO GERAL

Comparar parâmetros redox, no sistema cardiovascular, entre ratos suplementados e não suplementados com vitamina A por diferentes períodos de tratamento.

OBJETIVOS ESPECÍFICOS

Comparar parâmetros de estresse oxidativo, na artéria aorta e no coração, entre ratos suplementados e não suplementados com vitamina A por 3, 7 e 28 dias:

- Comparar os níveis de substâncias reativas ao ácido tiobarbitúrico;
- Comparar os níveis de sulfidril reduzido;
- Comparar a atividade da superóxido dismutase;
- Comparar a atividade da catalase.

Comparar parâmetros de estresse oxidativo, no coração, entre ratos suplementados e não suplementados com vitamina A por 3, 7 e 28 dias:

- Comparar os níveis de carbonilas;
- Comparar a reatividade antioxidante total (TAR);
- Comparar o potencial reativo antioxidante total (TRAP);

Comparar parâmetros de toxicidade, no coração, entre ratos suplementados e não suplementados com vitamina A 28 dias:

- Comparar a atividade da enzima glutationa S-transferase;

Comparar parâmetros de estresse nitrosativo, no coração, entre ratos suplementados e não suplementados com vitamina A por 3, 7 e 28 dias:

- Comparar os níveis de 3-nitrotirosina do tecido total;
- Comparar os níveis de 3-nitrotirosina da fração enriquecida em membrana mitocondrial.

Comparar parâmetros energéticos (atividade dos complexos da cadeia transferidora de elétrons), no coração, entre ratos suplementados e não suplementados com vitamina A por 28 dias:

- Comparar a atividade da transferência de elétrons do complexo I para o complexo III;
- Comparar a atividade da transferência de elétrons do complexo II para o complexo III;
- Comparar a atividade do complexo II;
- Comparar a atividade da succinato desidrogenase.

PARTE II

CAPÍTULO I

Vitamin A supplementation for different periods alters rat vascular redox parameters

Manuscrito (CBF-09-0273) submetido ao periódico:

Cell Biochemistry and Function

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CAPÍTULO II

Short-term vitamin A supplementation at therapeutic doses induces oxidative damage on rat heart

Cell Biochemistry & Function

Vitamin A supplementation for different periods alters rat vascular redox parameters

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Keywords:	Antioxidants, Aorta artery, Oxidative Stress, Reactive oxygen species, Retinol
Abstract:	Vitamin A plays physiological and antioxidants properties, but deleterious effects have been reported, including those observed by our group, which has demonstrated pro-oxidant properties. Our aim was to compare vascular redox parameters among animals supplemented or not with vitamin A. Adult male rats were treated with different retinyl palmitate doses (1000-9000 IU.Kg-1.day.-1) or saline for 3, 7 and 28 days periods, and aorta artery was surgically removed. It was evaluated thiobarbituric reactive species (TBARS), total reduced sulfhydryl (SH), and activities of superoxide dismutase (SOD) and catalase (CAT). Statistics were conducted by Anova one-way with Dunnet's pos hoc, and significance $\rho \leq 0.05$. 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.

Vitamin A supplementation for different periods alters rat vascular redox parameters

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ABSTRACT

Vitamin A plays physiological and antioxidants properties, but deleterious effects have been reported, including those observed by our group, which has demonstrated pro-oxidant properties. Our aim was to compare vascular redox parameters among animals supplemented or not with vitamin A. Adult male rats were treated with different retinyl palmitate doses (1000-9000 $IU.Kg^{-1}.day.^{-1}$) or saline for 3, 7 and 28 days periods, and aorta artery was surgically removed. It was evaluated thiobarbituric reactive species (TBARS), total reduced sulfhydryl (SH), and activities of superoxide dismutase (SOD) and catalase (CAT). Statistics were conducted by Anova one-way with Dunnet's *pos hoc*, and significance $\rho \leq 0.05$. 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.

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 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 until adult life. It has been well demonstrated its importance for eyes, reproduction, central nervous system (CNS) and others. Moreover, antioxidants properties are attributed to retinol and its related molecules [1]. 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 [2]. Moreover, it was demonstrated that retinoic acid inhibits angiotensin II actions on vascular smooth muscle cells (VSMC) [3].

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 [4-7]. 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 [8]. It has been reported that foods fortification with vitamin A is also associated to toxicological effects [9]. Interestingly, retinoids and its metabolites are nowadays not indicated to smokers, since it was demonstrated the risk for lung cancer development in this population [10].

Our research group has developing several experimental models to better understand the contradictory effects of retinoids. In 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, what lead us to believe that it can be one of

the cytosolic mechanism responsible for vitamin A pro-oxidant effects [11]. 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 lipids, proteins and deoxyribonucleic acid (DNA) oxidation, as well as antioxidant enzymatic impairment and mitochondrial dysfunction [12-17]. 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 [18-20].

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

MATERIALS AND METHODS

Animals and materials

Adult male rats (90 days old) were used in this work and were maintained on a 12 hours light-dark cycle with water and food *ad libitum*. All experiments 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 others 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 animal were randomly divided for the 3 treatments, and thereafter into the supplementation groups: Control (vehicle: saline solution 0.9 %) and 4 different vitamin A doses (1000, 2500, 4500 and 9000), 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 intra-gastric gavage, in a total 0.8 mL volume, always in the dark cycle beginning.

Sample preparation

After the respective treatments the rats were killed by decaptation 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 (PBS) pH 7.4, the homogenate was centrifuged at 700 x g to remove debris, and the supernatant was used as a mother solution. The protein content was quantified by Lowry method in order to correct the results [21].

Redox parameters

It was evaluated the thiobarbituric acid reactive species (TBARS) test as an index of lipids oxidation. The TBARS consists in 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 $protein^{-1}$ [22]. The total sulfhydryl (SH) content, present in proteins as well as glutathione, was quantified at 412 ηm by its reaction with 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB), and the results were expressed as ηmol.mg $protein^{-1}$ [23].

Antioxidant enzymes activity was also assessed. The superoxide dismutase (SOD) catalyzes 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 [24]. 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 ηm [25].

Statystical analysis

All data are presented as mean ± standard error of mean (SEM) and it was used Anova one way followed by Dunnet's *pos hoc* to determine the differences among goups 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 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 (figure 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 higher doses. No changes were seen on SH status after 7 and 28 days treatment period (figure 2).

Additionally, it was evaluated the activities of two important antioxidant enzymes, SOD (responsible for $^{\cdot}O_2^-$ dismutation) and CAT (responsible for H_2O_2 conversion to water). After 3 days treatment period, it was observed a decrease on SOD activity in three higher doses, after 7 days treatment period its activity did not change and after 28 days treatment period SOD activity decreased in all doses (figure 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 (figure 4).

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 showing negative effects of this compound using. Additionally, a few studies have regarded the redox effects of vitamin A at arterial system, probably due the difficult to hand 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 (3days) and chronic period [26, 27]. When evaluated in the hipothalamus, TBARS levels are also increased in a chronic treatment period [28]. However, it is needed to be regarded that these studies analyzed different tissues, what 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 [29].

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 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 [30]. 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 priors a decrease was found [26, 27]. 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 [30]. Obviously our results must 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 was reported increased SOD activity in liver (acutely and chronically) and hypothalamus (chronic period) [26-28]. 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 [29]. However, an experimental difference there is 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, as well as no modifications hypothalamus CAT activity after 28 days treatment period were also observed. The only exception is the CAT activity after 7 days treatment period, which in previous work did not change in the liver, but increased in aorta in the present work [26-28]. In response to retinol deficiency, CAT activity has played a diminished activity [29]. 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 (GPx), 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 (NFκB) activation, and this transcription factor is be related to antioxidant enzymes expression [31].

Taken together, the results presented here point to an antioxidant role of vitamin A at vascular level, what is in accordance to the related study with retinol deficiency model in spite of not be in agreement with previous studies from our research group for other tissues. Besides pro-oxidants effects demonstrated by the Gatica's work, in this same article they found increased nitrite levels associated to increased nitric oxide synthase (NOS) expression as result of the retinol deficiency [29]. However, these results about reactive nitrogen species need to be complemented to better clarify if it is indicating an improvement on nitric oxide (·NO) availability or is yielding increased nitration by peroxynitrite [32]. Furthermore, it was

also demonstrated that vitamin A can interfere on lipid metabolism. Using the same retinol deficiency model, for 3 months, changes on lipid metabolism were observed, such as diminished serum triacylglycerol (TAG) cholesterol levels and, in opposite, increased TAG and cholesterol levels on aorta [33].

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-oxidants effects on CNS, liver and lung. 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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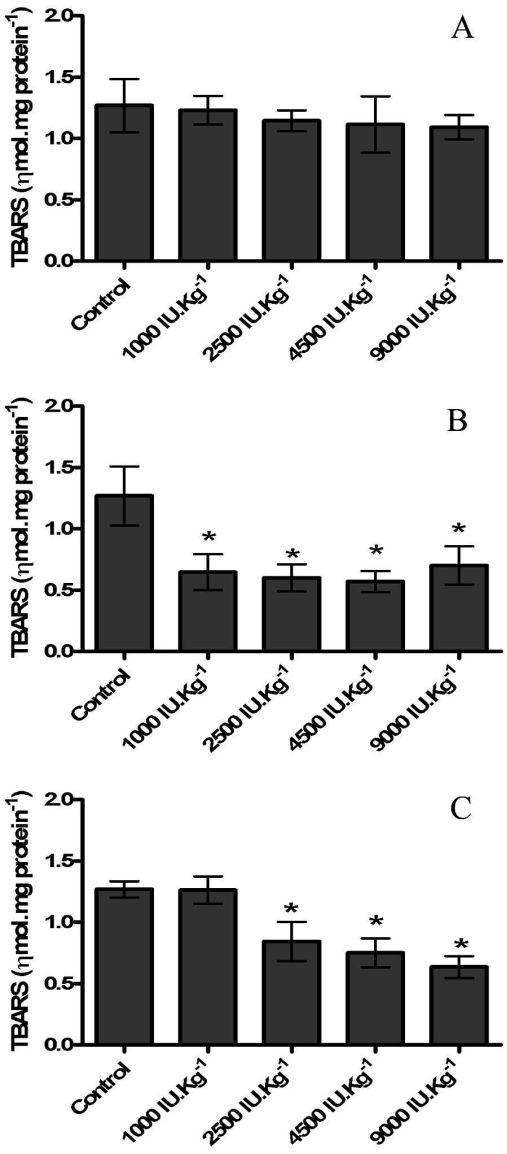
FIGURE CAPTIONS

Figure 1: TBARS levels. A. 3 days period (η = 5 for each group), B. 7 days period (η = 5 for each group) and C. 28 days period (η = 7 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's *pos hoc*, and the accepted significance level was $p \leq 0.05$.

Figure 2: SH content. A. 3 days period (η = 5 for each group), B. 7 days period (η = 5 for each group) and C. 28 days period (η = 7 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's *pos hoc*, and the accepted significance level was $p \leq 0.05$.

Figure 3: SOD activity. A. 3 days period (η = 5 for each group), B. 7 days period (η = 5 for each group) and C. 28 days period (η = 7 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's *pos hoc*, and the accepted significance level was $p \leq 0.05$.

Figure 4: CAT activity. A. 3 days period (η = 5 for each group), B. 7 days period (η = 5 for each group) and C. 28 days period (η = 7 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's *pos hoc*, and the accepted significance level was $p \leq 0.05$.



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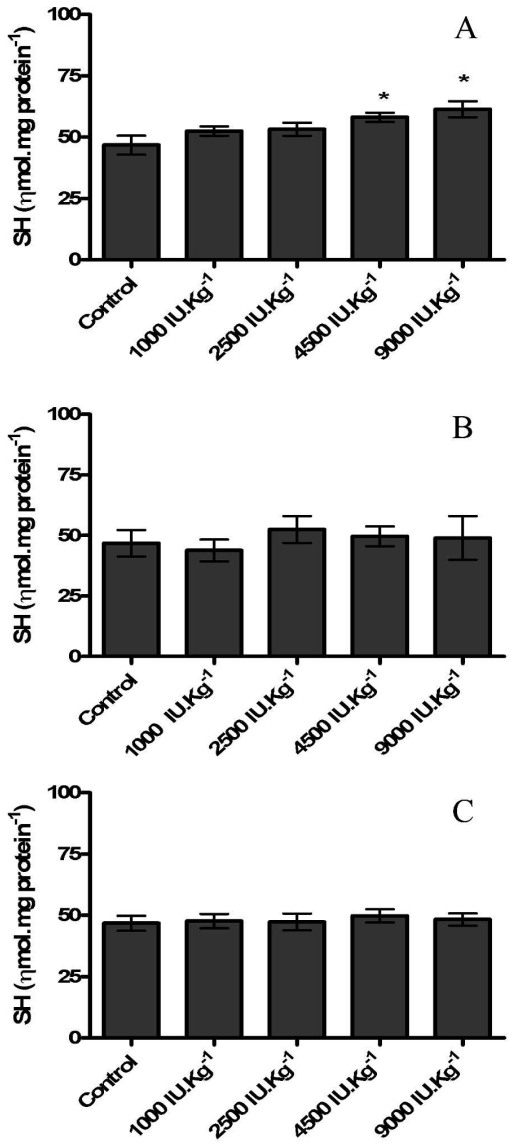

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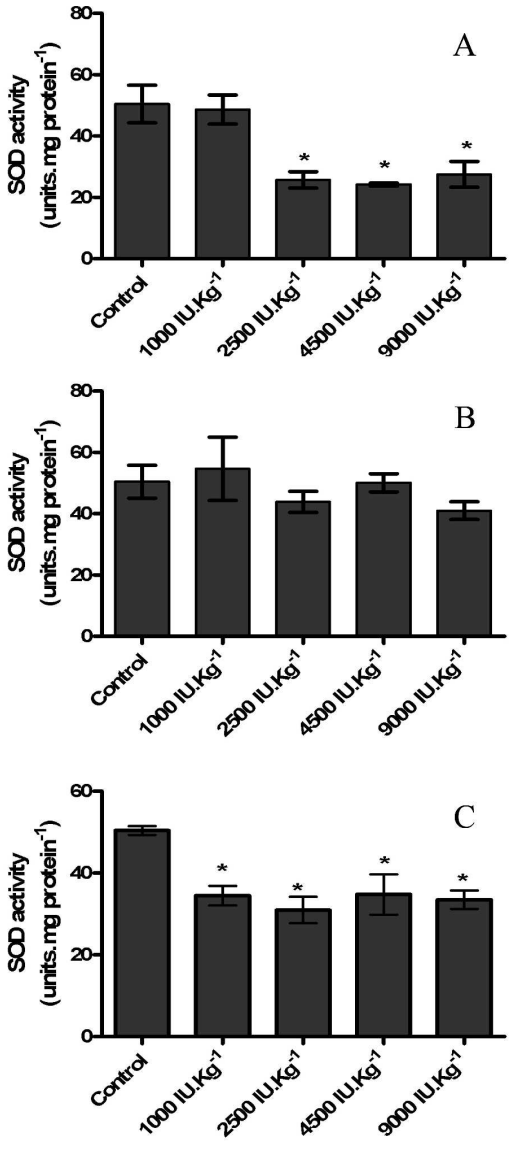

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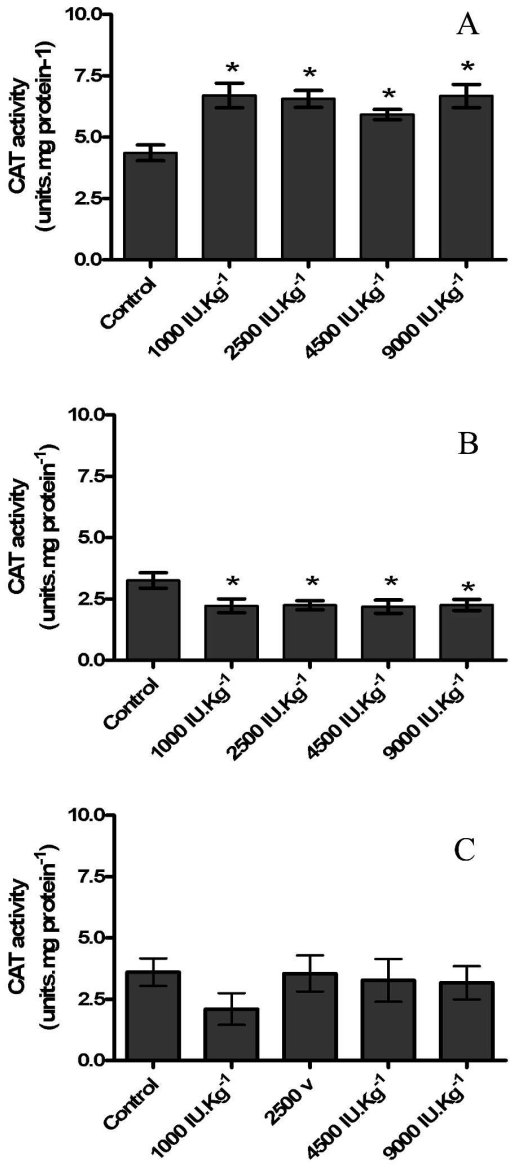

Figure 4: CAT activity. A. 3 days period (η = 5 for each group), B. 7 days period (η = 5 for each group) and C. 28 days period (η = 7 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's pos hoc, and the accepted significance level was $p \leq 0.05$.
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CAPÍTULO II

Short-term vitamin A supplementation at therapeutic doses induces oxidative damage on rat heart

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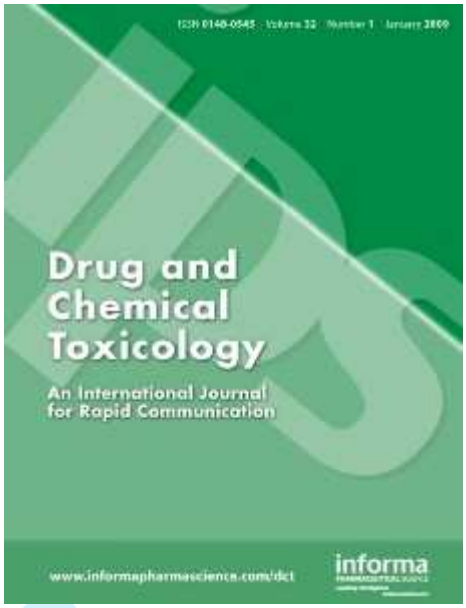

Short-term vitamin A supplementation at therapeutic doses induces oxidative damage on rat heart

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Keywords:	Antioxidants, Cardiovascular, Retinol

Short-term vitamin A supplementation at therapeutic doses induces oxidative damage on rat heart

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Keywords: Antioxidants. Cardiovascular. Damage. Oxidative Stress. Retinol.

ABSTRACT

Vitamin A plays several important roles on cardiovascular system, by regulating cell differentiation and proliferation. Antioxidant properties are also attributed to this vitamin. Thereby we aimed to compare heart oxidative stress parameters among rats supplemented or not with vitamin A. Male adult rats were treated for acute period (3 or 7 days) with different doses (1000, 2500, 4500 and 9000 $IU.Kg^{-1}.day^{-1}$) of retinyl palmitate, and further the heart was removed for analysis. As oxidative stress parameters were evaluated thiobarbituric acid reactive species (TBARS) levels, carbonyl content, sulfhydryl content, total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR) capacities, and the activities of superoxide dismutase (SOD) and catalase (CAT). Statistics were conducted by Anova one-way followed by Dunnet's *pos hoc*, and significance level was $\rho \leq 0.05$. About TBARS and carbonyl no differences were observed after 3 days, but in the highest dose there was an increase after 7 days. No modifications were found in sulfhydryl and TRAP, while a decrease was seen in TAR after 3 (all doses) and 7 (two higher doses) days. In regard of enzymatic activities, we could observe an increase in SOD after 3 (lowest dose) and 7 (highest dose) days, and a decrease in CAT after 3 days (all doses) and no changes after 7 days. Taken together, our results suggest that vitamin A supplementation at therapeutic doses induces redox imbalance and, consequently, oxidative damage. An interesting and important observation is that it happens in a short-term way.

INTRODUCTION

Vitamin A is the given name of retinol and any other molecule that plays retinol activity, and can be found on animal sources, such as liver meat, as retinyl palmitate/acetate form (pro-vitamin A) or on vegetal sources in retinol precursors, the carotenoids (pre-vitamin A). Several physiological functions are attributed to the retinoids, since the fetal until the adult life, mainly for its regulation on cell processes, such as maturation, differentiation and proliferation (Sporn 1994). Additionally, vitamin A presents important roles on cardiac development as well as it likes to have an importance for the heart after myocardial infarction, since there is a mobilization of retinol from resources to the affected organ (Hoover et al. 2008; Palace et al. 1999a; Palace et al. 1999b). In function of the retinoic acid ability in to inhibit some effects of angiotensin II on neonatal rat cardiac myocytes, it was suggested its therapeutic using for the prevention and treatment of cardiac hypertrophy and remodeling (Wang et al. 2002). Besides the related functional actions, antioxidants properties are also attributed to retinol and its precursors (Sporn 1994). Regarding these positive effects, many interventions with vitamin A have been largely used for treatment and prevention of several diseases and conditions, for instance psoriasis, cancer, cystic fibrosis and others (Montrone et al. 2009; O'Neil et al. 2008; Smith et al. 1992; Van Zander and Orlow 2005).

Despite all good effects of vitamin A, negative results have been also related. Actually, hepatic fibrosis, behavioral disturbance and risk for cancer development in smokers were related to high doses vitamin A intake (Lam et al. 2006; Myhre et al. 2003; Omenn et al. 1994). In addition, our research group has contributed to the better understanding of the dual retinoids roles, mainly by describing retinol pro-oxidants properties. By using an *in vitro* model, it was demonstrated that retinol can to compete with xanthyne by the enzyme xanthyne oxydase, so the catalyzed reaction produces superoxide radical (Zanotto-Filho et al.

2008). This mechanism is suggested as one responsible for superoxide radical production at cytosol level. A large literature has been produced with *ex vivo* models, by which was demonstrated oxidative damage on lipids, proteins and deoxyribonucleic acids (DNA), changes on antioxidants enzymes, as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and mitochondrial impairment induced by retinol treatment on Sertoli cells (Dal-Pizzol et al. 2001; Dal-Pizzol et al. 2000; Gelain et al. 2008; Klamt et al. 2003). In accordance to these cited results, it was reported that vitamin A generates DNA damage through increased superoxide production (Murata and Kawanishi 2000). Additionally, the control of cell proliferation/death pathways through nuclear factor kappa B (NFκB) was also observed in response to retinol treatment (Zanotto-Filho et al. 2009). Recently, *in vivo* studies have been also devoted to investigate the redox properties of retinoids, in which oxidative damage and antioxidant enzyme imbalance were found in rats' central nervous system (CNS) submitted to acute and chronic period treatments at pharmacological doses of retinyl palmitate. The reported CNS oxidative stress was related to behavior disturbances of the animals (De Oliveira and Moreira 2008; de Oliveira et al. 2008; de Oliveira et al. 2007).

Therefore, our aim in the present study was to compare heart oxidative stress parameters among rats supplemented with vitamin A and one not supplemented.

METHODS

Animals and materials

Adult male rats (90 days old) were used in this work and were maintained on a 12 hours light-dark cycle with water and food *ad libitum*. All experiments 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 others reagents were purchased from Sigma Chemicals (St. Louis, MO, USA).

Experimental design and drug administration

Two different periods (3 and 7 days) of acute treatment were performed. Initially, the animal were randomly divided for the 2 treatments, and thereafter into the supplementation groups: Control (vehicle: saline solution 0.9 %) and 4 different vitamin A doses (1000, 2500, 4500 and 9000), 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 intra-gastric gavage, in a total 0.8 mL volume always in the dark cycle beginning.

Sample preparation

After the respective treatments the rats were killed by decaptation and the heart was carefully removed and cleaned with iced saline solution to remove blood. The organ was homogenized in phosphate buffer for samples (PBS) pH 7.4, the homogenate was centrifuged at 700 x g to remove debris and the supernatant was used as mother solution. The protein content was measured by Lowry method in order to correct the results (Lowry et al. 1951).

Oxidative stress parameters

It was evaluated the thiobarbituric acid reactive species (TBARS) test as an index of lipids oxidation. The TBARS consists in 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 $protein^{-1}$ (Draper and Hadley 1990). For

protein oxidation analysis, it was measured the carbonyl content, which is based in the reaction of dinitrophenylhydrazine with protein carbonyl groups. The results are expressed as ηmol.mg protein^{-1} (Levine et al. 1990).

The total sulfhydryl (SH) content, present in proteins as well as 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^{-1} (Ellman 1959).

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 and added luminol (system), thereafter we waited for the system stabilization for 2 hours to do the first reading. After the sample to be added we analyze the readings for nearly 30 minutes (Lissi et al. 1992). The results were transformed in percentual and the area under curve (AUC) was calculated by software (*GraphPad Software Inc.®, San Diego, CA, USA –version 5.00*) as described (Dresch et al. 2009). The total antioxidant reactivity (TAR) was also analyzed on plasma and it is based in the same technical principles of TRAP, however TAR is more related to quality of samples antioxidants. The TAR results were calculated as the ratio of light in absence of samples (I^0)/ light intensity right after sample addition (I) (Lissi et al. 1995).

Antioxidant enzymes activity was also assessed. The superoxide dismutase (SOD) catalyzes 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 (Bannister and Calabrese 1987). 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 ηm (Aebi 1984).

Statystical analysis

All data are presented as mean ± standard error of mean (SEM) and it was used Anova one way followed by Dunnet's *pos hoc* to determine the differences among goups 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

As index of biomolecules oxidative damage parameters were evaluated TBARS levels (for lipid oxidation) and carbonyl content (for protein oxidation). These cited measurements played similar results, since anyone was modified after 3 days treatment period but both had an increase in the highest dose after 7 days treatment period (figure 1 and 2).

The SH groups have important role for controlling redox status in intracellular environment (by glutathione molecule) and plasma (mainly by albumin protein). Thereby the oxidation status of this molecule has been largely used as a redox status marker. However, no modifications were found in SH for any treatment period (figure 3).

For measure non-enzymatic capacity, the TRAP and TAR assays have been used. The TRAP is more related to the antioxidants amount in the samples, while the TAR is more related to the quality (reactivity) of sample antioxidants. No changes were observed for TRAP in any treatment period, but a decrease was seen in TAR after 3 (all doses) and 7 (two higher doses) days treatment periods (figure 4).

Regarding enzymatic activities, surprisingly only in the lowest dose was seen an increase in SOD activity after 3 days treatment period, while after 7 days treatment period the increase was observed at the highest dose. All doses played a decreased CAT activity after 3 days treatment period, but no modifications were seen after 7 days treatment period (figure 5).

DISCUSSION

In the present study we evaluated the effects of short-term vitamin A supplementation on oxidative stress parameters in the heart, since retinol using as a therapeutic molecule has been suggested to treat cardiovascular diseases, but negative effects are also attributed to this molecule and its derivates.

We could observe here that vitamin A supplementation even in a short-term period can yield an imbalance on redox system generating oxidative damage in macromolecules as lipids and proteins, what was demonstrated by the increase in TBARS levels and carbonyl content, respectively, after 7 days treatment period, but not after 3 days treatment period with 9000 $IU.Kg^{-1}.day^{-1}$. Our results are different from those previously published by us, which increased levels of TBARS were found on liver of rats submitted to retinyl palmitate supplementation after 3 days treatment period, but no changes after 7 days treatment period. However this same study presented similar results in carbonyl content, since an increase was demonstrated after both treatment periods (de Oliveira et al. 2009). Additionally, we demonstrated increase in TBARS levels and carbonyl content on rat hippocampus and *substantia nigra* after both acute treatment periods (de Oliveira et al. 2008; de Oliveira et al. 2007).

About SH status, we could not demonstrate any changes for any treatment period, what is different from our prior work with liver, which played diminished protein SH after 3

days treatment period and diminished non-protein SH after 7 days treatment period. It is important note that SH, from GSH, is considered the main redox buffer of the cell, but it is true only for cytosolic fraction and not for nucleous or mitochondria (Meyer and Hell 2005). Indeed, we demonstrated oxidative damage, imbalance redox, respiratory chain activity impairment and increased superoxide radical production on isolated liver mitochondria (de Oliveira et al. 2009).

Herein study we observed diminished antioxidant non-enzymatic reactivity, but no modifications in the amount of the antioxidant for both treatments, evidenced by decrease in TAR and unchanged TRAP. However it is difficult to know what exactly substance is being affected, because we could think on glutathione at a first time, but no changes were found in SH content. Previously, we found increase in TRAP of liver after 7 days treatment period (de Oliveira et al. 2009).

Surprinsingly, only for the lowest vitamin A dose was detected an increased SOD activity after 3 days treatment period, while after 7 days treatment period the higher dose was able to generate an increase. In our previous work all doses of vitamin A yielded increased SOD activity after 7 days treatment period, and only the highest dose increased the SOD activity after 3 days treatment period (de Oliveira et al. 2009). All doses of retinyl palmitate were able to diminish the CAT activity in the present work after 3 days treatment period, but its activity is recovered after 7 days treatment period. This result for CAT activity after 7 days treatment period is similar to our prior results with liver, but the modification after 3 days treatment period are very different to those results found for the liver, which CAT activity increased at the highest dose (de Oliveira et al. 2009). Before, we had demonstrated increased SOD and CAT activities after 3 and 7 days treatment period on rat hippocampus (de Oliveira et al. 2007).

Moreover, we had previously demonstrated that these same pharmacological doses of vitamin A were able to yield anxiety behavior and decrease the locomotion and exploration of rats (de Oliveira et al. 2008; de Oliveira et al. 2007).

Taken together our results suggest that short-term vitamin A supplementation is enough to generate oxidative damage on the heart, and it happens by using therapeutic doses. However the specific mechanisms by what retinol act is object of a future work, since we could not observe any modification on SH status, which taken together to previous works lead us to believe in a mechanism involving mitochondrial dysfunction. Furthermore, it was already demonstrated that retinol can inhibit the activity of respiratory chain complexes and generate superoxide radical, by using *ex vivo* and *in vivo* models (De Oliveira and Moreira 2008; de Oliveira et al. 2009; Klamt et al. 2003). Another interesting future goal is the relation of vitamin A to other non-enzimatic antioxidants, since the supplementation decreased the TAR, but it not likes be by modifications on GSH. The influence of retinol on SOD and CAT activity has being investigated, but better explanations about how it happens at myocardial level are needed yet (Dal-Pizzol et al. 2001; Gelain et al. 2008; Pasquali et al. 2009; Pasquali et al. 2008).

Therefore, in function of pro-oxidants and possible toxic effects of vitamin A, we suggest more attention when choice this molecule as an agent of prevention and treatment of diseases.

ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

Figure 1: TBARS levels. A. 3 days treatment period (η = 5 for each group) and B. 7 days treatment period (η = 6 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's *pos hoc*, and the accepted significance level was $p \leq 0.05$.

Figure 2: Carbonyl content. A. 3 days treatment period (η = 5 for each group) and B. 7 days treatment period (η = 6 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's *pos hoc*, and the accepted significance level was $p \leq 0.05$.

Figure 3: SH content. A. 3 days treatment period (η = 5 for each group) and B. 7 days treatment period (η = 6 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's *pos hoc*, and the accepted significance level was $p \leq 0.05$.

Figure 4: Non-enzymatic capacity. A. TRAP of 3 days treatment period (η = 5 for each group), B. TRAP of 7 days treatment period (η = 5 for each group), C. TAR of 3 days treatment period (η = 6 for each group) and D. TAR of 7 days treatment period (η = 6 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's *pos hoc*, and the accepted significance level was $p \leq 0.05$.

Figure 5: Enzymatic activities. A. SOD of 3 days treatment period (η = 5 for each group), B. SOD of 7 days treatment period (η = 5 for each group), C. CAT of 3 days treatment period (η = 6 for each group) and D. CAT of 7 days treatment period (η = 6 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were

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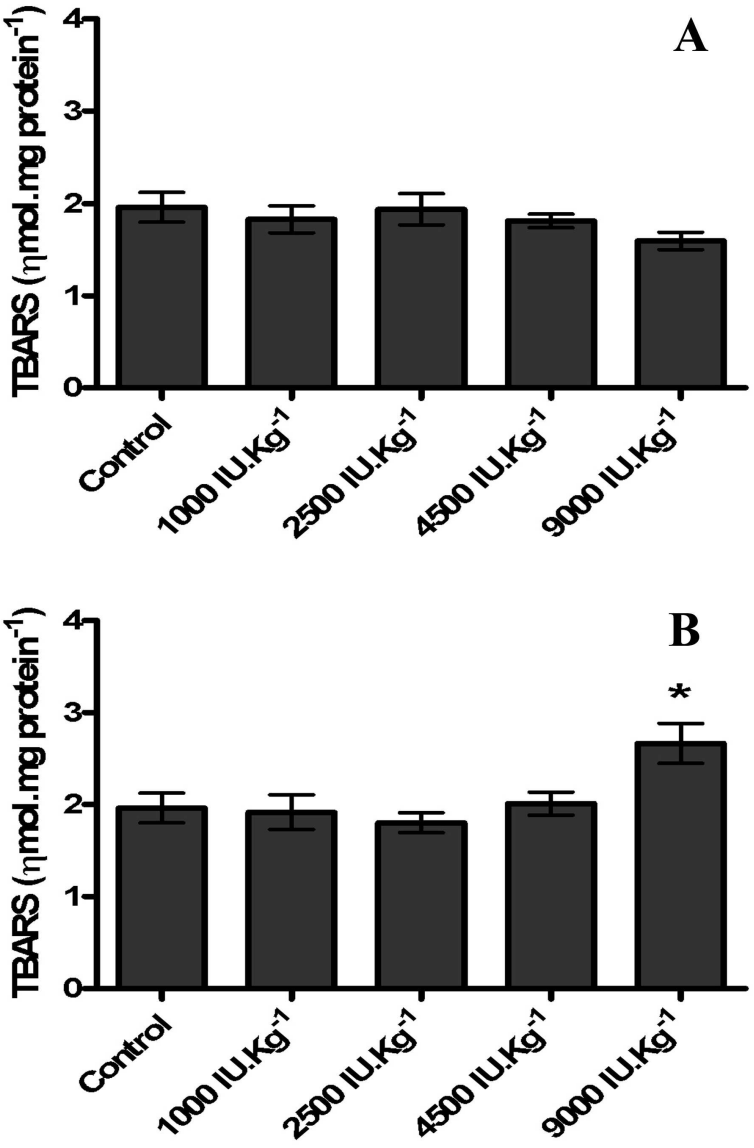

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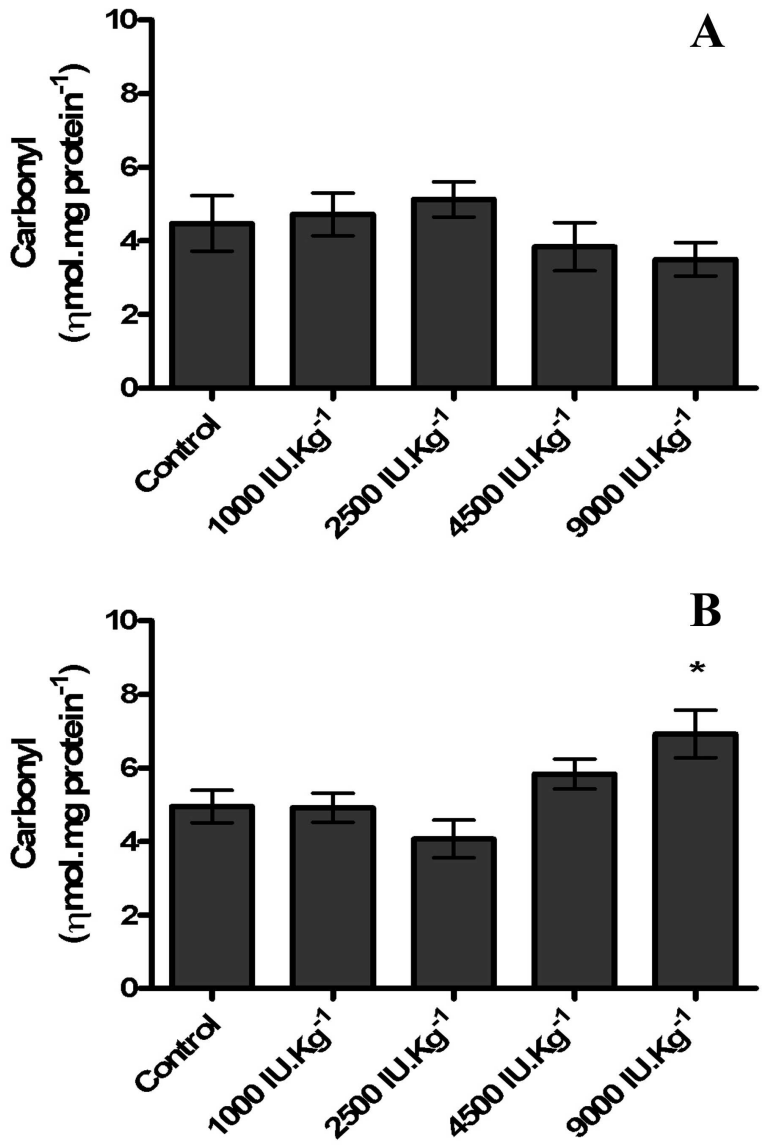

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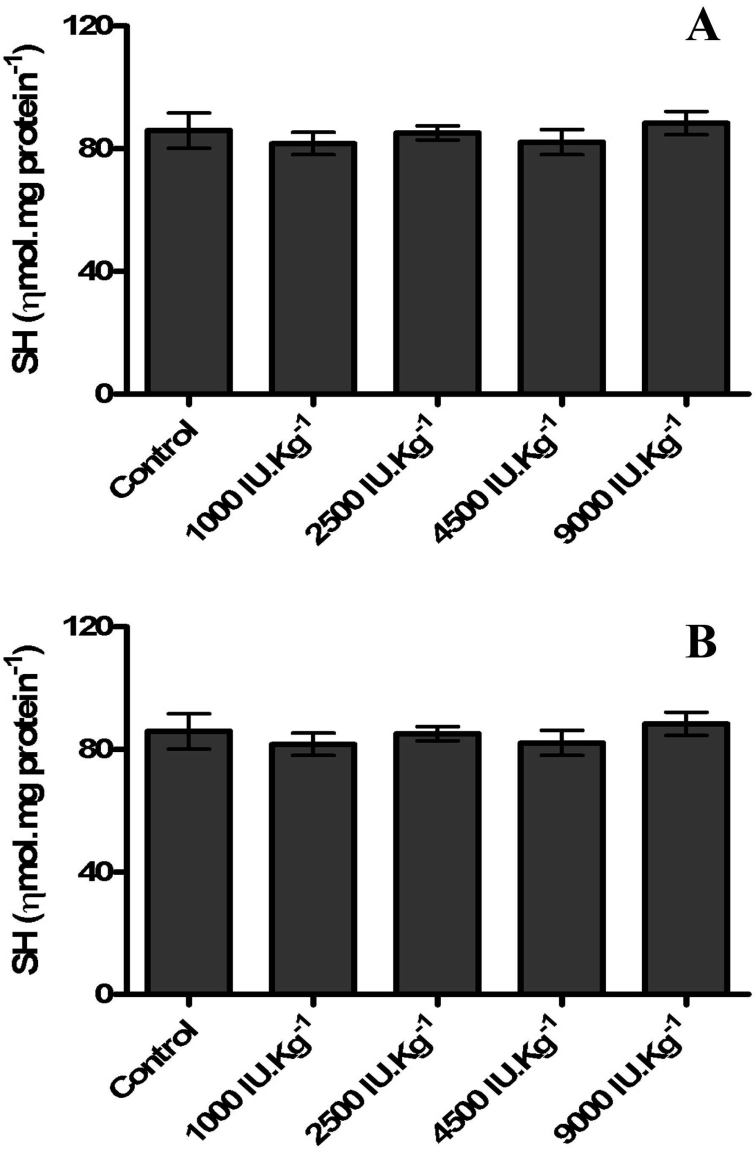

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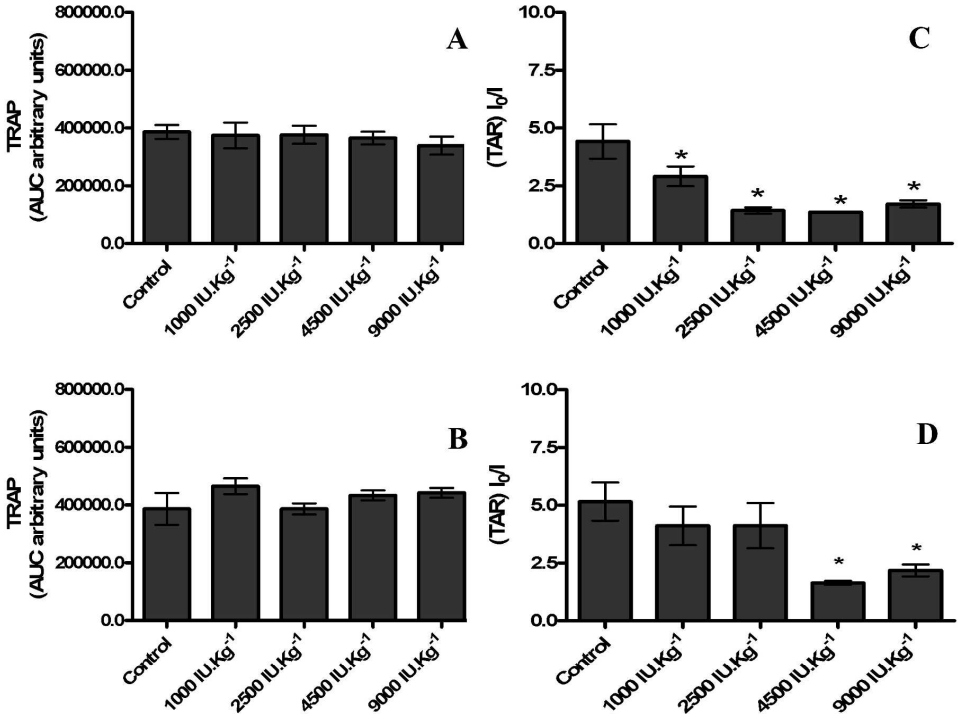

Figure 4: Non-enzymatic capacity. A. TRAP of 3 days treatment period (η = 5 for each group), B. TRAP of 7 days treatment period (η = 5 for each group), C. TAR of 3 days treatment period (η = 6 for each group) and D. TAR of 7 days treatment period (η = 6 for each group). Data are expressed as mean ± standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's pos hoc, and the accepted significance level was $p \leq 0.05$.
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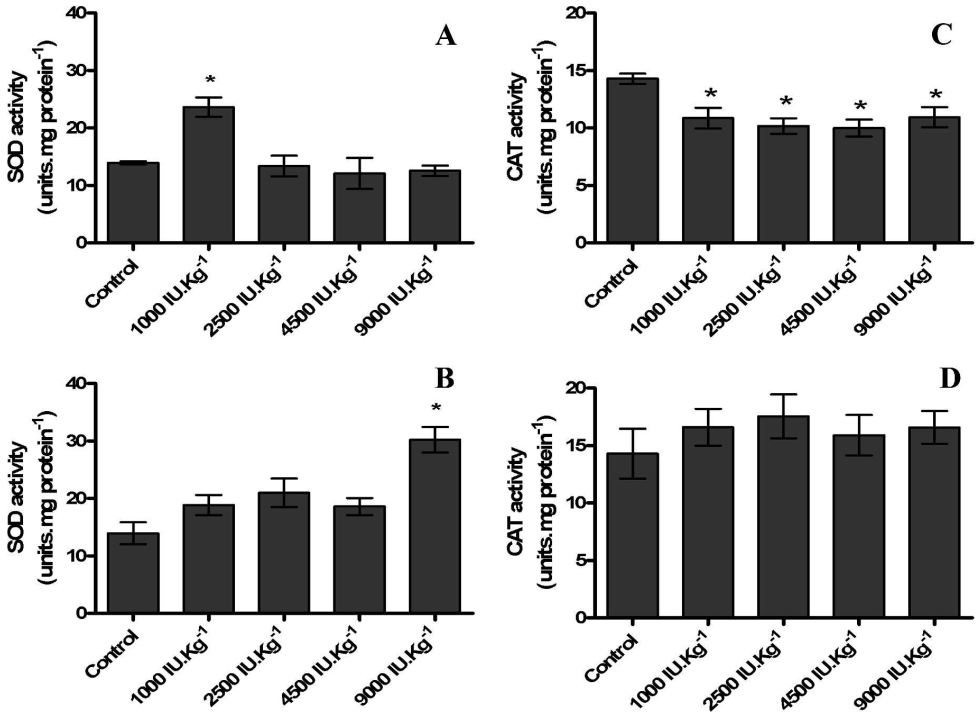

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CAPÍTULO III

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

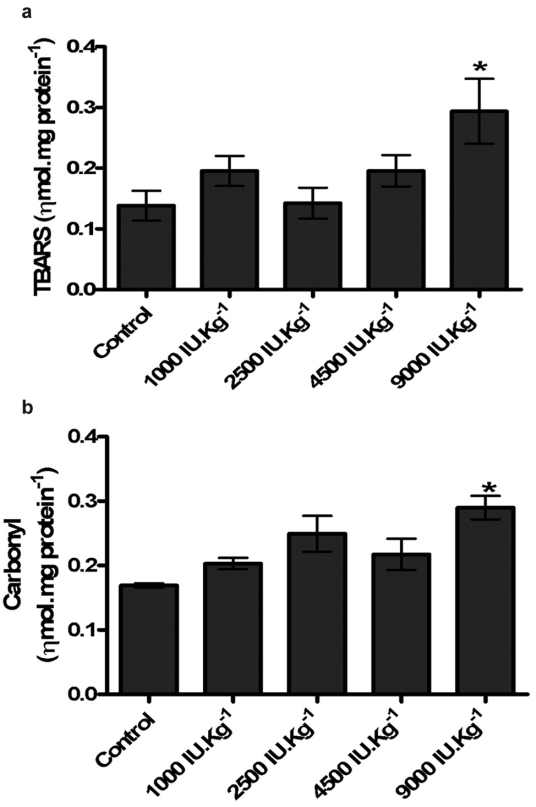

Figure 1. (A) TBARS levels ($n=7$ for each group) and (B) Carbonyl content ($n=7$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

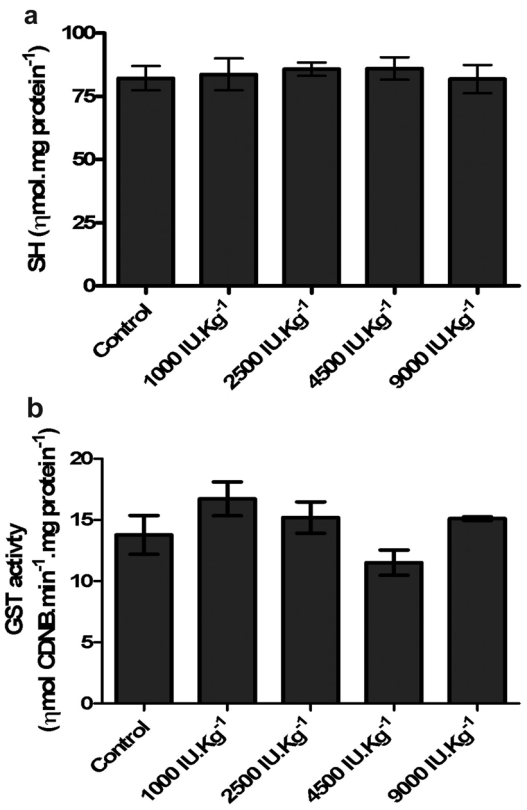

Figure 2. (A) SH content ($n=7$ for each group) and (B) GST activity ($n=5$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

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,

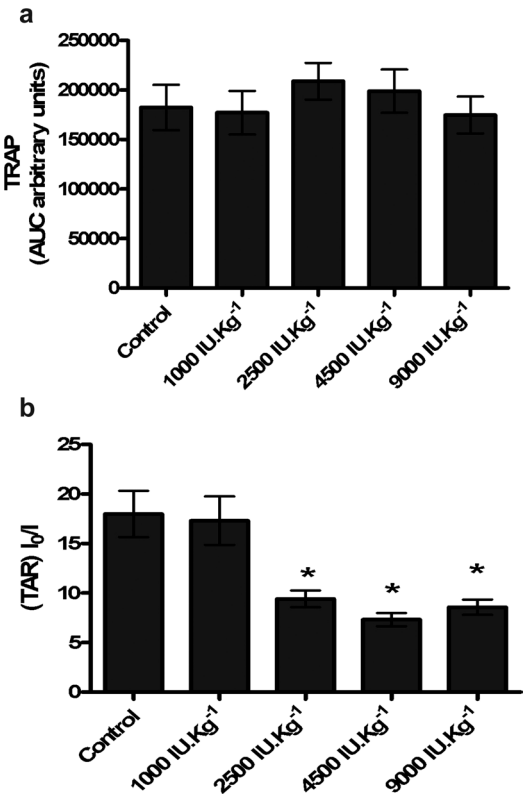

Figure 3. (A) TRAP ($n=7$ for each group) and (B) TAR ($n=7$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

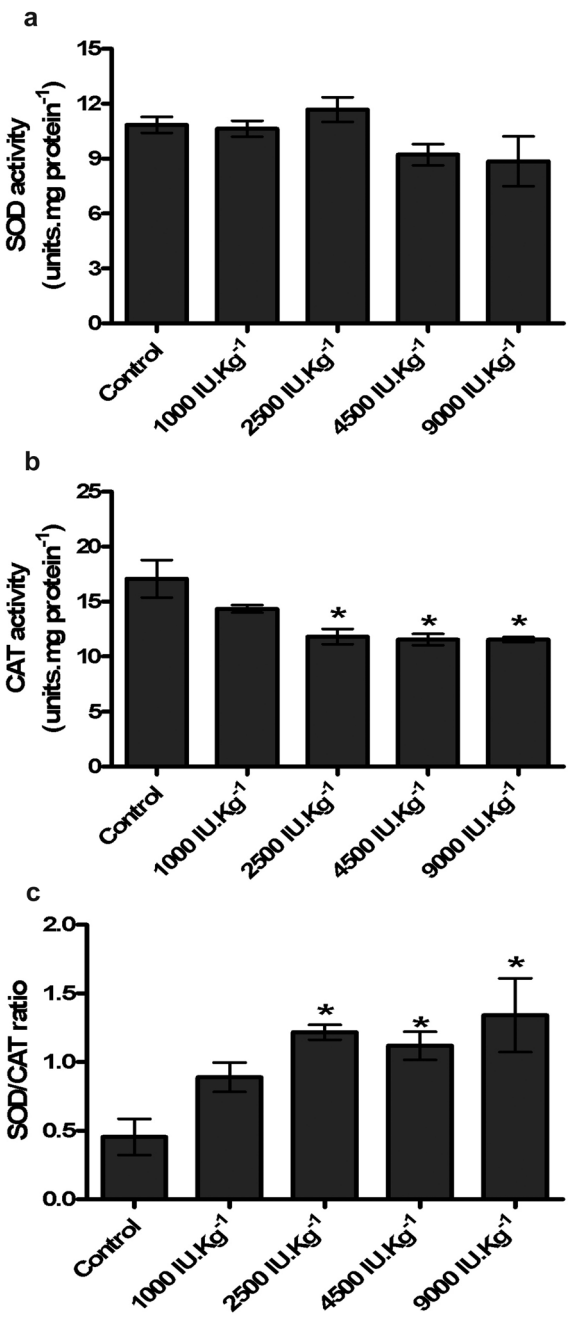

Figure 4. (A) SOD activity ($n=7$ for each group), (B) CAT activity ($n=7$ for each group) and (C) SOD/CAT ratio ($n=7$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted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

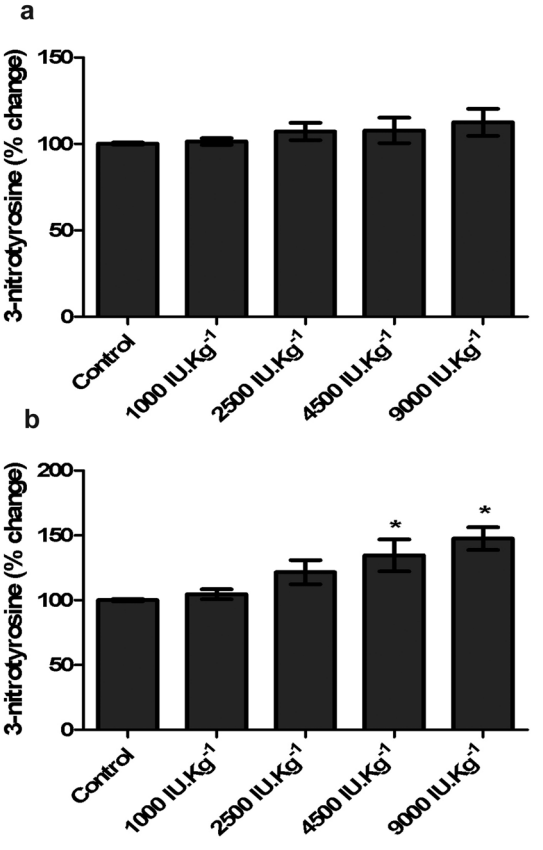

Figure 5. (A) Total 3-nitrotyrosine ($n=5$ for each group) and (B) MMEF nitrotyrosine ($n=5$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was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

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].

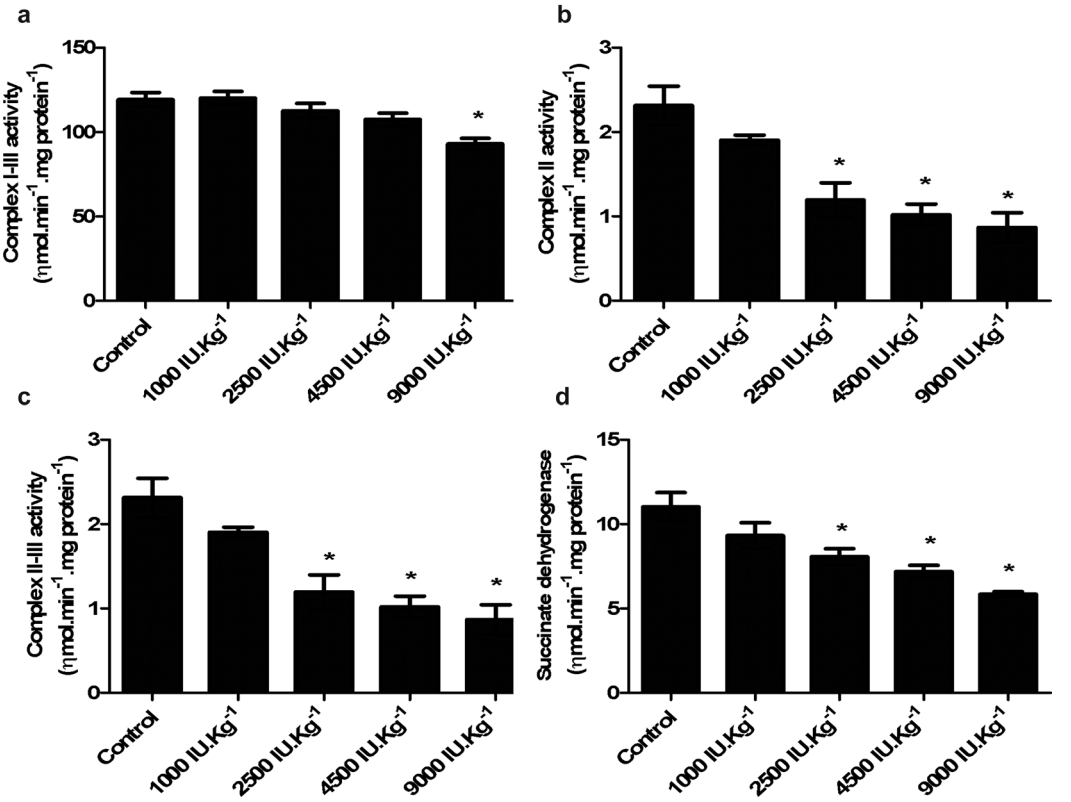

Figure 6. (A) Complex I–III activity ($n=5$ for each group), (B) Complex II–III activity ($n=5$ for each group), (C) Complex II activity ($n=5$ for each group) and (D) SDH activity ($n=5$ for each group). Data are expressed as mean ± standard error of mean.*Different to control. Differences were determined 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 prooxidants 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 ·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 ·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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PARTE III

DISCUSSÃO

O presente trabalho foi conduzido no sentido de melhor esclarecer os efeitos adversos do consumo da vitamina A, já que muitas intervenções utilizam essa vitamina para prevenção e tratamento de uma série de doenças e condições especiais (inclusive a forte sugestão de seu uso para fim de tratamento de doenças cardiovasculares) e ao mesmo tempo efeitos negativos são relacionados a mesma, incluindo as propriedades pró-oxidantes. De fato, este trabalho consegue demonstrar em um mesmo sistema (cardiovascular) efeitos opostos do retinol, já que um efeito antioxidante foi visto no sistema vascular e um efeito pró-oxidante no coração.

Os resultados encontrados a artéria aorta são surpreendentes, de certa forma, já que eles contrariam resultados prévios de nosso grupo que demonstram propriedades pró-oxidantes em resposta ao tratamento *in vivo* com palmitato de retinol (de Oliveira et al. 2009a; De Oliveira et al. 2009b; de Oliveira et al. 2009c; de Oliveira et al. 2009d). No entanto eles vão ao encontro de outros trabalhos da literatura com propósitos similares (Gatica et al. 2005). Foi possível observar no presente estudo uma resposta interessante em termos de estresse oxidativo, já que foi detectada uma diminuição da lipoperoxidação após 7 e 28 dias e nenhuma diferença no estado de oxidação dos grupamentos sulfidril nesses mesmo períodos. No entanto, houve um aumento de sulfidril em 3 dias, justamente onde não houve mudanças em termos de lipoperoxidação. Obviamente isso nos leva a propor uma seqüência no processo de adaptação, onde em um primeiro momento há um aumento na defesa, e em um segundo momento isso resulta numa maior proteção, já que o dano está diminuído. Mas é claro que essas suposições ficam ainda no campo das especulações, pois novos estudos devem ser realizados para melhor compreender qual seria essa "lógica" de adaptação. Esses resultados acabam representando um desafio ainda maior quando nos deparamos com nossos resultados prévios, onde encontramos aumento de TBARS no fígado e no hipotálamo de animais

tratados de forma aguda e crônica com palmitato de retinol (De Oliveira et al. 2009b; de Oliveira et al. 2009c; de Oliveira et al. 2009d). Em relação à oxidação dos grupos SH, os resultados do presente trabalho vão, em parte, ao encontro dos nossos trabalhos anteriores, já que não foram observadas modificações em nível hepático após 7 e 28 dias de tratamento (De Oliveira et al. 2009b; de Oliveira et al. 2009c; de Oliveira et al. 2009d). Quando comparado com outro estudo de propósito similar, que usou como modelo uma dieta deficiente em vitamina A ao longo de 3 meses, nossos resultados ganham um grande suporte, já que níveis aumentados de TBARS foram encontrados nos animais com dieta deficiente tanto na aorta quanto no soro (Gatica et al. 2005).

Considerando a atividade enzimática, nossos resultados apontam para uma lógica de efeito antioxidante, já que a diminuição da SOD, em 3 e 28 dias, são coerentes com um estado antioxidante, em que há uma menor formação de ROS. O mesmo se pode concluir em relação à CAT de 7 dias. O único resultado conflitante parece ser o da CAT de 3 dias, no entanto agudamente ela pode estar respondendo a uma ação genômica do retinol, que poderia induzir um aumento na quantidade (expressão) dessa enzima, pois o retinol está envolvido com modulação da ativação de fatores de transcrição como NrF2 (fator nuclear 2) e NFκB (fator nuclear kappa B) (Zanotto-Filho et al. 2009).

Quando analisamos os resultados de coração obtidos no presente estudo, podemos ver um perfil bem diferente dos resultados encontrados na artéria, porém altamente concordantes com outros trabalhos do grupo, já que aqui um perfil pró-oxidante é observado. Realmente, é possível observar um aumento tanto de lipoperoxidação como de oxidação de proteínas tanto em 7 quanto em 28 dias, o que vai ao encontro de nosso estudos anteriores em que resultados similares são vistos em fígado e estruturas do CNS (De Oliveira et al. 2009b; de Oliveira et al. 2009c; de Oliveira et al. 2008; de Oliveira et al. 2007; de Oliveira et al. 2009d). Além disso, desde 3 dias já é possível observar uma diminuição na reatividade antioxidante não-

enzimática, e isso permanece nos períodos mais longos. Em um primeiro momento, poderíamos imaginar que o tratamento estivesse influenciando no metabolismo da glutationa, principal tampão redox citosólico, entretanto nenhuma modificação no estado de oxidação dos grupamentos SH foi observada. Outros trabalhos precisam ser feitos para melhor entendermos a ação da vitamina A sobre a capacidade antioxidante não-enzimática da célula.

Interessantemente, no período crônico ocorre um desbalanço das enzimas antioxidantes, resultante de uma diminuição da atividade da CAT frente a manutenção da atividade da SOD. Esse aumento na razão SOD/CAT sugere um aumento de peróxido de hidrogênio, pois não há atividade de CAT suficiente para remover esse peróxido gerado pela dismutação do superóxido (catalisada pela SOD). Esse peróxido poderia então, em presença de metais de transição, gerar o radical hidroxila, o mais potente dos radicais e agente gerador de oxidação de lipídios e proteínas (Gutteridge 2001). Portanto, entendemos que esse desbalanço enzimático está associado com os danos observados em TBARS e carbonil. A ação do retinol sobre a atividade e o imunoconteúdo de enzimas com ação primária como antioxidante é alvo de interesse do nosso grupo, que utilizando modelos *ex vivo* e *in vivo* tem contribuído para o entendimento desse assunto, e de acordo com esses trabalhos parece que as adaptações nos sitemas enzimáticos variam de um tecido para o outro, já que em células de Sertoli há um aumento na atividade CAT (associado com aumento de imunoconteúdo), enquanto em pulmão de animais tratados com palmitato de retinol ocorre uma dimunição na atividade (também associada com diminuição do imunoconteúdo) (Dal-Pizzol et al. 2001; Gelain et al. 2008; Pasquali et al. 2009; Pasquali et al. 2008).

No momento em que começamos a aprofundar a investigação sobre os possíveis meios pelos quais ocorrem os danos no período crônico, nos deparamos com um interessante dado de nitração. Analisando os níveis de 3-nitrotirosina no tecido total não detectamos alterações, entretanto quando essa análise é feita na fração enriquecida em membrana mitocondrial, um

aumento de nitração é observado. Anteriormente, já havíamos demonstrado que o tratamento com palmitato de retinol pode gerar estresse nitrosativo (de Oliveira et al. 2009a; De Oliveira et al. 2009b). Considerando que o processo de nitração ocorre em resposta a um ataque do peroxinitrito aos resíduos tirosil de proteínas, que essa espécie reativa de nitrogênio é formada pela reação entre óxido nítrico e superóxido, que uma das mais importantes fontes de radical superóxido é a cadeia transferidora de elétrons, e que o aumento em 3-nitrotirosina observado aqui ocorre justamente na fração mitocondrial, optamos então em dosar a atividade dos complexos da cadeia respiratória (Ferrer-Sueta and Radi 2009; Poderoso et al. 1996). Como esperado, as atividades dos complexos analisados estavam diminuídas, sugerindo uma inibição induzida por retinol, o que pode levar ao aumento da produção de radical superóxido. Previamente, já havíamos demonstrado a capacidade do retinol em inibir a atividade dos complexos da cadeia respiratória e em induzir um aumento de radical superóxido via cadeia respiratória (de Oliveira et al. 2009a; de Oliveira et al. 2009c; Klamt et al. 2003).

Analisados em conjunto, os resultados de nitração, cadeia respiratória e danos oxidativos podem nos levar a supor algumas vias possíveis. De fato, duas hipóteses parecem ser as mais lógicas, a primeira pressupõe que o retinol inibiria diretamente a cadeia respiratória e, conseqüentemente, aumentaria a produção de radical superóxido (Klamt et al. 2003; Poderoso et al. 1996). O superóxido é conhecido por ativar a enzima óxido nítrico sintase, o que aumentaria então a síntese de óxido nítrico (Manning et al. 2001; McPherson et al. 2002; Mittal 1993). A segunda hipótese seria que o retinol ativaria diretamente a óxido nítrico sintase, o que aumentaria o radical óxido nítrico. O óxdio nítrico é um conhecido inibidor da cadeia respiratória, e inibindo ela favorece a produção de radical superóxido (Poderoso et al. 1996). Assim como na primeira hipótese, a segunda também leva a um aumento concomitante de superóxido e óxido nítrico. A partir desse ponto, as conseqüências seguiriam então uma via comum, pois tendo superóxido e óxido nítrico aumentados, eles

podem então dar origem ao peroxinitrito, que por sua vez é capaz tanto de gerar nitração, como também de induzir oxidação de biomoléculas, como lipídios (TBARS) e proteínas (carbonil) (Ferrer-Sueta and Radi 2009).

CONCLUSÃO

Portanto, concluímos que o coração e a aorta respondem de forma diferente ao tratamento com vitamina A, onde um efeito antioxidante é visto em nível arterial e um efeito pró-oxidante é observado em nível cardíaco, e que o dano no coração está associado a um estado de estresse nitrosativo mitocondrial, bem como a um bloqueio na cadeia transferidora de elétrons. Sugerimos que além de novos estudos para melhor compreender o impacto da vitamina A sobre o sistema cardiovascular, que se tenha também mais cautela nas propostas de uso dessa vitamina para prevenção e tratamento de doenças, principalmente aquelas do sistema circulatório.

PERSPECTIVAS

Certamente, este trabalho acaba despertando-nos uma grande curiosidade em descrever a fundo nos mecanismos envolvidos nas adaptações observadas. Em um primeiro momento seria importante um tratamento mais longo, com a intenção de verificar, de forma mais clara, possíveis efeitos nas doses mais baixas, já que nos períodos estudados as respostas delas foram mais discretas. No sistema arterial, ainda precisamos compreender um pouco melhor os mecanismos envolvidos com o efeito antioxidante verificado. No coração, dois pontos são intrigantes, um deles diz respeito a diminuição da reatividade não-enzimática, e o outro em relação ao esclarecimento das duas hipóteses levantadas sobre a relação entre cadeia respiratória e estresse nitrosativo. O primeiro passo para esclarecer essas hipóteses, seria o co-tratamento com um inibidor da NOS, bem como estudos *in vitro* e *ex vivo* que melhor esclareçam a parte de mecanismos da interação do retinol com a cadeia respiratória e a NOS. Além disso, análises fisiológicas do sistema cardiovascular (como a capacidade relaxamento da aorta) seriam fundamentais, pois os parâmetros aqui avaliados são caracterizados como bioquímicos, e para sugerirmos se eles são positivos ou negativos precisamos saber o quanto essas modificações influenciam na funcionalidade dos órgãos estudados. Pois apesar de mostrarmos efeitos bioquímicos opostos entre aorta e coração, não sabemos ainda se o impacto funcional também é oposto. Aliás, estudos anteriores de nosso grupo se preocuparam em aliar uma resposta bioquímica a uma resposta funcional, onde foi demonstrado que o estado de estresse oxidativo no CNS, induzido pelo tratamento com palmitato de retinol, estava associado com distúrbios comportamentais (de Oliveira et al. 2008; de Oliveira et al. 2007).

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ANEXOS

- Parecer favorável à aprovação do projeto, fornecido pelo Programa de Pós-Graduação em Ciências Biológicas: Bioquímica (UFRGS);
- Carta de recebimento da submissão do manuscrito ***Vitamin A supplementation for different periods alters rat vascular redox parameters***, por parte do periódico *Cell Biochemistry and Function, ISI JCR 2008: 1.333*;
- Carta de recebimento da submissão do manuscrito ***Short-term vitamin A supplementation at therapeutic doses induces oxidative damage on rat heart***, por parte do periódico *Drug na Chemical Toxicology, ISI JCR 2008: 1.409*;
- Carta de aceite do manuscrito ***Long-term vitamin A supplementation at therapeutic doses induces mitchondrial electrons transfer chain (METC) impairment and increased mitochondrial membrane-enriched fraction (MMEF)3-nitrotyrosine on rat heart***, por parte do periódico *Free Radical Research, ISI JCR 2008: 2.826*;
- Certificado da palestra intitulada **Papel das Espécies Reativas de Nitrogênio no Sistema Cardiovascular**, ministrada no curso de extensão universitária **Radicais Livres: Verdades, Falácias, Angústias e Expectativas**;
- Carta de aceite do manuscrito ***Vascular imbalance redox in rats submitted to chronic exercise***, aceito para publicação no periódico *Cell Biochemistry and Function, ISI JCR 2008: 1.333*. Estudo realizado como Trabalho de Conclusão de Curso (TCC) em Licenciatura em Educação Física – UFRGS, e aceito para publicação durante o período de realização do curso de mestrado;

PPG-Ciências Biológicas Bioquimica	UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PARECER CIRCUNSTANCIADO

O projeto de pesquisa intitulado "**Efeito do tratamento com palmitato de retinol sobre parâmetros de estresse oxidativo na artéria aorta e no coração de ratos Wistar**", do aluno do PPG-Ciências Biológicas-Bioquímica **Ricardo Fagundes da Rocha** sob orientação do PROF. Dr(a). José Cláudio Fonseca Moreira, matriculado ao PPG Ciências Biológicas – Boquímica desta Universidade, apresentado para apreciação da Comissão de Pós-Graduação dessa PPG, apresenta:

- Um tema relevante e com mérito científico uma vez que propõe **avaliar o efeito de diferentes doses de vitamina A sobre parâmetros oxidativos e fisiológicos no sistema cardiovascular de ratos adultos.**

- Uma introdução fundamentada e com objetivos adequados ao estudo proposto. A metodologia é coerente para que os objetivos sejam alcançados, havendo até este momento uma infra-estrutura adequada nos **Departamentos de Bioquímica da UFRGS** para sua realização.

- Um cronograma de execução que prevê o período de 24 **meses** para a realização do projeto.

O projeto atende a exigência de detalhamento do número da amostra.

Sendo assim, somos de parecer favorável à aprovação do presente projeto.

Parecer aprovado em 22/09/2008

24-Dec-2009

Dear Mr. Rocha,

Your manuscript entitled "Vitamin A supplementation for different periods alters rat vascular redox parameters" has been successfully submitted online and is presently being given full consideration for publication in Cell Biochemistry & Function.

Your manuscript number is CBF-09-0273. Please mention this number in all future correspondence regarding this submission.

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24-Dec-2009

Dear Mr Rocha:

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Drug and Chemical Toxicology Editorial Office

17-Jan-2010

Dear Mr. Rocha:

Your manuscript entitled "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" has been accepted for publication in Free Radical Research. The comments of the reviewer(s) who reviewed your manuscript are included below.

Thank you for your fine contribution. On behalf of the Editors of Free Radical Research, we look forward to your continued contributions to the Journal.

Sincerely,
Prof. Shinya Toyokuni
Editor, Free Radical Research
toyokuni@med.nagoya-u.ac.jp, shinya.toyokuni@gmail.com

Reviewer(s)' Comments to Author:
Reviewer: 1
Comments to the Author
The authors' responses are satisfactory and acceptable. Some parts were unfortunately modified, but there were some specific reasons. The content is worthy of publication in Free Radical Research.

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CERTIFICADO

Certificamos que **RICARDO FAGUNDES DA ROCHA**

Participou da ação de extensão universitária - modalidade CURSO PRESENCIAL,

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Porto Alegre, 19 de Maio de 2009

Sandra de Deus

SANDRA DE FATIMA BATISTA DE DEUS
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21-Nov-2009

Dear Mr. Rocha,

It is a pleasure to accept your manuscript entitled "Vascular imbalance redox in rats submitted to chronic exercise" in its current form for publication in Cell Biochemistry & Function. The comments of the referee(s) who reviewed your manuscript are included at the bottom of this letter.

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Sincerely,

Dr. Nigel Loveridge
Cell Biochemistry & Function
nl10003@cam.ac.uk

Referee(s)' Comments to Author:

Reviewing: 1
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Dear author,
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