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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 memoriam*), 1920 - 2008

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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 ($1000-9000 \text{ UI.Kg}^{-1}.\text{dia}^{-1}$) 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 $p \leq 0,05$. Na artéria aorta, foi observada uma diminuição na lipoperoxidação após 7 e 28 dias, bem como aumento de sulfidrila 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 diminuição na reatividade não enzimática nos 3 períodos. Alterações enzimáticas foram vistas nos 3 períodos. No período mais longo, foi observado aumento de nitratação 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 ($1000-9000 \text{ IU.Kg}^{-1}.\text{day}^{-1}$) 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 $p \leq 0.05$. 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-enzymatic 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 glutaciona (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

(H₂O₂), pouco reativo e muito solúvel, mas que pode dar origem ao mais potente dos radicais livres, o radical hidroxila ($\cdot\text{OH}$) (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 ($\cdot\text{O}_2^-$), por uma redução monovalente do oxigênio (O₂). 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 (H₂O), 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 glutathiona peroxidase (GPx), enzima que necessita da molécula de glutathiona (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 glutathiona, 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 glutatona 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 glutatona. 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 (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 aterosclerose, o retinol atua 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 glutathione 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

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Cell Biochemistry and Function

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CAPÍTULO II

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



Vitamin A supplementation for different periods alters rat vascular redox parameters

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Manuscript ID:	CBF-09-0273
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Date Submitted by the Author:	23-Dec-2009
Complete List of Authors:	Rocha, Ricardo; Universidade Federal do rio Grande do Sul, Bioquímica De Oliveira, Marcos Roberto; Universidade Federal do Rio Grande do Sul, Bioquímica Schonhofen, Patrícia; Universidade Federal do Rio Grande do Sul, Bioquímica Debastiani, Marco Antônio; Universidade Federal do Rio Grande do Sul, Bioquímica Schnorr, Carlos Eduardo; Universidade Federal do Rio Grande do Sul, Bioquímica Pasquali, Matheus Augusto; Universidade Federal do Rio Grande do Sul, Bioquímica Dal-Pizzol, Felipe; Universidade do Extremo-Sul Catarinense, Medicina Moreira, José Cláudio; Universidade Federal do Rio Grande do Sul, Bioquímica
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 $p \leq 0.05$. About TBARS, we observed no modifications after 3 days, but a decrease after 7 days in all doses and after 28 days in three higher doses. The two higher doses yielded an increase on SH only after 3 days. SOD activity decreased in three higher doses after 3 days and

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	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.



For Peer Review

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 $p \leq 0.05$. About TBARS, we observed no modifications after 3 days, but a decrease after 7 days in all doses and after 28 days in three higher doses. The two higher doses yielded an increase on SH only after 3 days. SOD activity decreased in three higher doses after 3 days and in all doses after 28 days, but no modifications after 7 days, while CAT activity increased in all doses after 3 days, decreased in all doses after 7 days, and did not change after 28 days. In conclusion, vitamin A induces antioxidant status on vascular level.

Keywords:

Antioxidants. Aorta artery. Oxidative Stress. Reactive oxygen species. Retinol.

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

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3 the cytosolic mechanism responsible for vitamin A pro-oxidant effects¹¹. Additionally, we
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5 previously described *ex vivo* pro-oxidant properties of retinol, as result of a little modification
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7 on its concentration, from 5 to 7 μM , on Sertoli cells culture. Our studies were able to
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9 demonstrate increased lipids, proteins and deoxyribonucleic acid (DNA) oxidation, as well as
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11 antioxidant enzymatic impairment and mitochondrial dysfunction¹²⁻¹⁷. Furthermore, *in vivo*
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13 models have been developed, and redox imbalance was found in response to vitamin A
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15 supplementation at therapeutical doses. Increased oxidative stress status was observed in
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17 cerebellum, hippocampus and *substantia nigra*, associated with anxiety behavior and
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19 decreased exploratory and locomotory activity¹⁸⁻²⁰.
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25 Therefore, putting the physiological importance of vitamin A in vascular level, its
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27 using for many treatments and its known antioxidant properties against its toxic and pro-
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29 oxidant effects, we aimed in the present study compare vascular redox parameters among
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31 animals supplemented or not with vitamin A.
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34 35 36 **MATERIALS AND METHODS** 37

38 39 40 **Animals and materials** 41

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43 Adult male rats (90 days old) were used in this work and were maintained on a 12
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45 hours light-dark cycle with water and food *ad libitum*. All experiments procedures were
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47 performed in accordance with the National Institute of Health Guide for Care and Use of
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49 Laboratory Animals. Retinyl palmitate (Arovit®), water soluble form, was purchased from
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51 Bayer, São Paulo, São Paulo, Brazil. All others reagents were purchased from Sigma
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53 Chemicals (St. Louis, MO, USA).
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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 ($\text{IU.Kg}^{-1}.\text{day}^{-1}$). 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 decapitation and thoracic aorta artery was surgically removed and cleaned with iced saline solution to remove blood. The vessel was homogenized in phosphate buffer for samples (PBS) pH 7.4, the homogenate was centrifuged at $700 \times g$ 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 nm and the results were expressed as $\text{nmol.mg protein}^{-1}$ ²². The total sulfhydryl (SH) content, present in proteins as well as glutathione, was quantified at 412 nm by its reaction with 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB), and the results were expressed as $\text{nmol.mg protein}^{-1}$ ²³.

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3 Antioxidant enzymes activity was also assessed. The superoxide dismutase (SOD)
4 catalyzes superoxide anion radical ($\cdot\text{O}_2^-$) dismutation to generate hydrogen peroxide (H_2O_2),
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7 and its activity measurement is based on the principle that adrenaline undergo auto-oxidation
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10 in $\cdot\text{O}_2^-$ presence, so at 480 nm we determine the SOD activity by the exogenous adrenaline
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12 auto-oxidation inhibition in sample presence ²⁴. The catalase (CAT) catalyzes the H_2O_2
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14 conversion to water (H_2O), and to determine its activity we added H_2O_2 and analyzed the
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16 capacity of sample to decrease the H_2O_2 amount at 240 nm ²⁵.
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20 21 22 Statistical analysis

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24 All data are presented as mean \pm standard error of mean (SEM) and it was used Anova
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26 one way followed by Dunnet's *pos hoc* to determine the differences among groups and the
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28 significance level considered was $p \leq 0.05$. The statistical analysis and the graphs making
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30 were conducted with *GraphPad Software Inc.®*, San Diego, CA, USA –version 5.00.
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34 35 36 RESULTS

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39 For measure oxidative damage we assessed TBARS, which has been used as an index
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41 of lipoperoxidation, and it was observed in the present work a decrease on this parameter in
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43 all doses after 7 days treatment period and after 28 days treatment period in the three higher
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45 doses, but no modifications after 3 days treatment period (figure 1). Regarding the sulfhydryl
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47 oxidation, which is present in proteins as well as glutathione and has been used as a marker of
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49 redox status, we could observe an increase on its reduced form after 3 days treatment period
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51 in two higher doses. No changes were seen on SH status after 7 and 28 days treatment period
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53 (figure 2).
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3 Additionally, it was evaluated the activities of two important antioxidant enzymes,
4 SOD (responsible for $\cdot\text{O}_2^-$ dismutation) and CAT (responsible for H_2O_2 conversion to water).
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6 After 3 days treatment period, it was observed a decrease on SOD activity in three higher
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8 doses, after 7 days treatment period its activity did not change and after 28 days treatment
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10 period SOD activity decreased in all doses (figure 3), while on CAT activity we detected an
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12 increase after 3 days treatment period in all doses, a decrease after 7 days treatment period in
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14 all doses and no modifications after 28 days treatment period (figure 4).
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22 DISCUSSION

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27 In the present work we have evaluated redox parameters on vascular level in response
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29 to vitamin A supplementation, since important roles are attributed to this vitamin at arterial
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31 level but growing literature has also showing negative effects of this compound using.
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33 Additionally, a few studies have regarded the redox effects of vitamin A at arterial system,
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35 probably due the difficult to hand the aorta and its low tissue yield.
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39 On lipoperoxidation terms we could observe a decrease on TBARS levels in response
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41 to the retinyl palmitate treatment, acutely (7 days) and chronically, what is not in agreement
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43 to previous results of our group for other tissues. In the liver of animals treated with retinyl
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45 palmitate, it was detected an increase on TBARS levels in one treated for acute (3days) and
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47 chronic period^{26,27}. When evaluated in the hipothalamus, TBARS levels are also increased in
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49 a chronic treatment period²⁸. However, it is needed to be regarded that these studies analyzed
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51 different tissues, what can be a strong point for these distinct results, in function of diverse
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53 physiological roles. Indeed, when we compare our data with another study that also evaluated
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55 TBARS in aorta, in response to vitamin A deficiency, our results make more sense. Gatica *et*
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60 *al* performed their study with a vitamin A-deficient diet model in rats for 3 months, and it

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3 resulted in increased TBARS levels at aorta and serum, as well as, serum retinol
4 concentrations in sub-clinical levels²⁹.
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8 In addition to lipoperoxidation, the SH content give us another important idea about
9 redox status, since it is present in proteins and glutathione molecules and, for this reason, is
10 considered the main intracellular redox buffer. Interestingly, SH is increased just after 3 days
11 treatment period, the unique period that TBARS had no changes, and did not play
12 modifications after 7 or 28 days treatment period, the same one in which TBARS is
13 diminished³⁰. The results presented here, in part, are in accordance to previous works, which
14 found no modifications on SH content after 7 or 28 days treatment period, with identical
15 doses to those tested here, but a difference is notated after 3 days, since in the present study
16 was observed an increase while in the priors a decrease was found^{26, 27}. Despite the opposite
17 results after 3 days, a coincidence is played, it is exactly in this period that occur the changes,
18 independently if it is to up or down. This coincidence point could be happening in function of
19 the SH redox buffer role³⁰. Obviously our results must carefully analyzed, however it is
20 possible that it represents a time-related adaptation mechanism, where in a first moment the
21 system responds to the new situation increasing its defenses, for instance SH groups, and as
22 consequence the damage to macromolecules decreases.
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43 For SOD activity, our results are not in agreement to other works, since we observed
44 decreased activity after 3 and 28 days treatment period (no modifications after 7 days) while
45 was reported increased SOD activity in liver (acutely and chronically) and hypothalamus
46 (chronic period)²⁶⁻²⁸. However, one more time we have to consider the organ analyzed and
47 the fact that a very few studies have done oxidative stress parameters analysis in vascular
48 level. Analyzing the study with diminished vitamin A intake, it can be seen lower SOD
49 activity on aorta in response to retinol deficiency²⁹. However, an experimental difference
50 there is here, since in the Gatica's study the cytosolic SOD activity was assessed, while in the
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3 present work we evaluated the total SOD activity. Moreover, the SOD activity observed in
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5 our study is in accordance with TBARS and SH results, because we believe that in this
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7 situation the retinol is acting as an antioxidant molecule, so less radical is offered, and SOD
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9 activity do not need be increased. Our results about CAT activity are in accordance with our
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11 previous works, which an increase on hepatic activity after 3 days treatment period, and no
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13 changes after 28 days treatment period were demonstrated, as well as no modifications
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15 hypothalamus CAT activity after 28 days treatment period were also observed. The only
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17 exception is the CAT activity after 7 days treatment period, which in previous work did not
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19 change in the liver, but increased in aorta in the present work ²⁶⁻²⁸. In response to retinol
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21 deficiency, CAT activity has played a diminished activity ²⁹. The differences on enzymatic
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23 activity results, mainly regarding CAT activity, can be attributed to the fact that in the present
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25 work we did not assess other peroxidases (enzymes group responsible for hydrogen peroxide
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27 removal, like the CAT), such as glutathione peroxidase (GPx), and thereby we do not know
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29 how is the balance of these enzymes. Another important consideration is if the observed
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31 modifications on enzymatic activities are related to changes on their immunocontent or not,
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33 since it has been demonstrated that retinol can interfere on nuclear factor kappa B (NFκB)
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35 activation, and this transcription factor is be related to antioxidant enzymes expression ³¹.
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44 Taken together, the results presented here point to an antioxidant role of vitamin A at
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46 vascular level, what is in accordance to the related study with retinol deficiency model in spite
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48 of not be in agreement with previous studies from our research group for other tissues.
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50 Besides pro-oxidants effects demonstrated by the Gatica's work, in this same article they
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52 found increased nitrite levels associated to increased nitric oxide synthase (NOS) expression
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54 as result of the retinol deficiency ²⁹. However, these results about reactive nitrogen species
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56 need to be complemented to better clarify if it is indicating an improvement on nitric oxide
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58 (NO) availability or is yielding increased nitration by peroxynitrite ³². Furthermore, it was
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3 also demonstrated that vitamin A can interfere on lipid metabolism. Using the same retinol
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5 deficiency model, for 3 months, changes on lipid metabolism were observed, such as
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7 diminished serum triacylglycerol (TAG) cholesterol levels and, in opposite, increased TAG
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9 and cholesterol levels on aorta³³.
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13 In conclusion, vitamin A plays an antioxidant effect on vascular level in our
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15 experimental model, what is the opposite observed in other *in vivo* works produced by our
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17 group, where were observed pro-oxidants effects on CNS, liver and lung. However, two
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19 points must be regarded, in one hand the different role of the studied organic system, and in
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21 another hand the real impact of this antioxidant adaptation, since it is a biochemical concept
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23 and cell function and physiological (vasorelaxation) parameters are needed to suggest if it is a
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25 positive or a negative adaptation. Another important consideration for future works will be to
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27 investigate the possible different effects among the cell types that form the vascular wall, for
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29 instance endothelial and smooth muscular cells.
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For Peer Review

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FIGURE CAPTIONS

Figure 1: TBARS levels. A. 3 days period ($\eta = 5$ for each group), B. 7 days period ($\eta = 5$ for each group) and C. 28 days period ($\eta = 7$ for each group). Data are expressed as mean \pm 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 ($\eta = 5$ for each group), B. 7 days period ($\eta = 5$ for each group) and C. 28 days period ($\eta = 7$ for each group). Data are expressed as mean \pm 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 ($\eta = 5$ for each group), B. 7 days period ($\eta = 5$ for each group) and C. 28 days period ($\eta = 7$ for each group). Data are expressed as mean \pm 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 ($\eta = 5$ for each group), B. 7 days period ($\eta = 5$ for each group) and C. 28 days period ($\eta = 7$ for each group). Data are expressed as mean \pm 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 $p \leq 0.05$.

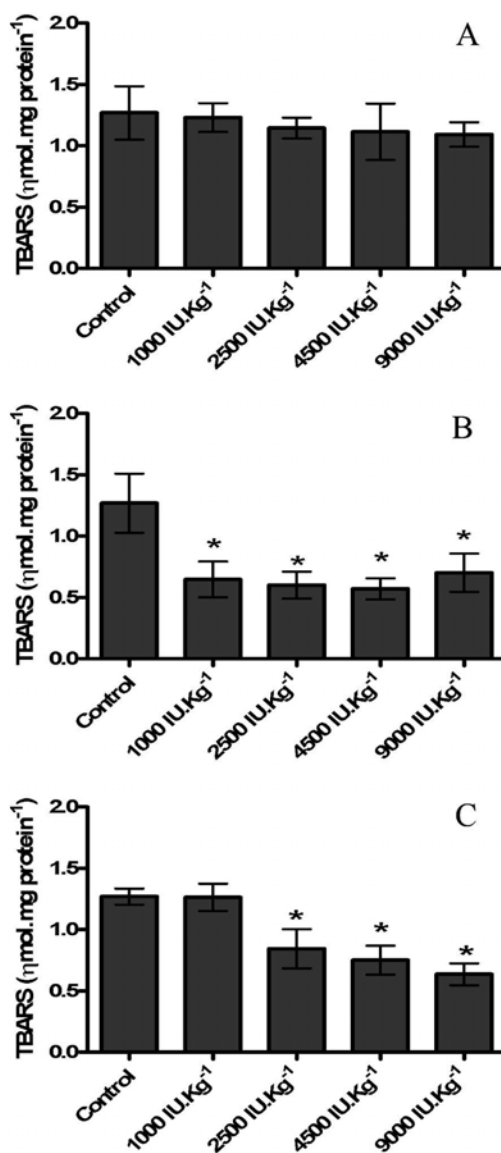


Figure 1: TBARS levels. A. 3 days period ($\eta = 5$ for each group), B. 7 days period ($\eta = 5$ for each group) and C. 28 days period ($\eta = 7$ for each group). Data are expressed as mean \pm standard error of mean. *different of control. Differences were determined by Anova one-way followed by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

80x180mm (600 x 600 DPI)

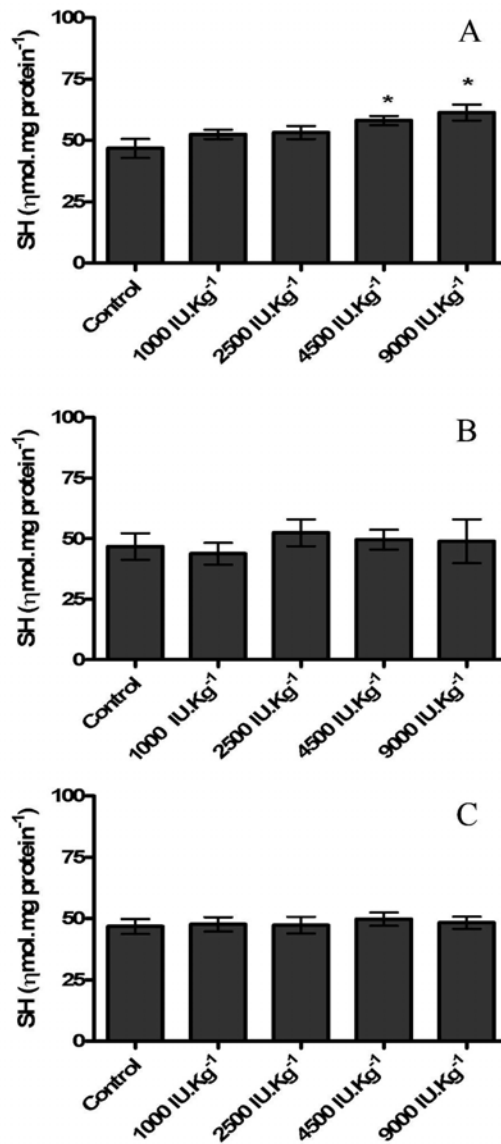


Figure 2: SH content. A. 3 days period ($n = 5$ for each group), B. 7 days period ($n = 5$ for each group) and C. 28 days period ($n = 7$ for each group). Data are expressed as mean \pm standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's pos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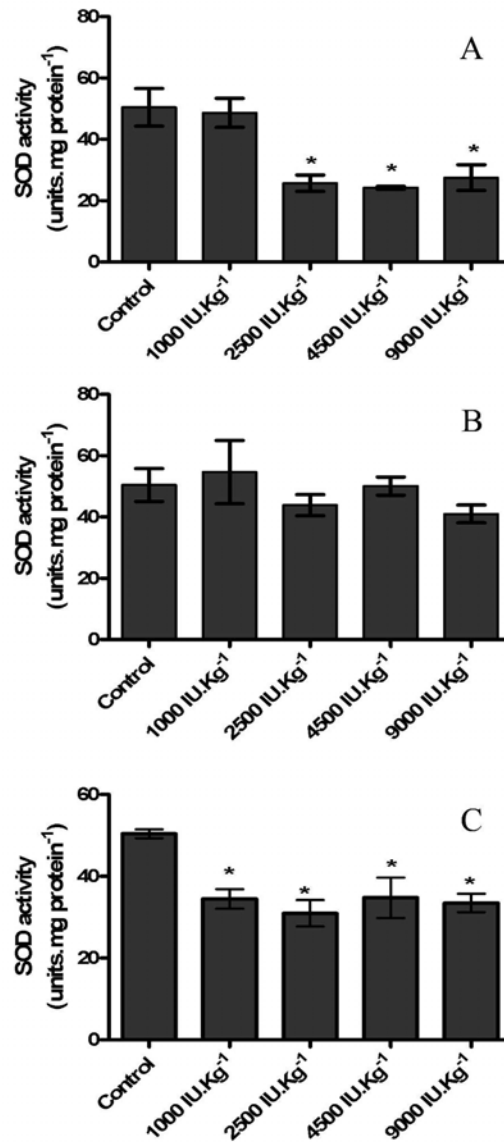


Figure 3: SOD activity. A. 3 days period ($\eta = 5$ for each group), B. 7 days period ($\eta = 5$ for each group) and C. 28 days period ($\eta = 7$ for each group). Data are expressed as mean \pm standard error of mean. *different of control. Differences were determined by Anova one-way followed by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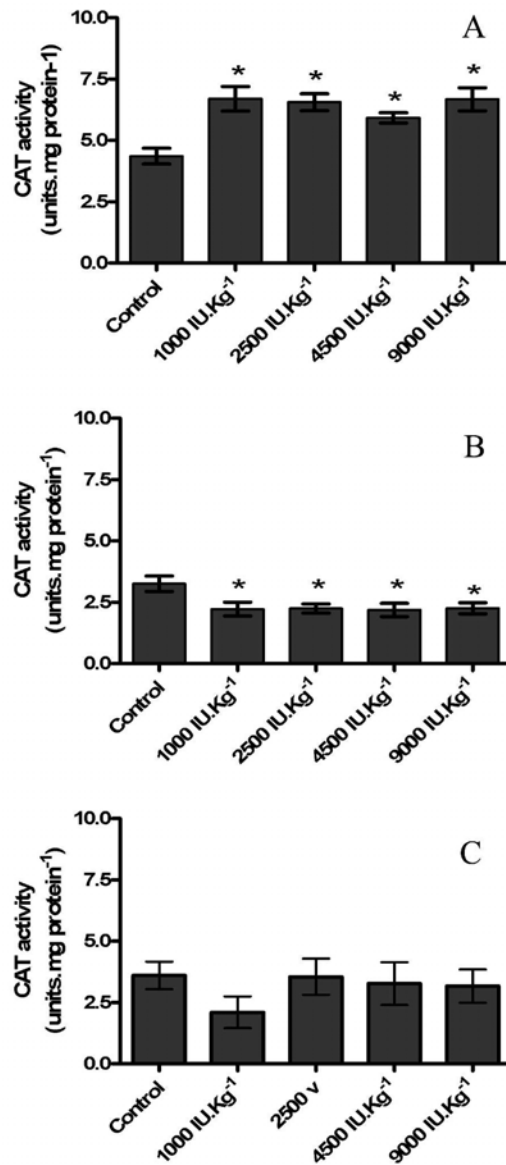


Figure 4: CAT activity. A. 3 days period ($\eta = 5$ for each group), B. 7 days period ($\eta = 5$ for each group) and C. 28 days period ($\eta = 7$ for each group). Data are expressed as mean \pm standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's pos hoc, and the accepted significance level was $p \leq 0.05$.
80x180mm (600 x 600 DPI)

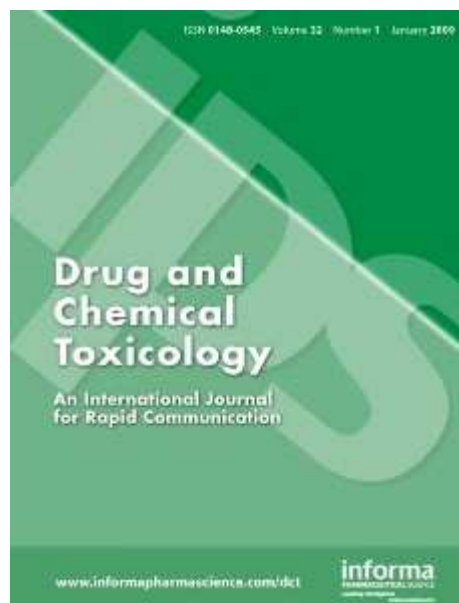
CAPÍTULO II

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

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Keywords:	Antioxidants, Cardiovascular, Retinol



For Peer Review Only

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3 **Short-term vitamin A supplementation at therapeutic doses induces oxidative damage**
4 **on rat heart**
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11

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14 MAURÍLIO DA SILVA MORENO EDAL PIZZOLÉ CLÁUDIO FONSECA MOREIRA
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55 KEYWORDS: ANTIOXIDANTS. CARDIOVASCULAR. DAMAGE. OXIDATIVE STRESS. RETINOL.
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ABSTRACT

VITAMIN A PLAYS SEVERAL IMPORTANT ROLES ON CARDIOVASCULAR SYSTEM, BY DIFFERENTIATION AND PROLIFERATION. ANTIOXIDANT PROPERTIES ARE ALSO ATTRIBUTED TO IT. THEREBY WE AIMED TO COMPARE HEART OXIDATIVE STRESS PARAMETERS AMONG RATS WITH AND WITHOUT VITAMIN A. MALE ADULT RATS WERE TREATED FOR ACUTE PERIOD (3 OR 7 DAYS) WITH DIFFERENT DOSES (1000, 2500, 4500 AND 9000 IU/KG) OF RETINYL PALMITATE, AND FURTHER THE HEART WAS REMOVED FOR ANALYSIS. AS OXIDATIVE STRESS PARAMETERS WERE EVALUATED THROUGH TBARS, REACTIVE SPECIES (TBARS) LEVELS, CARBONYL CONTENT, SULFHYDRYL CONTENT, ANTIOXIDANT POTENTIAL (TRAP) AND TOTAL ANTIOXIDANT REACTIVITY (TAR) CAPACITY. ENZYME ACTIVITIES OF SUPEROXIDE DISMUTASE (SOD) AND CATALASE (CAT). STATISTICS WERE PERFORMED BY ANOVA ONE-WAY FOLLOWED BY DUNN'S TEST AND SIGNIFICANCE LEVEL WAS ABOUT 0.05. NO DIFFERENCES IN TBARS AND CARBONYL NO DIFFERENCES WERE OBSERVED AFTER 3 DAYS, BUT IN THE HIGH DOSE GROUP WAS AN INCREASE AFTER 7 DAYS. NO MODIFICATIONS WERE FOUND IN SULFHYDRYL AND ANTIOXIDANT POTENTIAL. DECREASE WAS SEEN IN TAR AFTER 3 (ALL DOSES) AND 7 (TWO HIGHER DOSES) DAYS. IN ENZYME 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 HIGH DOSES INDUCES REDOX IMBALANCE AND, CONSEQUENTLY, OXIDATIVE DAMAGE. AN INTERESTING OBSERVATION IS THAT IT HAPPENS IN A SHORT-TERM WAY.

INTRODUCTION

VITAMIN A IS THE GIVEN NAME OF RETINOL AND ANY OTHER MOLECULE THAT ACTIVITY, AND CAN BE FOUND ON ANIMAL SOURCES, SUCH AS LIVER MEAT, AS RETINYL FORM (PRO-VITAMIN A) OR ON VEGETAL SOURCES IN RETINOL PRECURSORS, THE CAROTE A). SEVERAL PHYSIOLOGICAL FUNCTIONS ARE ATTRIBUTED TO THE RETINOIDS, SINCE ADULT LIFE, MAINLY FOR ITS REGULATION ON CELL PROCESSES, SUCH AS MATURATION PROLIFERATION (SPORN 1994). ADDITIONALLY, VITAMIN A PRESENTS IMPORTANT RO DEVELOPMENT AS WELL AS IT LIKES TO HAVE AN IMPORTANCE FOR THE HEART AFTER MY SINCE THERE IS A MOBILIZATION OF RETINOL FROM RESOURCES TO THE AFFECTED OR 2008; PALACE ET AL. 1999A; PALACE ET AL. 1999B). IN FUNCTION OF THE RETINOIC ACID INHIBIT SOME EFFECTS OF ANGIOTENSIN II ON NEONATAL RAT CARDIAC MYOCYTES, IT THERAPEUTIC USING FOR THE PREVENTION AND TREATMENT OF CARDIAC HYPERTROP (WANG ET AL. 2002). BESIDES THE RELATED FUNCTIONAL ACTIONS, ANTIOXIDANTS PRO ATTRIBUTED TO RETINOL AND ITS PRECURSORS (SPORN 1994). REGARDING THESE POSITI INTERVENTIONS WITH VITAMIN A HAVE BEEN LARGELY USED FOR TREATMENT AND PRE DISEASES AND CONDITIONS, FOR INSTANCE PSORIASIS, CANCER, CYSTIC FIBROSIS AND OT 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 ACTUALLY, HEPATIC FIBROSIS, BEHAVIORAL DISTURBANCE AND RISK FOR CANCER DEVE WERE RELATED TO HIGH DOSES VITAMIN A INTAKE (LAM ET AL. 2006; MYHRE ET AL. 2003; 1994). IN ADDITION, OUR RESEARCH GROUP HAS CONTRIBUTED TO THE BETTER UNDERST RETINOIDS ROLES, MAINLY BY DESCRIBING RETINOL PRO-OXIDANTS ~~PROPE~~ PROPERTIES. BY U MODEL, IT WAS DEMONSTRATED THAT RETINOL CAN TO COMPETE WITH XANTHYNE XANTHYNE OXYDASE, SO THE CATALYZED REACTION PRODUCES SUPEROXIDE RADICAL (

1
2
3 2008). THIS MECHANISM IS SUGGESTED AS ONE RESPONSIBLE FOR SUPEROXIDE
4
5 CYTOSOL LEVEL. A LARGE LITERATURE HAS BEEN PROMOTEDS WITH WHICH WAS
6
7 DEMONSTRATED OXIDATIVE DAMAGE ON LIPIDS, PROTEINS AND DEOXYRIBONUCLEIC
8
9 CHANGES ON ANTIOXIDANTS ENZYMES, AS SUPEROXIDE DISMUTASE (SOD), CATALASE
10
11 GLUTATHIONE PEROXIDASE (GPX), AND MITOCHONDRIAL IMPAIRMENT INDUCED BY RETINOL
12
13 SERTOLI CELLS (DAL-PIZZOL ET AL. 2001; DAL-PIZZOL ET AL. 2000; GELAIN ET AL. 2008; KAWA
14
15 2003). IN ACCORDANCE TO THESE CITED RESULTS, IT WAS REPORTED THAT VITAMIN A
16
17 DAMAGE THROUGH INCREASED SUPEROXIDE PRODUCTION (MURATA AND KAWA
18
19 ADDITIONALLY, THE CONTROL OF CELL PROLIFERATION/DEATH PATHWAYS THROUGH NUCLEAR
20
21 (NF κ B) WAS ALSO OBSERVED IN RESPONSE TO RETINOL TREATMENT (ZANOTTO-FILHO
22
23 RECENTLY, *vivo* STUDIES HAVE BEEN ALSO DEVOTED TO INVESTIGATE THE REDOX PR
24
25 RETINOIDS, IN WHICH OXIDATIVE DAMAGE AND ANTIOXIDANT ENZYME IMBALANCE WE
26
27 CENTRAL NERVOUS SYSTEM (CNS) SUBMITTED TO ACUTE AND CHRONIC PERIOD
28
29 PHARMACOLOGICAL DOSES OF RETINYL PALMITATE. THE REPORTED CNS OXIDATIVE STR
30
31 BEHAVIOR DISTURBANCES OF THE ANIMALS (DE OLIVEIRA AND MOREIRA 2008; DE OLIVE
32
33 DE OLIVEIRA ET AL. 2007).

34
35 THEREFORE, OUR AIM IN THE PRESENT STUDY WAS TO COMPARE HEART OXI
36
37 PARAMETERS AMONG RATS SUPPLEMENTED WITH VITAMIN A AND ONE NOT SUPPLEMENT
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39

40 41 42 43 44 45 46 47 48 **METHODS**

49 50 51 52 53 **ANIMALS AND MATERIALS**

54
55 ADULT MALE RATS (90 DAYS OLD) WERE USED IN THIS WORK AND WERE MAINTAIN
56
57 HOURS LIGHT-DARK CYCLE WITH WATER AND FOOD. EXPERIMENTS PROCEDURES WERE
58
59 PERFORMED IN ACCORDANCE WITH THE NATIONAL INSTITUTE OF HEALTH GUIDE FOR
60

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2
3 LABORATORY ANIMALS. RETINYL PALMITATE (AROVIT~~FORM, WAS PURCHASED~~ FROM
4 BAYER, SÃO PAULO, SÃO PAULO, BRAZIL. ALL OTHERS REAGENTS WERE PURCHASED
5
6
7
8 CHEMICALS (ST. LOUIS, MO, USA).
9

10 11 12 EXPERIMENTAL DESIGN AND DRUG ADMINISTRATION

13
14 TWO DIFFERENT PERIODS (3 AND 7 DAYS) OF ACUTE TREATMENT WERE PERFORMED
15
16 ANIMAL WERE RANDOMLY DIVIDED FOR THE 2 TREATMENTS, AND THEREAFTER INTO TWO
17
18 GROUPS: CONTROL (VEHICLE: SALINE SOLUTION 0.9 %) AND 4 DIFFERENT VITAMIN A DOSE
19
20 4500 AND 9000), EXPRESSED AS INTERNATIONAL UNITS (IU) FOR BODY MASS KILOGRAM (1
21
22 (IU.KG¹.DAY). RETINYL PALMITATE SOLUTION WAS PREPARED DAILY USING SALINE SOLUTION
23
24 (%) AS VEHICLE, AND IT WAS ADMINISTRATION VIA ORAL, BY INTRA-GASTRIC GAVAGE, IN
25
26 VOLUME ALWAYS IN THE DARK CYCLE BEGINNING.
27
28
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34 35 SAMPLE PREPARATION

36
37 AFTER THE RESPECTIVE TREATMENTS THE RATS WERE KILLED BY DECAPITATION AND
38
39 CAREFULLY REMOVED AND CLEANED WITH ICED SALINE SOLUTION TO REMOVE BLOOD
40
41 HOMOGENIZED IN PHOSPHATE BUFFER FOR SAMPLES (PBS) PH 7.4, THE HOMOGENATE WAS
42
43 AT 700 X G TO REMOVE DEBRIS AND THE SUPERNATANT WAS USED AS MOTHER SOLUTION
44
45 CONTENT WAS MEASURED BY LOWRY METHOD IN ORDER TO CORRECT THE RESULTS (LOWRY
46
47
48
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51 52 OXIDATIVE STRESS PARAMETERS

53
54 IT WAS EVALUATED THE THIOBARBITURIC ACID REACTIVE SPECIES (TBARS) TEST AND
55
56 LIPIDS OXIDATION. THE TBARS CONSISTS IN AN ACID-HEATING REACTION OF THE LIPID
57
58 PRODUCT, MALONDIALDEHYDE, WITH THIOBARBITURIC ACID (TBA). THE TBARS WERE
59
60 532 η M AND THE RESULTS WERE EXPRESSED AS PROTEIN (FRANCO AND HADLEY 1990). FOR

1
2
3 PROTEIN OXIDATION ANALYSIS, IT WAS MEASURED THE CARBONYL CONTENT, WHICH
4
5 REACTION OF DINITROPHENYLHYDRAZINE WITH PROTEIN CARBONYL GROUPS. THE RESULT
6
7 η MOL.MG PROTEIN (LIVINE ET AL. 1990).

8
9
10 THE TOTAL SULFHYDRYL (SH) CONTENT, PRESENT IN PROTEINS AS WELL AS GLYCOPROTEIN
11
12 MEASURED AT η M BY ITS REACTION WITH 5,5'-DITHIO-BIS 2-NITROBENZOIC ACID (DTNB), AND
13
14 RESULTS WERE EXPRESSED AS μ MOL/MG PROTEIN (HENLMAN 1959).

15
16
17 THE TOTAL REACTIVE ANTIOXIDANT POTENTIAL (TRAP) HAS BEEN USED AS AN INDICATOR OF
18
19 ENZYMATIC ANTIOXIDANT CAPACITY, BASED ON THE PEROXYL RADICAL (GENERATED BY
20
21 2,2'-AZOBIS[2-AMIDINOPROPANE], WITH LUMINOL) QUENCHING BY SAMPLE COMPOUNDS. THIS
22
23 IS DONE BY CHEMILUMINESCENCE EMISSION. BRIEFLY WE PREPARED AAPH SOLUTION AND
24
25 LUMINOL (SYSTEM), THEREAFTER WE WAITED FOR THE SYSTEM STABILIZATION FOR 2 HOURS
26
27 READING. AFTER THE SAMPLE TO BE ADDED WE ANALYZE THE READINGS FOR NEARLY 30
28
29 AL. 1992). THE RESULTS WERE TRANSFORMED IN PERCENTUAL AND THE AREA UNDER CURVE
30
31 CALCULATED BY SOFTWARE (Graware Software Inc.®, San Diego, CA, USA –version 5.00) AS
32
33 DESCRIBED (DRESCH ET AL. 2009). THE TOTAL ANTIOXIDANT REACTIVITY (TAR) WAS ALSO
34
35 PLASMA AND IT IS BASED IN THE SAME TECHNICAL PRINCIPLES OF TRAP, HOWEVER THIS
36
37 RELATED TO QUALITY OF SAMPLES ANTIOXIDANTS. THE TAR RESULTS WERE CALCULATED AS
38
39 LIGHT IN ABSENCE OF SAMPLES INTENSITY RIGHT AFTER SAMPLE ADDITION (I) (LISSA ET AL.
40
41 1995).

42
43
44 ANTIOXIDANT ENZYMES ACTIVITY WAS ALSO ASSESSED. THE SUPEROXIDE DISMUTASE (SOD)
45
46 CATALYZES SUPEROXIDE ANION RADICALS TO GENERATE HYDROGEN PEROXIDE (H₂O₂)
47
48 AND ITS ACTIVITY MEASUREMENT IS BASED ON THE PRINCIPLE THAT ADRENALINE UNDERGOES
49
50 IN O₂ PRESENCE, SO THAT WE DETERMINE THE SOD ACTIVITY BY THE EXOGENOUS ADRENALINE
51
52 AUTO-OXIDATION INHIBITION IN SAMPLE PRESENCE (BANNISTER AND CALABRESE 1987).
53
54 (CAT) CATALYZES THE CONVERSION TO WATER AND O₂ TO DETERMINE ITS ACTIVITY WE ADDED
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3 H₂O₂ AND ANALYZED THE CAPACITY OF SAMPLE TO DECREASE THE AEBI
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5
6 1984).

7 8 9 10 STATYSTICAL ANALYSIS

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12 ALL DATA ARE PRESENTED AS MEAN ± STANDARD ERROR OF MEAN (SEM) AND IT WAS
13
14 ONE WAY FOLLOWED BY DUNNETTS TO DETERMINE THE DIFFERENCES AMONG GOUPS AND
15
16 SIGNIFICANCE LEVEL CONSIDERED 0.05. ALL STATISTICAL ANALYSIS AND THE GRAPHS MADE
17
18 WERE CONDUCTED WITH *GraphPad Software Inc.®, San Diego, CA, USA –version 5.00.*

19 20 21 22 23 24 25 RESULTS

26
27
28 AS INDEX OF BIOMOLECULES OXIDATIVE DAMAGE PARAMETERS WERE EVALUATED
29
30 (FOR LIPID OXIDATION) AND CARBONYL CONTENT (FOR PROTEIN OXIDATION). THESE CITED
31
32 PLAYED SIMILAR RESULTS, SINCE ANYONE WAS MODIFIED AFTER 3 DAYS TREATMENT PERIOD
33
34 AN INCREASE IN THE HIGHEST DOSE AFTER 7 DAYS TREATMENT PERIOD (FIGURE 1 AND 2).

35
36
37 THE SH GROUPS HAVE IMPORTANT ROLE FOR CONTROLLING REDOX STATUS IN
38
39 ENVIRONMENT (BY GLUTATHIONE MOLECULE) AND PLASMA (MAINLY BY ALBUMIN PROTEIN).
40
41 OXIDATION STATUS OF THIS MOLECULE HAS BEEN LARGELY USED AS A REDOX STATUS MARKER.
42
43 MODIFICATIONS WERE FOUND IN SH FOR ANY TREATMENT PERIOD (FIGURE 3).

44
45
46 FOR MEASURE NON-ENZYMATIC CAPACITY, THE TRAP AND TAR ASSAYS HAVE BEEN USED.
47
48 TRAP IS MORE RELATED TO THE ANTIOXIDANTS AMOUNT IN THE SAMPLES, WHILE THE TAR IS
49
50 RELATED TO THE QUALITY (REACTIVITY) OF SAMPLE ANTIOXIDANTS. NO CHANGES WERE OBSERVED
51
52 IN ANY TREATMENT PERIOD, BUT A DECREASE WAS SEEN IN TAR AFTER 3 (ALL DOSES) AND 7
53
54 (ALL DOSES) DAYS TREATMENT PERIODS (FIGURE 4).

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3 REGARDING ENZYMATIC ACTIVITIES, SURPRISINGLY ~~ON DOSE IN THIS LOWEST~~
4
5 INCREASE IN SOD ACTIVITY AFTER 3 DAYS TREATMENT PERIOD, WHILE AFTER 7 DAYS TRE
6
7 INCREASE WAS OBSERVED AT THE HIGHEST DOSE. ALL DOSES PLAYED A DECREASED CA
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9 DAYS TREATMENT PERIOD, BUT NO MODIFICATIONS WERE SEEN AFTER 7 DAYS TREATMEN
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14 15 **DISCUSSION**

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20 IN THE PRESENT STUDY WE EVALUATED THE EFFECTS OF SHORT-TERM VITAMIN A S
21
22 ON OXIDATIVE STRESS PARAMETERS IN THE HEART, SINCE RETINOL USING AS A THERAP
23
24 BEEN SUGGESTED TO TREAT CARDIOVASCULAR DISEASES, BUT NEGATIVE EFFECTS ARE A
25
26 MOLECULE AND ITS DERIVATES.
27
28

29
30 WE COULD OBSERVE HERE THAT VITAMIN A SUPPLEMENTATION EVEN IN A SHORT-T
31
32 YIELD AN IMBALANCE ON REDOX SYSTEM GENERATING OXIDATIVE DAMAGE IN MACROM
33
34 AND PROTEINS, WHAT WAS DEMONSTRATED BY THE INCREASE IN TBARS LEVELS AND CA
35
36 RESPECTIVELY, AFTER 7 DAYS TREATMENT PERIOD, BUT NOT AFTER 3 DAYS TREATMEN
37
38 IU.KG¹.DAY¹. OUR RESULTS ARE DIFFERENT FROM THOSE PREVIOUSLY PUBLISHED BY
39
40 INCREASED LEVELS OF TBARS WERE FOUND ON LIVER OF RATS SUBMITTED TO RET
41
42 SUPPLEMENTATION AFTER 3 DAYS TREATMENT PERIOD, BUT NO CHANGES AFTER 7 DAYS
43
44 HOWEVER THIS SAME STUDY PRESENTED SIMILAR RESULTS IN CARBONYL CONTENT, SIN
45
46 DEMONSTRATED AFTER BOTH TREATMENT PERIODS (DE OLIVEIRA ET AL. 2009). AD
47
48 DEMONSTRATED INCREASE IN TBARS LEVELS AND CARBONYL CONTENT ON RAT HIP
49
50 *substantia nigra* AFTER BOTH ACUTE TREATMENT PERIODS (DE OLIVEIRA ET AL. 2008; DE OL
51
52 2007).
53
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56

57 ABOUT SH STATUS, WE COULD NOT DEMONSTRATE ANY CHANGES FOR ANY TRE
58
59 WHAT IS DIFFERENT FROM OUR PRIOR WORK WITH LIVER, WHICH PLAYED DIMINISHED P
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3 DAYS TREATMENT PERIOD AND DIMINISHED NON-PROTEIN SHANTERPERIODYST TREA
4
5 IMPORTANT NOTE THAT SH, FROM GSH, IS CONSIDERED THE MAIN REDOX BUFFER OF TH
6
7 TRUE ONLY FOR CYTOSOLIC FRACTION AND NOT FOR NUCLEOUS OR MITOCHONDRIA (ME
8
9
10 INDEED, WE DEMONSTRATED OXIDATIVE DAMAGE, IMBALANCE REDOX, RESPIRATOR
11
12 IMPAIRMENT AND INCREASED SUPEROXIDE RADICAL PRODUCTION ON ISOLATED LIVER
13
14
15 OLIVEIRA ET AL. 2009).

16
17
18 HEREIN STUDY WE OBSERVED DIMINISHED ANTIOXIDANT NON-ENZYMATIC REAC
19
20 MODIFICATIONS IN THE AMOUNT OF THE ANTIOXIDANT FOR BOTH TREATMENTS, EVIDEN
21
22 TAR AND UNCHANGED TRAP. HOWEVER IT IS DIFFICULT TO KNOW WHAT EXACTLY SUBS
23
24 AFFECTED, BECAUSE WE COULD THINK ON GLUTATHIONE AT A FIRST TIME, BUT NO CHA
25
26 SH CONTENT. PREVIOUSLY, WE FOUND INCREASE IN TRAP OF LIVER AFTER 7 DAYS TREAT
27
28 OLIVEIRA ET AL. 2009).

29
30
31
32 SURPRINSINGLY, ONLY FOR THE LOWEST VITAMIN A DOSE WAS DETECTED AN IN
33
34 ACTIVITY AFTER 3 DAYS TREATMENT PERIOD, WHILE AFTER 7 DAYS TREATMENT PERIOD
35
36 ABLE TO GENERATE AN INCREASE. IN OUR PREVIOUS WORK ALL DOSES OF VITAMIN A Y
37
38 SOD ACTIVITY AFTER 7 DAYS TREATMENT PERIOD, AND ONLY THE HIGHEST DOSE INC
39
40 ACTIVITY AFTER 3 DAYS TREATMENT PERIOD (DE OLIVEIRA ET AL. 2009). ALL DOSES OF I
41
42 WERE ABLE TO DIMINISH THE CAT ACTIVITY IN THE PRESENT WORK AFTER 3 DAYS TREA
43
44 ITS ACTIVITY IS RECOVERED AFTER 7 DAYS TREATMENT PERIOD. THIS RESULT FOR CAT AC
45
46 TREATMENT PERIOD IS SIMILAR TO OUR PRIOR RESULTS WITH LIVER, BUT THE MODIFIC
47
48 TREATMENT PERIOD ARE VERY DIFFERENT TO THOSE RESULTS FOUND FOR THE LIVER,
49
50 INCREASED AT THE HIGHEST DOSE (DE OLIVEIRA ET AL. 2009). BEFORE, WE HAD DEMONST
51
52 SOD AND CAT ACTIVITIES AFTER 3 AND 7 DAYS TREATMENT PERIOD ON RAT HIPPOCAMPU
53
54 ET AL. 2007).

1
2
3 MOREOVER, WE HAD PREVIOUSLY DEMONSTRATED THAT THESE LOW DOSES OF PHARMACO
4
5 VITAMIN A WERE ABLE TO YIELD ANXIETY BEHAVIOR AND DECREASE THE LOCOMOTION
6
7
8 RATS (DE OLIVEIRA ET AL. 2008; DE OLIVEIRA ET AL. 2007).

9
10 TAKEN TOGETHER OUR RESULTS SUGGEST THAT SHORT-TERM VITAMIN A SUPP
11
12 ENOUGH TO GENERATE OXIDATIVE DAMAGE ON THE HEART, AND IT HAPPENS BY USING T
13
14 HOWEVER THE SPECIFIC MECHANISMS BY WHAT RETINOL ACT IS OBJECT OF A FUTURE
15
16
17 COULD NOT OBSERVE ANY MODIFICATION ON SH STATUS, WHICH TAKEN TOGETHER TO PR
18
19 US TO BELIEVE IN A MECHANISM INVOLVING MITOCHONDRIAL DYSFUNCTION. FURTH
20
21 ALREADY DEMONSTRATED THAT RETINOL CAN INHIBIT THE ACTIVITY OF RESPIRATORY
22
23 GENERATE SUPEROXIDE RADICAL, BY USING *in vivo* MODELS (DE OLIVEIRA AND MOREIRA
24
25 2008; DE OLIVEIRA ET AL. 2009; KLAMT ET AL. 2003). ANOTHER INTERESTING FUTURE
26
27 RELATION OF VITAMIN A TO OTHER NON-ENZYMATIC ANTIOXIDANTS, SINCE THE S
28
29 DECREASED THE TAR, BUT IT NOT LIKES BE BY MODIFICATIONS ON GSH. THE INFLUENCE
30
31 SOD AND CAT ACTIVITY HAS BEING INVESTIGATED, BUT BETTER EXPLANATIONS ABOUT H
32
33 MYOCARDIAL LEVEL ARE NEEDED YET (DAL-PIZZOL ET AL. 2001; GELAIN ET AL. 2008; P
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FIGURE CAPTIONS

Figure 1: TBARS LEVELS. A. 3 DAYS TREATMENT PERIOD (EACH GROUP) AND B. 7 DAYS TREATMENT PERIOD FOR EACH GROUP). DATA ARE EXPRESSED AS MEAN \pm STANDARD ERROR OF MEAN. *DIFFERENT OF CONTROL. DIFFERENCES WERE DETERMINED BY ANOVA ONE-WAY FOLLOWED BY *post hoc*, AND THE ACCEPTED SIGNIFICANCE LEVEL WAS $P \leq 0.05$

Figure 2: CARBONYL CONTENT. A. 3 DAYS TREATMENT PERIOD (GROUP) AND B. 7 DAYS TREATMENT PERIOD FOR EACH GROUP). DATA ARE EXPRESSED AS MEAN \pm STANDARD ERROR OF MEAN. *DIFFERENT OF CONTROL. DIFFERENCES WERE DETERMINED BY ANOVA ONE-WAY FOLLOWED BY *post hoc*, AND THE ACCEPTED SIGNIFICANCE LEVEL WAS $P \leq 0.05$

Figure 3: SH CONTENT. A. 3 DAYS TREATMENT PERIOD (EACH GROUP) AND B. 7 DAYS TREATMENT PERIOD FOR EACH GROUP). DATA ARE EXPRESSED AS MEAN \pm STANDARD ERROR OF MEAN. *DIFFERENT OF CONTROL. DIFFERENCES WERE DETERMINED BY ANOVA ONE-WAY FOLLOWED BY *post hoc*, AND THE ACCEPTED SIGNIFICANCE LEVEL WAS $P \leq 0.05$

Figure 4: NON-ENZYMATIC CAPACITY. A. TRAP OF 3 DAYS TREATMENT PERIOD (EACH GROUP), B. TRAP OF 7 DAYS TREATMENT PERIOD (EACH GROUP), C. TAR OF 3 DAYS TREATMENT PERIOD FOR EACH GROUP) AND D. TAR OF 7 DAYS TREATMENT PERIOD (EACH GROUP). DATA ARE EXPRESSED AS MEAN \pm STANDARD ERROR OF MEAN. *DIFFERENT OF CONTROL. DIFFERENCES WERE DETERMINED BY ANOVA ONE-WAY FOLLOWED BY DUNN'S TEST, AND THE ACCEPTED SIGNIFICANCE LEVEL WAS $P \leq 0.05$

Figure 5: ENZYMATIC ACTIVITIES. A. SOD OF 3 DAYS TREATMENT PERIOD (EACH GROUP), B. SOD OF 7 DAYS TREATMENT PERIOD (EACH GROUP), C. CAT OF 3 DAYS TREATMENT PERIOD (EACH GROUP) = 6 FOR EACH GROUP) AND D. CAT OF 7 DAYS TREATMENT PERIOD (EACH GROUP). DATA ARE EXPRESSED AS MEAN \pm STANDARD ERROR OF MEAN. *DIFFERENT OF CONTROL. DIFFERENCES WERE DETERMINED BY ANOVA ONE-WAY FOLLOWED BY DUNN'S TEST, AND THE ACCEPTED SIGNIFICANCE LEVEL WAS $P \leq 0.05$

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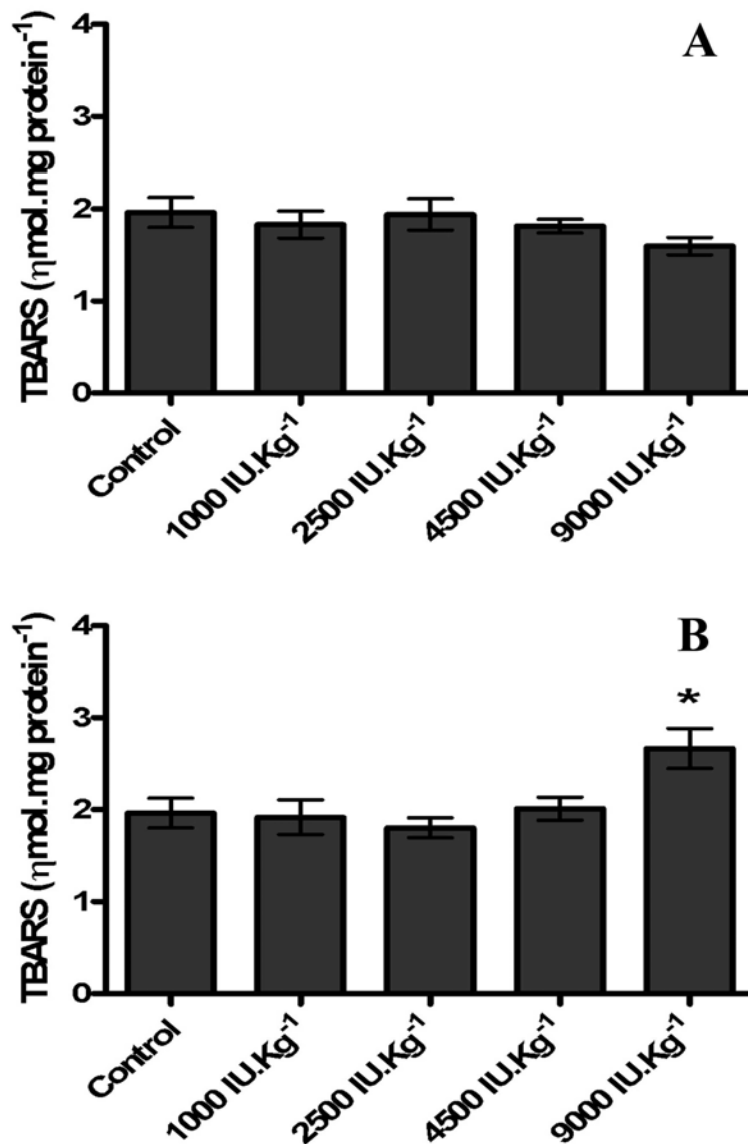


Figure 1: TBARS levels. A. 3 days treatment period ($n = 5$ for each group) and B. 7 days treatment period ($n = 6$ for each group). Data are expressed as mean \pm standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's pos hoc, and the accepted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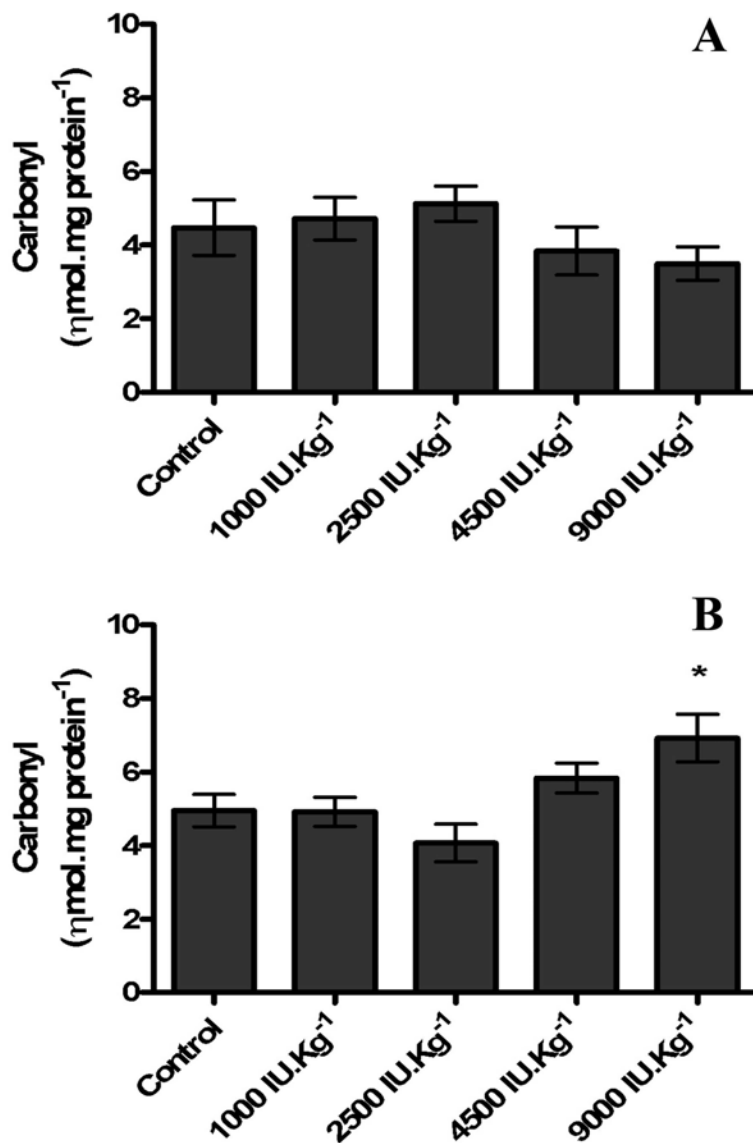


Figure 2: Carbonyl content. A. 3 days treatment period ($n = 5$ for each group) and B. 7 days treatment period ($n = 6$ for each group). Data are expressed as mean \pm standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's pos hoc, and the accepted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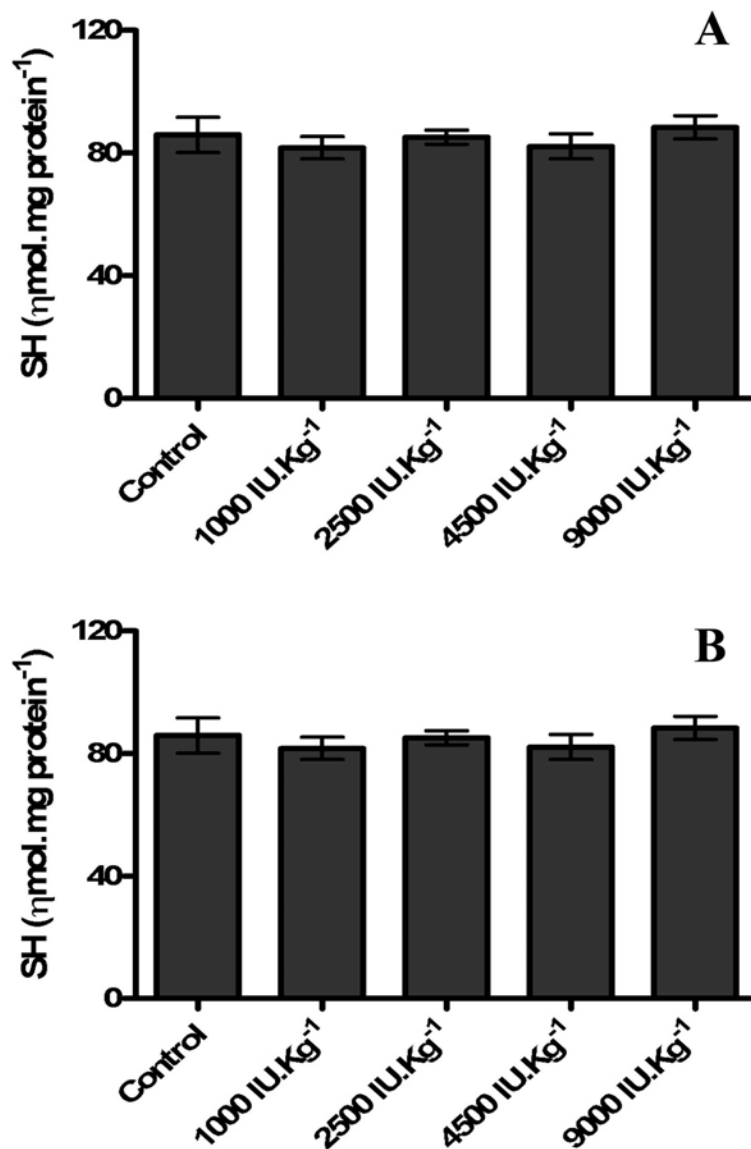


Figure 3: SH content. A. 3 days treatment period ($n = 5$ for each group) and B. 7 days treatment period ($n = 6$ for each group). Data are expressed as mean \pm standard error of mean. *different of control. Differences were determined by Anova one-way followed by Dunnet's pos hoc, and the accepted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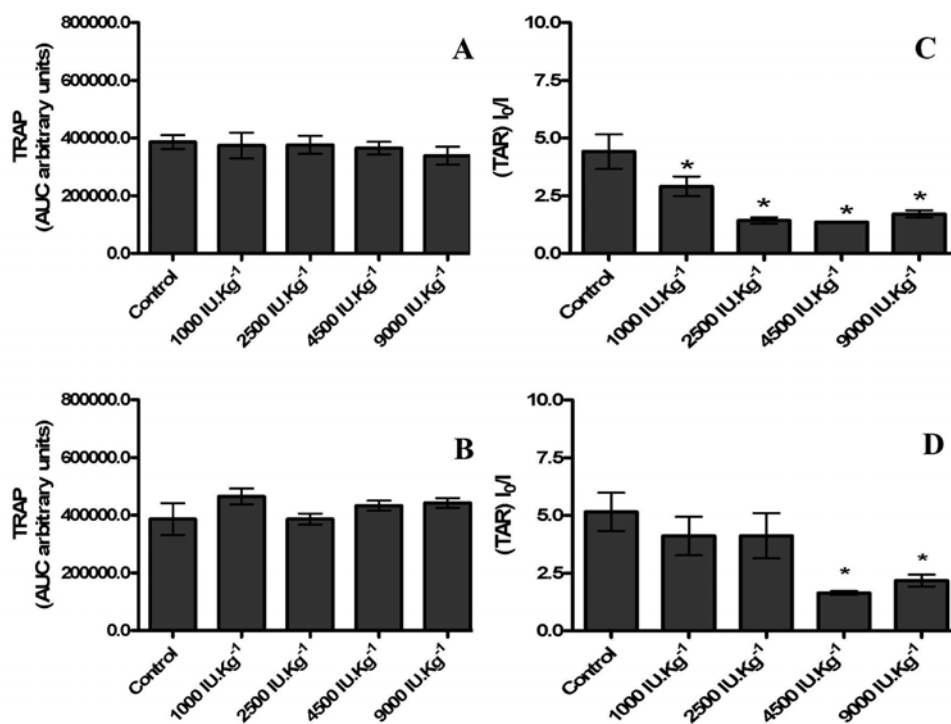


Figure 4: Non-enzymatic capacity. A. TRAP of 3 days treatment period ($\eta = 5$ for each group), B. TRAP of 7 days treatment period ($\eta = 5$ for each group), C. TAR of 3 days treatment period ($\eta = 6$ for each group) and D. TAR of 7 days treatment period ($\eta = 6$ for each group). Data are expressed as mean \pm 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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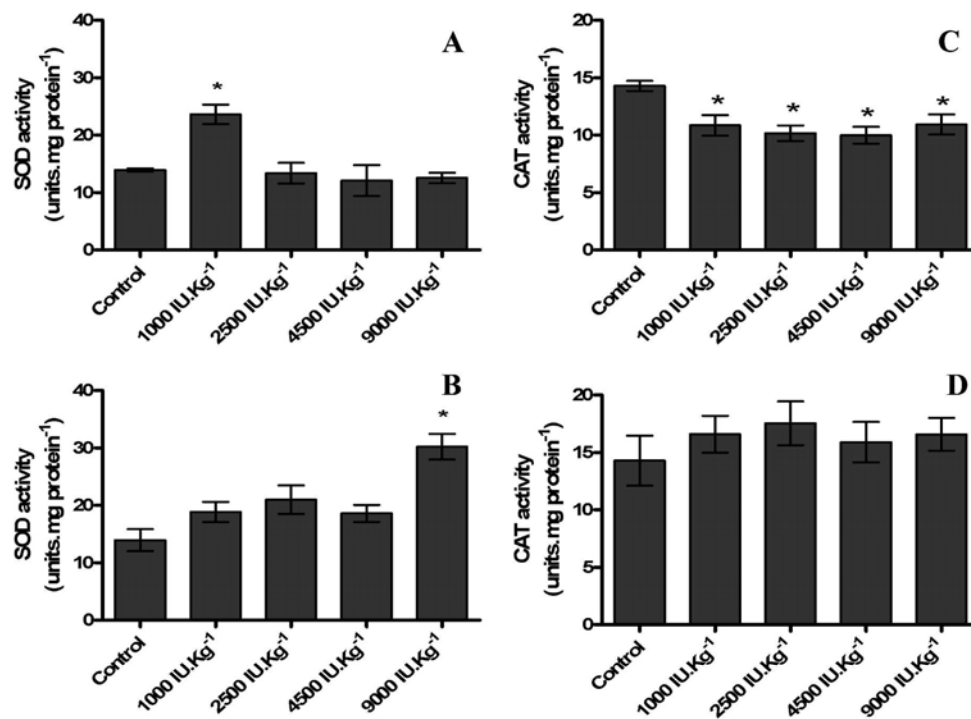


Figure 5: Enzymatic activities. A. SOD of 3 days treatment period ($\eta = 5$ for each group), B. SOD of 7 days treatment period ($\eta = 5$ for each group), C. CAT of 3 days treatment period ($\eta = 6$ for each group) and D. CAT of 7 days treatment period ($\eta = 6$ for each group). Data are expressed as mean \pm 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 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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PARTE III

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Article Title 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

Author Name Ricardo F. Da Rocha, Marcos Roberto De Oliveira, Patrícia Schonhofen, Carlos Eduardo Schnorr, Felipe Dal Pizzol & José Cláudio F. Moreira

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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

RICARDO F. DA ROCHA¹, MARCOS ROBERTO DE OLIVEIRA¹, PATRÍCIA SCHONHOFEN¹, CARLOS EDUARDO SCHNORR¹, FELIPE DAL PIZZOL², & JOSÉ CLÁUDIO F. MOREIRA¹

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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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1 through nuclear factor kappa B (NFκB) in a Sertoli
2 cells culture model [2–5]. Thereafter, using an *in vivo*
3 model, we found oxidative and nitrosative stress in
4 several structures of the central nervous system, as
5 well as behaviour disorders and bioenergetic impair-
6 ment [6–10]. Recently, we gave a good contribution
7 for the understanding of retinoids pro-oxidants effects,
8 suggesting a cytosolic source of superoxide radical
9 ($\cdot\text{O}_2^-$) by xanthine oxidase, since retinol competes
10 with xanthine for this enzyme and the given reaction
11 release ($\cdot\text{O}_2^-$) [11].

12 Other groups have also demonstrated negative results
13 about vitamin A usage, which give support to our find-
14 ings. Interestingly, a study that treated smokers with
15 vitamin A was discontinued because the intervention
16 raised the lung cancer incidence among the participants
17 [12]. Indeed, many other dysfunctions are attributed
18 to the elevated vitamin A intake, such as behavioural
19 disturbance and hepatic fibrosis [13,14].

20 Therefore, it is a worry if vitamin A usage is really
21 safe, since it is used for many treatments, such as
22 psoriasis, cancer, cystic fibrosis and others [15–18].
23 Additionally, vitamin A is suggested as a candidate for
24 treatment of myocardial hypertrophy and remodel-
25 ling, because retinoic acid inhibits angiotensin II
26 effects on neonatal rat cardiac myocytes [19]. More-
27 over, it was demonstrated that there is a mobilization
28 of retinol from its physiological resources to the myo-
29 cardium after infarction, what also indicates this
30 molecule as a possible therapeutic agent [20,21].

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

39 **Materials and methods**

40 *Animals*

41 Adult male rats (90 days old) were used and were main-
42 tained on a 12 h light–dark cycle with water and food
43 *ad libitum*. All experimental procedures were performed
44 in accordance to the National Institute of Health Guide
45 for Care and Use of Laboratory Animals. Retinyl palm-
46 itate (Arovit[®]), water soluble form, was purchased from
47 Bayer (São Paulo, Brazil). All others reagents, when
48 not specified, were purchased from Sigma Chemicals
49 (St. Louis, MO).

52 *Experimental design and treatment*

53 Initially, the animals were randomly divided into the
54 supplementation groups: Control (vehicle: saline solution
55 0.9%) and four different vitamin A doses (1000, 2500,
56 4500 and 9000), expressed as International Units (IU)
57 per body mass Kilogram (Kg) per day (IU/Kg/day)

58 and thereafter were treated for 28 days with retinyl
59 palmitate. The retinyl palmitate solution was prepared
60 daily using saline solution (NaCl 0.9%) as a vehicle
61 and was administrated orally, by intra-gastric gavage,
62 in a total 0.8 mL volume, always in the dark cycle
63 beginning.

66 *Samples preparation*

67 After the respective treatments, the rats were killed by
68 decapitation and the heart was carefully removed and
69 then cleaned with iced saline solution to remove blood
70 excess contamination. For general analysis, the organ
71 was homogenized in phosphate buffer for samples (PBS)
72 pH 7.4, so the homogenate was centrifuged at $700 \times$
73 g to remove debris and the resulting supernatant was
74 used as the mother solution. To obtain the cardiac
75 mitochondrial membrane-enriched fraction (MMEF),
76 in order to assess the mitochondrial electron transfer
77 chain (METC) complexes activities, the tissue was
78 homogenized in a buffer containing sucrose 250 mM,
79 EDTA 2 mM, Tris 10 mM pH7.4 and heparin 50 IU/
80 mL. The samples were then centrifuged at $1000 \times$ g
81 and the supernatants were collected. Thereafter the
82 samples were frozen and thawed three times. The pro-
83 tein content of general analysis and MMEF was mea-
84 sured by the Lowry et al. [22] method in order to
85 correct the results.

88 *Redox status and damage*

89 The thiobarbituric acid reactive species (TBARS) test
90 were evaluated as an index of lipids oxidation. The
91 TBARS consists of an acid-heating reaction of the lipid
92 peroxidation end product, malondialdehyde, with thio-
93 barbituric acid (TBA). The TBARS were determined
94 at 532 nm and the results were expressed as nmol/mg
95 protein [23]. For protein oxidation analysis the car-
96 bonyl content was measured, which is based on the
97 reaction of dinitrophenylhydrazine with protein car-
98 bonyl groups. The results are expressed as nmol/mg
99 protein [24]. The total sulphhydryl (SH) content, pres-
100 ent in proteins as well as in glutathione, was measured
101 at 412 nm by its reaction with 5,5'-dithio-bis 2-nitroben-
102 zoic acid (DTNB) and the results were expressed as
103 nmol/mg protein [25].

107 *Non-enzymatic antioxidant potential*

108 The total reactive antioxidant potential (TRAP) has been
109 used as an index of the non-enzymatic antioxidant capa-
110 city, based on the peroxy radical (generated by AAPH
111 solution, 2,2'-azobis[2-amidinopropane], with luminol)
112 quenching by sample compounds. The reading is done
113 by chemiluminescence emission. Briefly, we prepared
114 AAPH solution, added luminol (AAPH + luminol,
115 radical generating system) and then we waited for the
116

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 (I⁰)/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 ($\cdot\text{O}_2^-$) dismutation to generate hydrogen peroxide (H_2O_2) and its activity measurement is based on the principle that adrenaline undergo auto-oxidation in $\cdot\text{O}_2^-$ presence, so at 480 nm 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 nm [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 nm [31].

Indirect enzyme-linked immunosorbent 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 nm, 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 nm 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 nm 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 nm as a reference range, in a

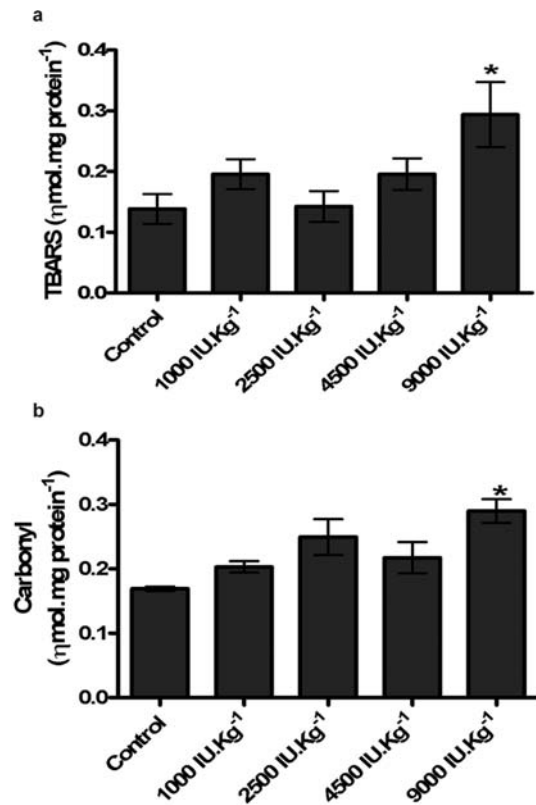


Figure 1. (A) TBARS levels (n=7 for each group) and (B) Carbonyl content (n=7 for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was p≤0.05.

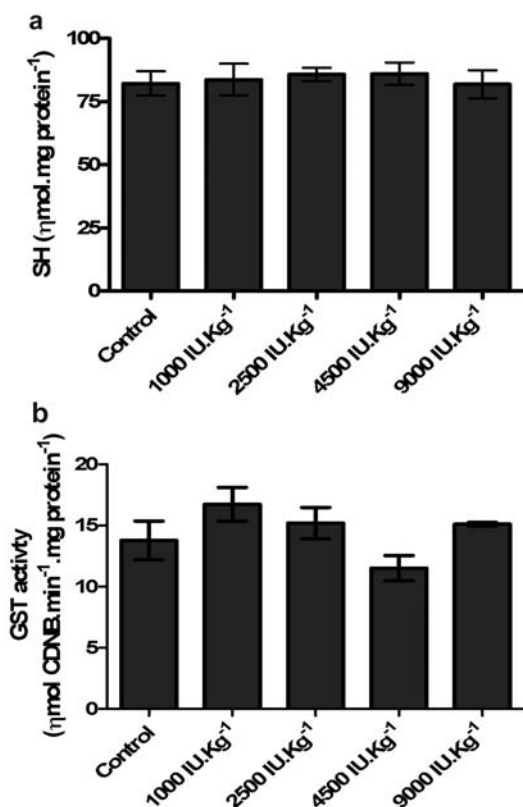


Figure 2. (A) SH content ($n=7$ for each group) and (B) GST activity ($n=5$ for each group). Data are expressed as mean \pm standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

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 nm as a reference range, in a reaction started by succinate [33].

Statistical analysis

All data are presented as mean \pm 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).

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,

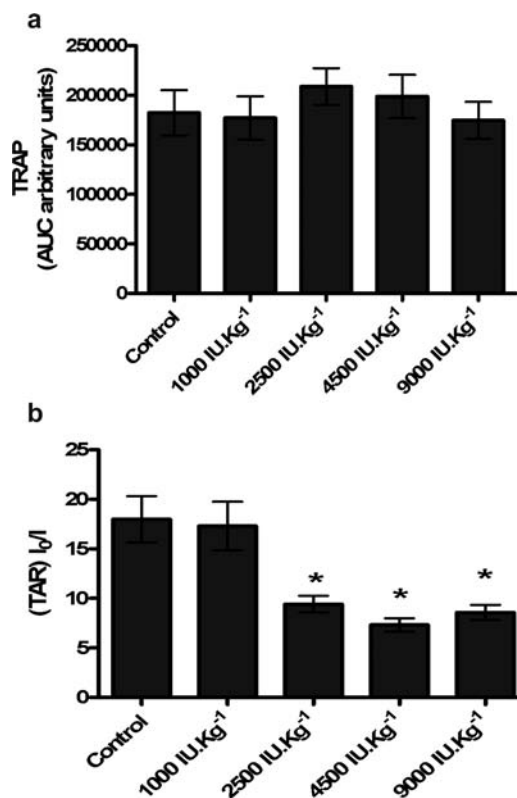


Figure 3. (A) TRAP ($n=7$ for each group) and (B) TAR ($n=7$ for each group). Data are expressed as mean \pm standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

[AQ4]

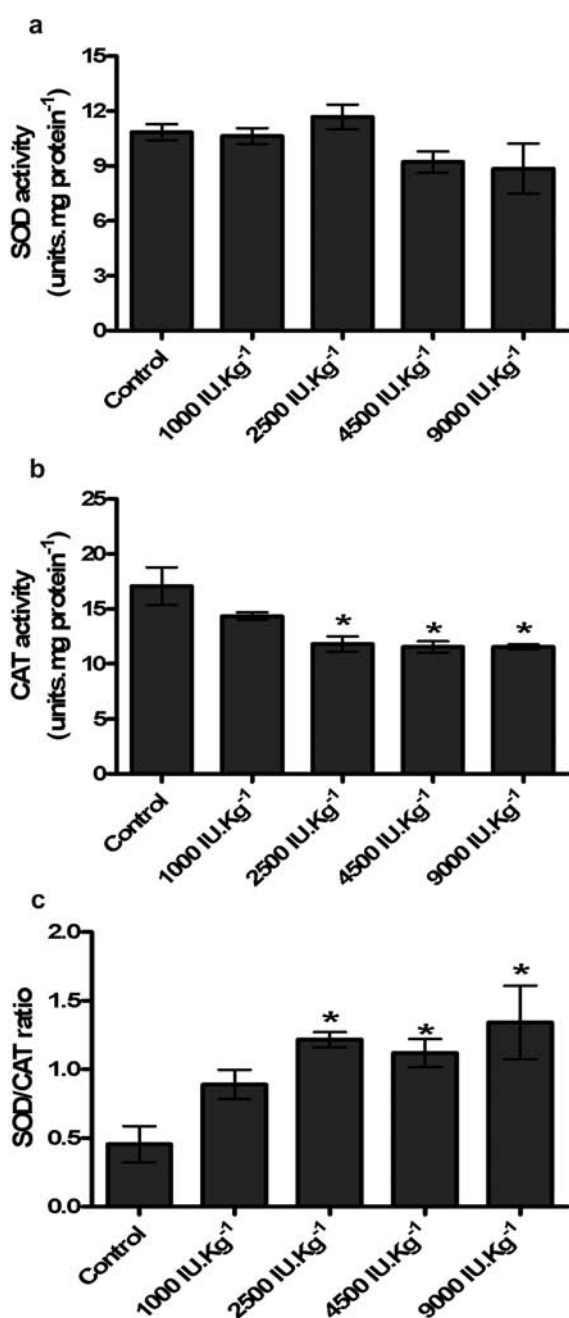


Figure 4. (A) SOD activity ($n=7$ for each group), (B) CAT activity ($n=7$ for each group) and (C) SOD/CAT ratio ($n=7$ for each group). Data are expressed as mean \pm standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted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

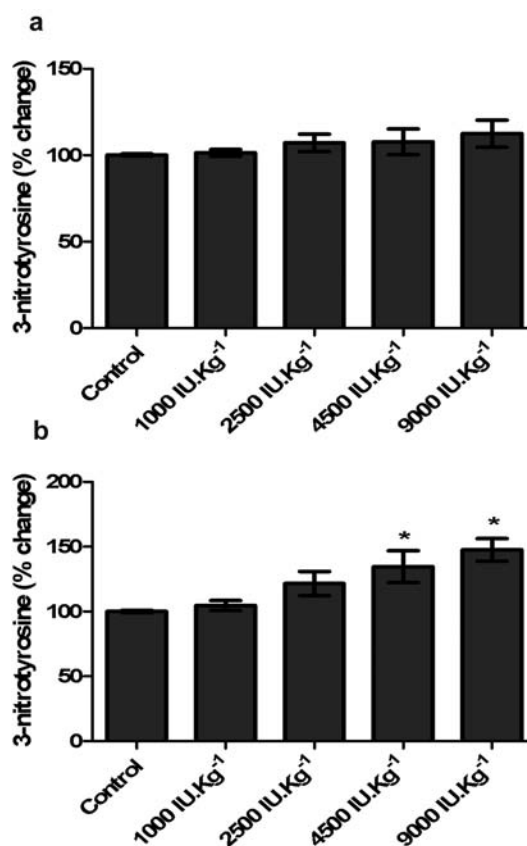


Figure 5. (A) Total 3-nitrotyrosine ($n=5$ for each group) and (B) MMEF nitrotyrosine ($n=5$ for each group). Data are expressed as mean \pm standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level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

Discussion

The present work evaluated the effects of a long-term retinyl palmitate supplementation on rat heart, regarding oxidative/nitrosative 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].

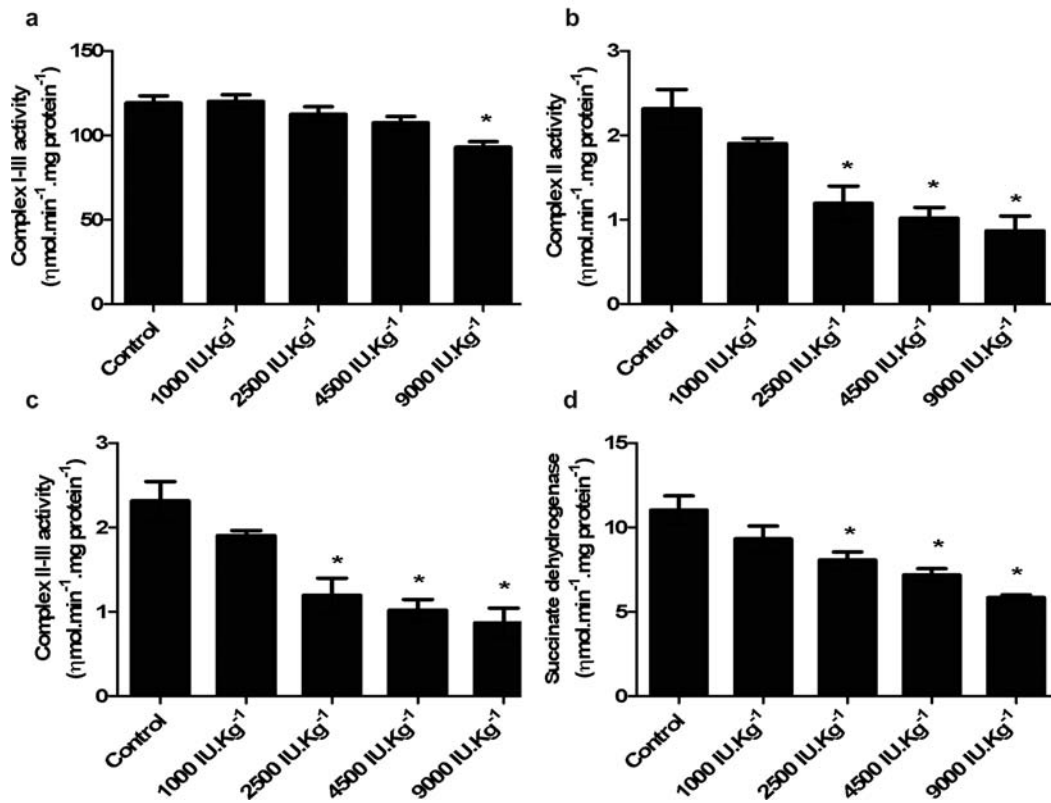


Figure 6. (A) Complex I–III activity ($n=5$ for each group), (B) Complex II–III activity ($n=5$ for each group), (C) Complex II activity ($n=5$ for each group) and (D) SDH activity ($n=5$ for each group). Data are expressed as mean \pm standard error of mean. *Different to control. Differences were determined 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 $\cdot\text{O}_2^-$ 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 its presence can generate the most potent free radical, the $\cdot\text{OH}$ (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 $\cdot\text{O}_2^-$ and the radical nitric oxide ($\cdot\text{NO}$) [39]. Prior studies have also demonstrated nitrosative stress induced by retinol treatment [6,7].

Additionally, as the respiratory chain is an important source of $\cdot\text{O}_2^-$, 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 $\cdot\text{O}_2^-$ [37,41].

Taken together, our results suggest that the pro-oxidants effects of vitamin A are related to an impairment on mitochondrial level, since the respiratory chain is inhibited and there is an increase on MMEF 3-nitrotyrosine. However, we have here a possible cycle, in which we do not know where it begins. As a first hypothesis, we believe that retinol can directly inhibit the respiratory chain complexes, because it was previously demonstrated that their decreased activities are associated to increased $\cdot\text{O}_2^-$ production. The $\cdot\text{O}_2^-$, generated by complexes inhibition, could then activate the nitric oxide synthase (NOS), increasing $\cdot\text{NO}$ production. So, these two

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1 molecules ($\cdot\text{O}_2^-$ and $\cdot\text{NO}$) can react, forming ONOO^-
 2 [42–44]. The second hypothesis is that the cycle begin-
 3 ning would be the direct activation of NOS by retinol
 4 and the resulting $\cdot\text{NO}$ could inhibit the respiratory
 5 chain complexes activities [41,45–48]. However, more
 6 studies are needed to confirm one of these theories.

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

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23
 24
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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 nossos 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 glutathione, 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 imunoc conteúdo 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 sistemas enzimáticos variam de um tecido para o outro, já que em células de Sertoli há um aumento na atividade CAT (associado com aumento de imunoc conteúdo), enquanto em pulmão de animais tratados com palmitato de retinol ocorre uma diminuição na atividade (também associada com diminuição do imunoc conteúdo) (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 óxido 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 circulatorio.

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 mitochondrial 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 Bioquímica	UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
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PARECER CIRCUNSTANCIADO

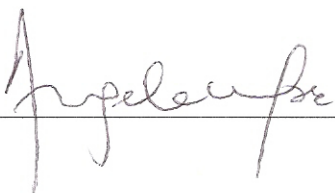
O projeto de pesquisa intitulado “**Efeito do tratamento com palmitato de retinol sobre parâmetros de estresse oxidativo na artéria aorta e no coração de ratos Wistar**”, do aluno do PPG-Ciências Biológicas-Bioquímica **Ricardo Fagundes da Rocha** sob orientação do PROF. Dr(a). José Cláudio Fonseca Moreira, matriculado ao PPG Ciências Biológicas – Bioquímica 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.

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24-Dec-2009

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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.

CERTIFICADO

Certificamos que **RICARDO FAGUNDES DA ROCHA**

Participou da ação de extensão universitária - modalidade CURSO PRESENCIAL,

RADICAIS LIVRES : VERDADES, FALÁCIAS, ANGUSTIAS E EXPECTATIVAS

Transcorrida no período de **08/08/2008 a 31/10/2008** com carga horária total de **225h**

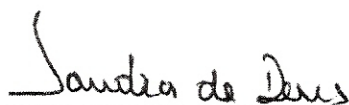
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Porto Alegre, 19 de Maio de 2009



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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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Thank you for your fine contribution.

Sincerely,

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

Referee(s)' Comments to Author:

Reviewing: 1

Comments to the Author

Dear author,

Analysing your modifications and answers to my comments I have suggested the

manuscript to be accepted by the editor.
regards

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