Free Radical Research, May 2010; 44(5): 505–512

informa
healthcare

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 ROCHA[1], MARCOS ROBERTO DE OLIVEIRA[1], PATRÍCIA SCHONHOFEN[1], CARLOS EDUARDO SCHNORR[1], FELIPE DAL PIZZOL[2] & JOSÉ CLÁUDIO F. MOREIRA[1]

[1]*Center of Oxidative Stress Research, Professor Tuiskon Dick Departament of Biochemistry, Institute of Health Basic Sciences, Federal University of the Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil, and* [2]*Laboratory of Experimental Physiopathology, Departament of Medicine, University of the Extremo-Sul Catarinense (UNESC), Criciúma, Santa Catarina, Brazil*

(Received date: 24 December 2009; In revised form date: 17 January 2010)

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

Correspondence: Ricardo F. Da Rocha, Laboratório 32, Departamento de Bioquímica, Campus da Saúde, Rua Ramiro Barcelos, número 2600, prédio Anexo, bairro Santa Cecília, CEP 90035-003, Brazil. Tel: +55 051 33085577; +55 051 33085578. Fax: +55 051 33085540. Email: ricardorocha21@hotmail.com; 00140244@ufrgs.br

ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK Ltd. (Informa Healthcare, Taylor Francis AS)
DOI: 10.3109/10715761003636849

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

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

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

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

Materials and methods

Animals

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

Experimental design and treatment

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

Samples preparation

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

Redox status and damage

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

Non-enzymatic antioxidant potential

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

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

Enzymes activity

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

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

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

Mitochondrial electrons transfer chain (METC)

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

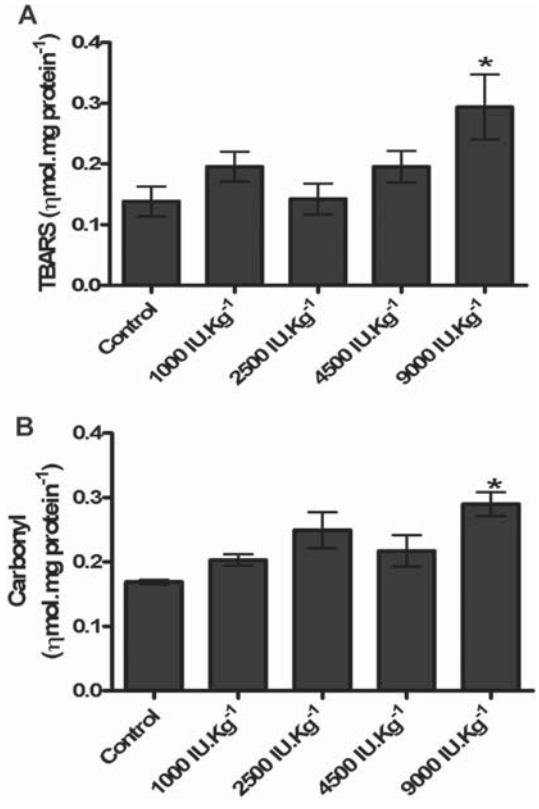

Figure 1. (A) TBARS levels ($n=7$ for each group) and (B) Carbonyl content ($n=7$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

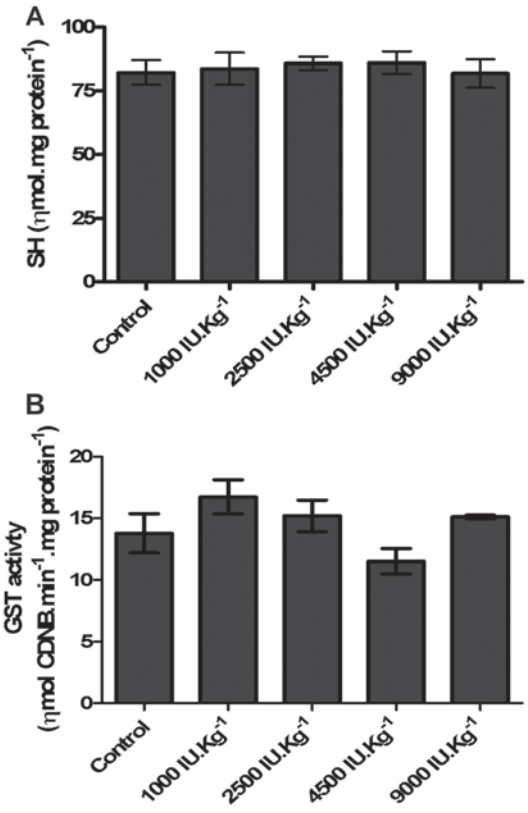

Figure 2. (A) SH content ($n=7$ for each group) and (B) GST activity ($n=5$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

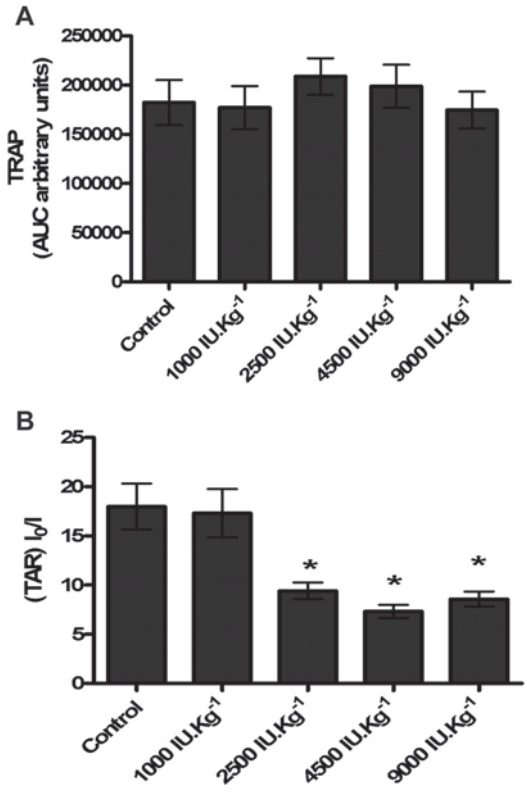

Figure 3. (A) TRAP ($n=7$ for each group) and (B) TAR ($n=7$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was $p \leq 0.05$.

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

Statistical analysis

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

Results

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

The non-enzymatic antioxidant properties were evaluated by TRAP (antioxidant capacity more related to antioxidants amount) and TAR (antioxidant capacity more related to antioxidant quality), but only for TAR were differences observed, which happened at the three higher doses (Figure 3). Antioxidants enzymes activities were also analysed and no modifications were observed for SOD activity, while CAT activity decreased at the three higher doses, which yielded an enzymatic imbalance that can be seen by the increased SOD/CAT ratio (Figure 4).

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,

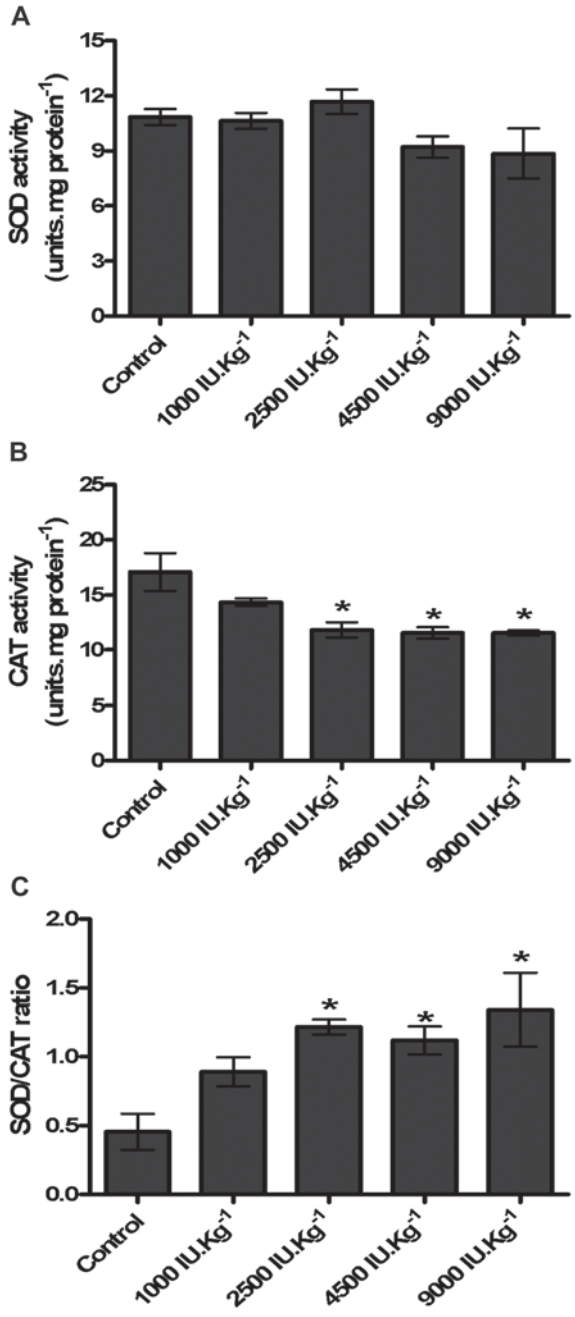

Figure 4. (A) SOD activity ($n=7$ for each group), (B) CAT activity ($n=7$ for each group) and (C) SOD/CAT ratio ($n=7$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted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

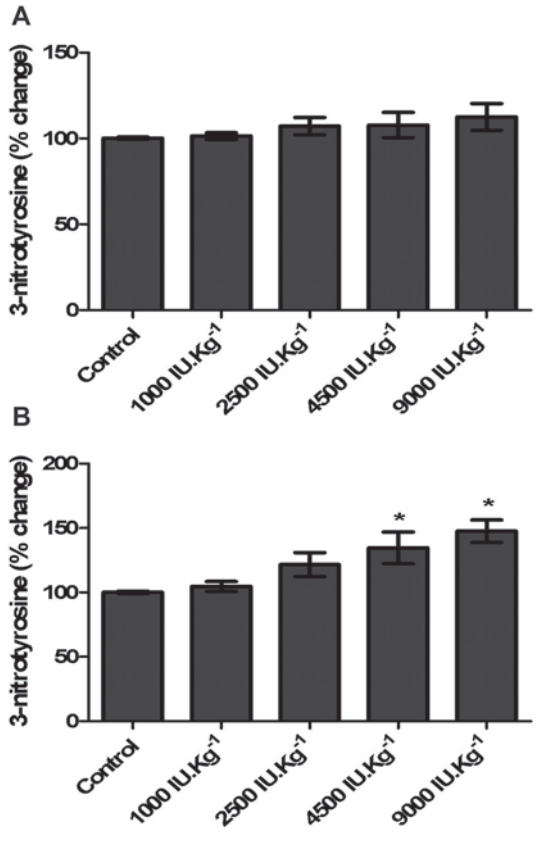

Figure 5. (A) Total 3-nitrotyrosine ($n=5$ for each group) and (B) MMEF nitrotyrosine ($n=5$ for each group). Data are expressed as mean ± standard error of mean. *Different to control. Differences were determined by Anova one-way followed by Dunnet's *post hoc* and the accepted significance level was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

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).

Discussion

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

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

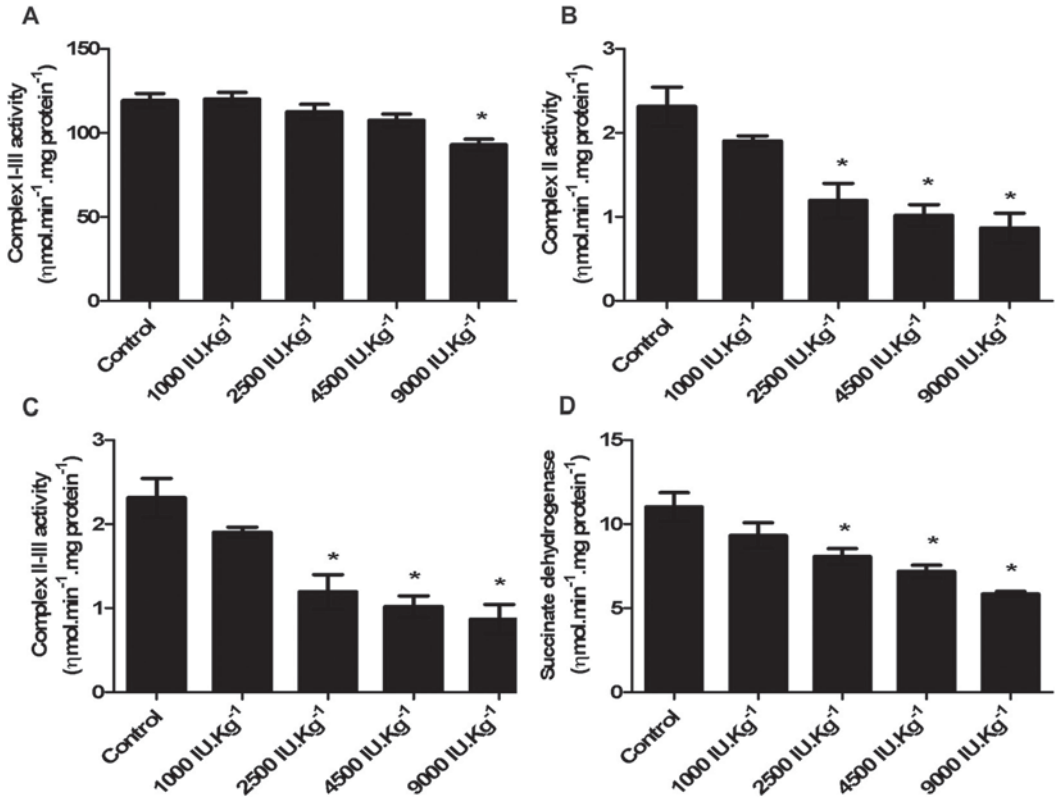

Figure 6. (A) Complex I–III activity ($n=5$ for each group), (B) Complex II–III activity ($n=5$ for each group), (C) Complex II activity ($n=5$ for each group) and (D) SDH activity ($n=5$ for each group). Data are expressed as mean ± standard error of mean.*Different to control. Differences were determined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

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

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

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

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

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

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

Acknowledgements

This work was supported by grants from a Brazilian public agency, *Conselho Nacional de Desenvolvimento Científico e Tecnológico*/National Council for Research and Development (CNPq) and from *Rede Instituto Brasileiro de Neurociência*/Net Brazilian Institute of Neuroscience (IBN-Net) – 01.06.0842-00. We would also like to thank the UFRGS, a public university from Brazil, where the study was performed.

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

References

[1] Sporn MBRAB, Goodman DS. The retinoids: Biology, chemistry and medicine. New York: Raven Press; 1994.

[2] Dal-Pizzol F, Klamt F, Benfato MS, Bernard EA, Moreira JC. Retinol supplementation induces oxidative stress and modulates antioxidant enzyme activities in rat sertoli cells. Free Radic Res 2001;34:395.

[3] Dal-Pizzol F, Klamt F, Frota ML, Jr., Moraes LF, Moreira JC, Benfato MS. Retinol supplementation induces DNA damage and modulates iron turnover in rat Sertoli cells. Free Radic Res 2000;33:677.

[4] Klamt F, Dal-Pizzol F, Bernard EA, Moreira JC. Enhanced UV-mediated free radical generation; DNA and mitochondrial damage caused by retinol supplementation. Photochem Photobiol Sci 2003;2:856.

[5] Zanotto-Filho A, Gelain DP, Schroder R, Souza LF, Pasquali MA, Klamt F, Moreira JC. The NF kappa B-mediated control of RS and JNK signaling in vitamin A-treated cells: duration of JNK-AP-1 pathway activation may determine cell death or proliferation. Biochem Pharmacol 2009;77:1291.

[6] De Oliveira MR, Oliveira MW, Da Rocha RF, Moreira JC. Vitamin A supplementation at pharmacological doses induces nitrosative stress on the hypothalamus of adult Wistar rats. Chem Biol Interact 2009;180:407.

[7] de Oliveira MR, Oliveira MW, Behr GA, Hoff ML, da Rocha RF, Moreira JC. Evaluation of the effects of vitamin A supplementation on adult rat substantia nigra and striatum redox and bioenergetic states: mitochondrial impairment, increased 3-nitrotyrosine and alpha-synuclein, but decreased D2 receptor contents. Prog Neuropsychopharmacol Biol Psychiatry 2009; 33:353.

[8] De Oliveira MR, Moreira JC. Impaired redox state and respiratory chain enzyme activities in the cerebellum of vitamin A-treated rats. Toxicology 2008;253:125.

[9] de Oliveira MR, Silvestrin RB, Mello e Souza T, Moreira JC. Therapeutic vitamin A doses increase the levels of markers of oxidative insult in substantia nigra and decrease locomotory and exploratory activity in rats after acute and chronic supplementation. Neurochem Res 2008;33:378.

[10] de Oliveira MR, Silvestrin RB, Mello EST, Moreira JC. Oxidative stress in the hippocampus, anxiety-like behavior and decreased locomotory and exploratory activity of adult rats: effects of sub acute vitamin A supplementation at therapeutic doses. Neurotoxicology 2007;28:1191.

[11] Zanotto-Filho A, Schroder R, Moreira JC. Xanthine oxidase-dependent ROS production mediates vitamin A prooxidant effects in cultured Sertoli cells. Free Radic Res 2008; 42:593.

[12] Omenn GS, Goodman G, Thornquist M, Grizzle J, Rosenstock L, Barnhart S, Balmes J, Cherniack MG, Cullen MR, Glass A, et al. The beta-carotene and retinol efficacy trial (CARET) for chemoprevention of lung cancer in high risk populations: smokers and asbestos-exposed workers. Cancer Res 1994;54:2038s.

[13] Lam HS, Chow CM, Poon WT, Lai CK, Chan KC, Yeung WL, Hui J, Chan AY, Ng PC. Risk of vitamin A toxicity from candy-like chewable vitamin supplements for children. Pediatrics 2006;118:820.

[14] Myhre AM, Carlsen MH, Bohn SK, Wold HL, Laake P, Blomhoff R. Water-miscible, emulsified, and solid forms of retinol supplements are more toxic than oil-based preparations. Am J Clin Nutr 2003;78:1152.

[15] Montrone M, Martorelli D, Rosato A, Dolcetti R. Retinoids as critical modulators of immune functions: new therapeutic perspectives for old compounds. Endocr Metab Immune Disord Drug Targets 2009;9:113.

[16] O'Neil C, Shevill E, Chang AB. Vitamin A supplementation for cystic fibrosis. Cochrane Database Syst Rev 2008; CD006751.

[17] Smith MA, Parkinson DR, Cheson BD, Friedman MA. Retinoids in cancer therapy. J Clin Oncol 1992;10:839.

[18] Van Zander J, Orlow SJ. Efficacy and safety of oral retinoids in psoriasis. Expert Opin Drug Saf 2005;4:129.

[19] Wang HJ, Zhu YC, Yao T. Effects of all-trans retinoic acid on angiotensin II-induced myocyte hypertrophy. J Appl Physiol 2002;92:2162.

[20] Palace VP, Hill MF, Farahmand F, Singal PK. Mobilization of antioxidant vitamin pools and hemodynamic function after myocardial infarction. Circulation 1999;99:121.

[21] Palace VP, Hill MF, Khaper N, Singal PK. Metabolism of vitamin A in the heart increases after a myocardial infarction. Free Radic Biol Med 1999;26:1501.

[22] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265.

[23] Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 1990;186:421.

[24] Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990;186:464.

[25] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70.

[26] Lissi E, Pascual C, Del Castillo MD. Luminol luminescence induced by 2,2'-Azo-bis(2-amidinopropane) thermolysis. Free Radic Res Commun 1992;17:299.

[27] Dresch MT, Rossato SB, Kappel VD, Biegelmeyer R, Hoff ML, Mayorga P, Zuanazzi JA, Henriques AT, Moreira JC. Optimization and validation of an alternative method to evaluate total reactive antioxidant potential. Anal Biochem 2009;385:107.

[28] Lissi E, Salim-Hanna M, Pascual C, del Castillo MD. Evaluation of total antioxidant potential (TRAP) and total antioxidant

reactivity from luminol-enhanced chemiluminescence measurements. Free Radic Biol Med 1995;18:153.
[29] Bannister JV, Calabrese L. Assays for superoxide dismutase. Methods Biochem Anal 1987;32:279.
[30] Aebi H. Catalase *in vitro*. Methods Enzymol 1984;105:121.
[31] Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130.
[32] Schapira AH, Mann VM, Cooper JM, Dexter D, Daniel SE, Jenner P, Clark JB, Marsden CD. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 1990;55:2142.
[33] Fischer JC, Ruitenbeek W, Berden JA, Trijbels JM, Veerkamp JH, Stadhouders AM, Sengers RC, Janssen AJ. Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin Chim Acta 1985;153:23.
[34] Pasquali MA, Gelain DP, Oliveira MR, Behr GA, Motta LL, Rocha RF, Klamt F, Moreira JC. Vitamin a supplementation induces oxidative stress and decreases the immunocontent of catalase and superoxide dismutase in rat lungs. Exp Lung Res 2009;35:427.
[35] de Oliveira MR, Soares Oliveira MW, Muller Hoff ML, Behr GA, da Rocha RF, Fonseca Moreira JC. Evaluation of redox and bioenergetics states in the liver of vitamin A-treated rats. Eur J Pharmacol 2009;610:99.
[36] Meyer AJ, Hell R. Glutathione homeostasis and redox-regulation by sulfhydryl groups. Photosynth Res 2005; 86:435.
[37] Gutteridge BHAJMC. Free radicals in biology and medicine. New York: Oxford University Press Inc.; 2001.
[38] Peluffo G, Radi R. Biochemistry of protein tyrosine nitration in cardiovascular pathology. Cardiovasc Res 2007;75:291.
[39] Ferrer-Sueta G, Radi R. Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. ACS Chem Biol 2009;4:161.
[40] De Oliveira MR, Oliveira MW, Behr GA, Moreira JC. Vitamin A supplementation at clinical doses induces a dysfunction in the redox and bioenergetics states, but did change neither caspases activities nor TNF-alpha levels in the frontal cortex of adult Wistar rats. J Psychiatr Res 2009;43:754.
[41] Poderoso JJ, Carreras MC, Lisdero C, Riobo N, Schopfer F, Boveris A. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. Arch Biochem Biophys 1996; 28:85.
[42] McPherson DB, Kilker RP, Foley TD. Superoxide activates constitutive nitric oxide synthase in a brain particulate fraction. Biochem Biophys Res Commun 2002;296:413.
[43] Mittal CK. Nitric oxide synthase: involvement of oxygen radicals in conversion of L-arginine to nitric oxide. Biochem Biophys Res Commun 1993;193:126.
[44] Manning P, Cookson MR, McNeil CJ, Figlewicz D, Shaw PJ. Superoxide-induced nitric oxide release from cultured glial cells. Brain Res 2001;911:203.
[45] Achan V, Tran CT, Arrigoni F, Whitley GS, Leiper JM, Vallance P. All-trans-Retinoic acid increases nitric oxide synthesis by endothelial cells: a role for the induction of dimethylarginine dimethylaminohydrolase. Circ Res 2002; 90:764.
[46] Boveris A, Costa LE, Poderoso JJ, Carreras MC, Cadenas E. Regulation of mitochondrial respiration by oxygen and nitric oxide. Ann NY Acad Sci 2000;899:121.
[47] Riobo NA, Clementi E, Melani M, Boveris A, Cadenas E, Moncada S, Poderoso JJ. Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. Biochem J 2001;359:139.
[48] Carreras MC, Franco MC, Peralta JG, Poderoso JJ. Nitric oxide, complex I, and the modulation of mitochondrial reactive species in biology and disease. Mol Aspects Med 2004;25:125.