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Evaluation of redox and bioenergetics states in the liver of vitamin A-treated rats

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ABSTRACT

Vitamin A is normally stored in the mammalian liver and is physiologically released depending on the need of the organism for the vitamin. However, there is a compelling evidence showing that even the liver is affected by conditions of high vitamin A intake. Based on these previously reported findings showing negative effects of vitamin A on mammalian tissues, we have investigated the effects of a supplementation with vitamin A at clinical doses (1000–9000 IU/kg day^{-1}) on some rat liver parameters. We have analyzed hepatic redox environment, as well as the activity of the mitochondrial electron transfer chain in vitamin A-treated rats. Additionally, activity of the detoxifying enzyme glutathione S-transferase was checked. Also, caspase-3 and caspase-8 and tumor necrosis factor-α levels were quantified to assess either cell death or inflammation effects of vitamin A on rat liver. We found increased free radical production and, consequently, increased oxidative damage in biomolecules in the liver of vitamin A-treated rats. Interestingly, we found increased mitochondrial electron transfer chain activity, as well as glutathione-S-transferase enzyme activity. Neither caspases activity, nor tumor necrosis factor-α levels change in this experimental model. Our results suggest a pro-oxidant, but not pro-inflammatory effect of vitamin A on rat liver.

1. Introduction

The liver is the main site of vitamin A storage in mammalian organisms (Napoli, 1999; Ross and Zolfaghari, 2004). Its function in regulating vitamin A metabolism is crucial to the physiology of other tissues. On the other hand, vitamin A is equally important to hepatic homeostasis. It was demonstrated, for example, that vitamin A normal levels are required to maintain rat liver mitochondria morphology, which is closely related to the mitochondrial electron transfer chain activity (Seward et al., 1966; Esteronell et al., 2000). Furthermore, it was reported that either vitamin A deficiency or hypervitaminosis A (200,000 IU/kg/day) impairs cell cycle in adult rat liver and lung, the former inducing cell cycle arrest and the later favoring a proliferative state in both liver and lung cells (Borrás et al., 2003). Indeed, in patients submitted to chronic vitamin A treatment, it was observed that hepatotoxicity is sometimes followed by mild cognitive impairments (Myhre et al., 2003). These findings suggest that vitamin A levels modulate a wide range of hepatic parameters that could culminate in toxicity.

The ability to alter the redox environment in *in vitro* and *in vivo* experimental models is also a consequence of increasing vitamin A concentration. It was demonstrated that retinol (vitamin A) induces oxidative DNA damage through increased superoxide anion radical ($O_2^{-\bullet}$) production (Murata and Kawanishi, 2000). We have reported that vitamin A induces lipid peroxidation, protein carbonylation, DNA oxidative damage, and modulates the activity of antioxidants enzymes in cultured Sertoli cells (Dal-Pizzol et al., 2001; Pasquali et al., 2008). Additionally, $O_2^{-\bullet}$ production was found in rat liver mitochondria treated with increasing doses of vitamin A, consequently inducing mitochondrial lipid peroxidation and swelling of the organelle, which resulted in an increase in mitochondrial cytochrome *c* release, a pro-apoptotic factor (Klamt et al., 2005). Recently, it has been shown that retinol induces apoptotic cell death in cultured Sertoli cells through an oxidative stress-related process (Klamt et al., 2008).

Based on previously reported works demonstrating the pro-oxidant effects of vitamin A on rat tissues, we investigated here the effects of a chronic supplementation (28 days) with retinol palmitate, a vitamin A supplement commercially available at drug stores, at pharmacological doses (1000–9000 IU/kg day^{-1}) on the hepatic redox state, mitochondrial electron transfer chain activity, caspases-3 and -8 enzymes activities, and tumor necrosis factor-α levels in adult Wistar rats. The retinol palmitate doses tested here are within a therapeutic range commonly applied in cancer treatment in both children and adult humans (Fenaux et al., 2001; Allen and Haskell, 2002; Myhre et al., 2003; Mactier and Weaver, 2005).

2. Experimental procedures

2.1. Animals

Adult male Wistar rats (290–320 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle

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(7:00–19:00 h), at a temperature-controlled colony room (23 ± 1 °C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80–23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. Our research protocol was approved by the Ethical Committee for animal experimentation of the Federal University of Rio Grande do Sul.

2.2. Drugs and reagents

Arovit® (retinol palmitate, a water-soluble form of vitamin A) was purchased from Roche, Sao Paulo, SP, Brazil. Caspase-8 activity assay kit was purchased from Biotium, Inc., Hayward, CA, USA. Tumor necrosis factor-α assay kit was obtained from BD Biosciences, San Diego, CA, USA. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A treatment was prepared daily and it occurred by protecting vitamin A from light.

2.3. Treatment

The animals were treated once a day for 28 days. All treatments were carried out at night (*i.e.* when the animals are more active and take a greater amount of food) in order to ensure maximum vitamin A absorption, since this vitamin is better absorbed during or after a meal. The animals were treated with vehicle (0.15 M saline; $n = 8$–10 animals), 1000 ($n = 10$), 2500 ($n = 10$), 4500 ($n = 10$), or 9000 IU/kg ($n = 10$) of retinol palmitate (vitamin A) orally via a metallic gastric tube (gavage) in a maximum volume of 0.6 ml. Adequate measures were taken to minimize pain or discomfort.

2.4. Oxidative stress and antioxidant enzyme activities analyses

Before sacrifice, the animals were anesthetized with ketamine plus xylazine (100 mg/kg and 14 mg/kg, respectively). The animals were sacrificed by decapitation at 24 h after the last vitamin A administration. The liver was dissected out in ice immediately after the rat was sacrificed and stored at −80 °C for posterior oxidative stress analyses. The homogenates were centrifuged (700 *g*, 5 min at 4 °C) to remove cellular debris. Supernatants were used to all biochemical assays described herein. All the results were normalized by the protein content using bovine albumin as standard (Lowry et al., 1951).

2.4.1. Thiobarbituric acid reactive species

As an index of lipid peroxidation, we used the formation of thiobarbituric acid reactive species during an acid-heating reaction, which is widely adopted for measurement of lipid redox state, as previously described (Draper and Hadley, 1990). The samples were mixed with 0.6 ml of 10% trichloroacetic acid and 0.5 ml of 0.67% thiobarbituric acid, and then heated in a boiling water bath for 25 min. Thiobarbituric acid reactive species were determined by the absorbance in a spectrophotometer at 532 nm. Results are expressed as thiobarbituric acid reactive species /mg protein.

2.4.2. Measurement of protein carbonyls

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhidrazine as previously described (Levine et al., 1990). Proteins were precipitated by the addition of 20% trichloroacetic acid and redissolved in dinitrophenylhidrazine and the absorbance read in a spectrophotometer at 370 nm. Results are expressed as nmol carbonyl/mg protein.

2.4.3. Measurement of protein thiol content

Briefly, an aliquot was diluted in SDS 0.1% and 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid in ethanol was added and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min (Ellman, 1959). Results are expressed as μmol SH/mg protein.

2.4.4. Enzyme-linked immunosorbent assay (ELISA) to 3-nitrotyrosine

Indirect ELISA assay was performed to analyze changes in the content of nitrotyrosine by utilizing a polyclonal antibody to nitrotyrosine (Calbiochem) diluted 1:5000 in phosphate-buffered saline (PBS) pH 7.4 with 5% albumin. Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in PBS with 5% albumin. Plates were then washed four times with wash buffer (PBS with 0.05% Tween-20), and the specific antibodies were added to the plates for 2 h at room temperature. After washing (seven times) with wash buffer, a second incubation with anti-rabbit antibody peroxidase conjugated (diluted 1:10,000) for 1 h at room temperature was carried out. After addition of substrates (hydrogen peroxide and 3, 3′, 5, 5′-tetramethylbenzidine 1:1 v:v), the samples were read at 450 nm in a plate spectrophotometer. Results are expressed as changes in percentage among the groups (compared to control group).

2.4.5. Antioxidant enzyme activities estimations

Superoxide dismutase enzyme activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described (Misra and Fridovich, 1972), and the results are expressed as U/mg protein. Catalase activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm (Aebi, 1984), and the results are expressed as U/mg protein. A ratio between superoxide dismutase and catalase enzyme activities were applied to better understand the effect of vitamin A-supplementation upon these two oxidant-detoxifying enzymes that work in sequence converting superoxide anion to water (Halliwell and Gutteridge, 1999). An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

2.5. Oxidative parameters in submitochondrial particles

To obtain submitochondrial particles, liver was dissected and homogenized in 230 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl and 1 mM EDTA (pH 7.4). Freezing and thawing (three times) the mitochondrial solution gave rise to superoxide dismutase-free submitochondrial particles. The submitochondrial particles solution was also washed (twice) with 140 mM KCl, 20 mM Tris–HCl (pH 7.4) to ensure Mn-superoxide dismutase release from mitochondria. To quantify superoxide ($O_2^{-\bullet}$) production, submitochondrial particles was incubated in reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4, 0.1 μM catalase, and 1 mM epinephrine, and the increase in the absorbance (auto-oxidation of adrenaline to adrenochrome) was read in a spectrophotometer at 480 nm at 32 °C, as previously described (Poderoso et al., 1996; De Oliveira and Moreira, 2007). As a marker of lipid peroxidation, we measured the formation of thiobarbituric acid reactive species during an acid-heating reaction, as previously described (Draper and Hadley, 1990). The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine as previously described above (Levine et al., 1990). Protein thiol content in liver submitochondrial particles samples was determined as described above. Briefly, an aliquot was diluted in SDS 0.1% and 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid in ethanol was added and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min (Ellman, 1959).

2.6. Total radical-trapping antioxidant parameter

The non-enzymatic antioxidant cellular defenses were estimated by the total radical-trapping antioxidant parameter, which determines

the non-enzymatic antioxidant potential of the sample, as previously described (Wayner et al., 1985). Briefly, the reaction was initiated by injecting luminol and 2,2′-Azobis[2-methylpropionamidine]dihydrochloride–a free radical source that produces peroxyl radical at a constant rate–in glycine buffer (0.1 M, pH 8.6), resulting in a steady luminescent emission. Hepatic samples (30 μg of protein) were mixed in glycine buffer in the reaction vial and the decrease in luminescence monitored in a liquid scintillation counter for 60 min after the addition of the sample homogenates. The area under the curve obtained of the chemiluminescence values were transformed to percentage values and compared against the control values.

2.7. Mitochondrial electron transfer chain activity

To obtain submitochondrial particles from rat liver in order to assess mitochondrial electron transfer chain activity, we have homogenized the tissue in 250 mM sucrose, 2 mM EDTA, 10 mM Tris, pH 7.4, and 50 IU/ml heparin buffer. The samples were centrifuged 1000×*g* and the supernatants were colleted. Then, the samples were frozen and thawed three times, and mitochondrial electron transfer chain activity detection was performed as described below.

2.7.1. Complex I-CoQ-III activity

Complex I-CoQ-III activity was determined by following the increase in absorbance due to reduction of cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\in = 19.1\ mM^{-1}\ cm^{-1}$). The reaction buffer contained 20 mM potassium phosphate, pH 8.0, 2.0 mM KCN, 10 μM EDTA, 50 μM cytochrome *c*, and 20–45 μg supernatant protein. The reaction started by addition of 25 μM NADH and was monitored at 30 °C for 3 min before the addition of 10 μM rotenone, after the which the activity was monitored for an additional 3 min. Complex I–III activity was the rotenone-sensitive NADH:cytochrome *c* oxidoreductase activity (Shapira et al., 1990).

2.7.2. Complex II and succinate dehydrogenase activities

Complex II (succinate-DCPIP-oxidoreductase) activity was measured by following the decrease in absorbance due to the reduction of 2,6-dichloroindophenol at 600 nm with 700 nm as reference wavelength ($\in = 19.1\ mM^{-1}\ cm^{-1}$). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8.0 μM 2,6-dichloroindophenol was preincubated with 48–80 μg supernatant protein at 30 °C for 20 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction was started by addition of 40 μM 2,6-dichloroindophenol and was monitored for 5 min at 30 °C. Succinate dehydrogenase activity was assessed by adding 1 mM phenazine methasulphate to the reaction mixture. Then, succinate dehydrogenase activity was monitored for 5 min at 30 °C at 600 nm with 700 nm as reference wavelength (Fischer et al., 1985).

2.7.3. Complex II-CoQ-III activity

Complex II-CoQ-III activity was measured by following the increase in absorbance due to the reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength ($\in = 21\ mM^{-1}\ cm^{-1}$). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate was preincubated with 50–100 μg supernatant protein at 30 °C for 30 min. Subsequently, 4.0 mM sodium azide and 7.0 μM rotenone were added and the reaction started by the addition of 0.6 μg/ml cytochrome *c* and monitored for 5 min at 30 °C (Fischer et al., 1985).

2.7.4. Complex IV activity

Complex IV activity was measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* at 550 nm with 580 nm as reference wavelength ($\in = 19.15\ mM^{-1}\ cm^{-1}$). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl-β-D-maltoside, 2–4 μg supernatant protein and the reaction was started with addition of 0.7 μg reduced cytochrome *c*. The activity of complex IV was measured at 25 °C for 10 min (Rustin et al., 1994).

2.8. Glutathione-S-transferase activity

Glutathione-S-transferase activity was determined spectrophotometrically according to the method of Habig et al., 1974. Glutathione-S-transferase activity was quantified in liver homogenates in a reaction mixture containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 1 mM glutathione as substrates in 0.1 M sodium phosphate buffer, pH 6.5, at 37 °C. Enzyme activity was calculated by the change in the absorbance value from the slope of the initial linear portion of the absorbance time curve at 340 nm for 5 min. Enzyme activity was expressed as nmol of CDNB conjugated with glutathione/min mg^{-1} protein.

2.9. Caspase-3 activity

Caspase-3 activity was determined through a fluorimetric commercial kit according manufacturer's instructions (Sigma). Briefly, the samples were homogenized in lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT), and centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatants were used to determine caspase-3 assay in a microplate fluorimeter at 360 nm excitation and 460 nm emission for 180 min at 25 °C. Results are expressed as nmol AMC produced/min mg^{-1} protein.

2.10. Caspase-8 activity

Caspase-8 activity was determined through a colorimetric commercial kit according manufacturer's instructions (Biotium). The samples were prepared as described to investigate caspase-3 activity. However, caspase-8 activity was monitored in a microplate spectrophotometer at 495 nm for 180 min at 25 °C. Results are expressed as nmol R110 produced/min mg^{-1} protein.

2.11. Tumor necrosis factor-α quantification

We have measured tumor necrosis factor-α through commercial kit for enzyme-linked immunosorbent assay according manufacturer's instructions (BD Biosciences). Briefly, tissue samples were collected and suspended in lysis buffer containing protease inhibitors. Following cell lysis, the homogenate was centrifuged, and a portion of the

Table 1
The effects of vitamin A supplementation on rat liver oxidative stress markers levels.

Groups	Lipid peroxidation (nmol TBARS/mg protein)	Protein carbonylation (nmol/mg protein)	Protein thiol content (μmol sulfhydryl/mg protein)	3-Nitrotyrosine content (% change)
Control	7.33 ± 1.05	1.84 ± 0.18	160.5 ± 26.35	100.7 ± 5.5
1000 IU/kg day^{-1}	7.72 ± 1.83	1.92 ± 0.2	181.3 ± 16.2	114.0 ± 7.81
2500 IU/kg day^{-1}	8.0 ± 1.16	1.79 ± 0.22	166.2 ± 20.8	118.9 ± 20.1
4500 IU/kg day^{-1}	11.0 ± 1.34	1.79 ± 0.18	164.3 ± 22.3	141.0 ± 17.7[a]
9000 IU/kg day^{-1}	12.11 ± 1.79[a]	2.23 ± 0.3	173.7 ± 22.2	145.2 ± 7.8[a]

Values are means ± S.D. of 8–10 animals per group.
[a] $P<0.05$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

Table 2
The effects of vitamin A supplementation on rat liver antioxidant and detoxification enzyme activities.

Groups	Superoxide dismutase (U/mg protein)	Catalase (U/mg protein)	SOD/CAT ration (arbitrary units)	Glutathione-S-transferase (nmol CDNB/min mg protein)
Control	29.9 ± 5.1	39.2 ± 3.11	0.76 ± 0.07	372.15 ± 24.9
1000 IU/kg day^{-1}	36.0 ± 7.51	50.5 ± 6.7	0.72 ± 0.2	440.1 ± 153.9
2500 IU/kg day^{-1}	40.21 ± 9.42	49.0 ± 10.1	0.85 ± 0.3	485.3 ± 189.1
4500 IU/kg day^{-1}	55.8 ± 12.2[a]	54.07 ± 10.1	1.02 ± 0.03	553.2 ± 98.2[a]
9000 IU/kg day^{-1}	58.2 ± 4.9[a]	55.9 ± 2.94	1.04 ± 0.14	575.4 ± 28.4[a]

Values are means ± S.D. of 8–10 animals per group.
[a] $P<0.05$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

supernatant was reserved for protein concentration measurement, and the remaining was stored at − 80 °C for posterior tumor necrosis factor-α levels quantification. The samples were read in a microplate spectrophotometer at 450 nm.

2.12. Statistical analyses

Data are expressed as means ± standard deviation (S.D.); p values were considered significant when $p<0.05$. Differences in experimental groups were determined by one-way ANOVA followed by the post hoc Tukey's test whenever necessary.

3. Results

3.1. Oxidative stress markers and antioxidant enzyme activities analyses

3.1.1. Oxidative/nitrosative damage markers

In this experimental model, only vitamin A supplementation at 9000 IU/kg day^{-1} induced an increase in lipid peroxidation levels in rat liver ($p<0.05$; Table 1). Protein carbonylation levels, as well as protein thiol content, did not change in the liver of vitamin A-treated rats (Table 1). However, we observed increased 3-nitrotyrosine content in the liver of the rats that received vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Table 1).

3.1.2. Antioxidant and detoxifying enzyme activities

Vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} induced a 1.7–1.9-fold increase of rat liver superoxide dismutase enzyme activity ($p<0.05$; Table 2). Catalase enzyme activity did not change in this experimental model (Table 2). Consequently, the hepatic superoxide dismutase/catalase ratio did not change in vitamin A-treated rats (Table 2). Glutathione-S-transferase activity was found increased in the liver of the rats that received vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Table 2).

3.1.3. Superoxide anion radical ($O_2^{-\bullet}$) production and oxidative damage in submitochondrial particles

As depicted in Fig. 2A, vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} induced a 1.8–2-fold increase of $O_2^{-\bullet}$ production in hepatic submitochondrial particles ($p<0.05$; Table 3). Lipid peroxidation levels were found increased in submitochondrial particles isolated of the rats that received vitamin A supplementation at 9000 IU/kg day^{-1} ($p<0.05$; Table 3). Protein carbonylation levels and protein thiol content did not change in the liver of vitamin A-treated rats (Table 3).

3.1.4. Non-enzymatic antioxidant capacity

Chemiluminescence emitted from samples at 1 min of experiment (total antioxidant reactivity–TAR) did not change in the hepatic homogenates from the rats that received vitamin A supplementation (Fig. 1A). Also, TroloxÔ equivalents, an antioxidant parameter, did not change in this experimental model (Fig. 1B). Consequently, area under the curve, which represents a measure of the pro-oxidant capacity of the sample tested, was found unaltered in the liver of the rats that received vitamin A supplementation (Fig. 1C).

3.2. Mitochondrial electron transfer chain activity

Vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} induced a 1.5–1.7-fold increase of complex I–III activity in rat liver ($p<0.05$; Fig. 2A). Complex II–III activity was found increased only in the liver of the rats that were administrated with vitamin A at 9000 IU/kg day^{-1} ($p<0.05$; Fig. 2B). Similarly, complex II activity, as well as succinate dehydrogenase enzyme activity, was found increased only in the liver of the rats that received vitamin A supplementation at 9000 IU/kg day^{-1} ($p<0.05$; Fig. 2C and D). Complex IV activity was found increased in the liver of the rats that were exposed to vitamin A supplementation at 4500 or 9000 IU/kg day^{-1} ($p<0.05$; Fig. 2E).

3.3. Caspase-3 and caspase-8 activities and tumor necrosis factor-α levels

Vitamin A supplementation did change neither caspase-3 activity, nor caspase-8 activity in rat liver. Similarly, tumor necrosis factor-α levels did not vary in this experimental model (Table 4).

4. Discussion

In the herein presented work, we have shown that vitamin A supplementation at clinical doses commonly used in the treatment of life-threatening maladies induces an increase in lipid peroxidation, but not protein carbonylation, in rat liver. In addition, we found increased $O_2^{-\bullet}$ production in hepatic submitochondrial particles of vitamin A-treated animals. Increased $O_2^{-\bullet}$ production may be the result of an uncoupled or increased electron flux during oxidative phosphorylation, as previously reviewed (Halliwell, 2006). Indeed, we have found

Table 3
The effects of vitamin A supplementation on oxidative damage levels and superoxide anion radical ($O_2^{-\bullet}$) production in rat liver submitochondrial membranes.

Groups	Lipid peroxidation (nmol TBARS/mg protein)	Protein carbonylation (nmol/mg protein)	Protein thiol content (nmol sulfhydryl/mg protein)	$O_2^{-\bullet}$ production (nmol/min mg protein^{-1})
Control	4.0 ± 0.33	1.24 ± 0.2	111.0 ± 18.27	4.1 ± 0.4
1000 IU/kg day^{-1}	5.47 ± 0.6	1.46 ± 0.37	112.0 ± 11.85	6.1 ± 1.31
2500 IU/kg day^{-1}	5.66 ± 1.42	1.35 ± 0.22	100.7 ± 9.55	7.52 ± 1.21
4500 IU/kg day^{-1}	6.26 ± 1.3	1.28 ± 0.4	107.7 ± 15.3	8.38 ± 2.1[a]
9000 IU/kg day^{-1}	7.7 ± 1.08[a]	1.31 ± 0.62	109.6 ± 12.4	8.45 ± 1.7[a]

Values are means ± S.D. of 8–10 animals per group.
[a] $P<0.05$ vitamin A vs. control (one-way ANOVA followed by the post hoc Tukey's test).

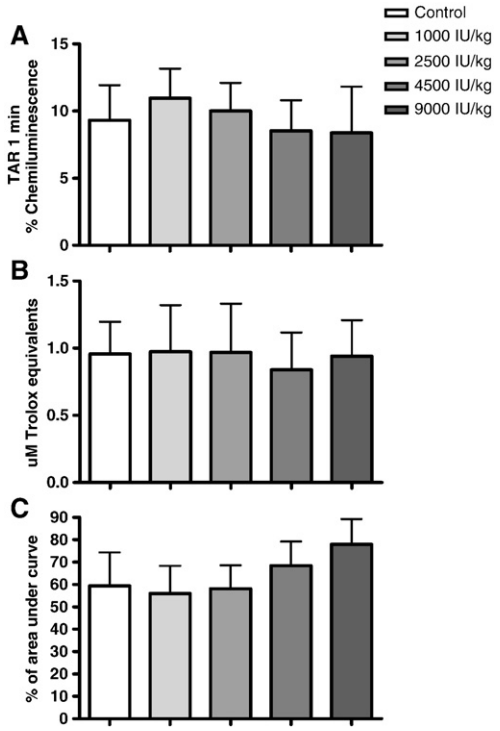

Fig. 1. Effects of vitamin A supplementation on hepatic non-enzymatic antioxidant defenses. The total antioxidant reactivity (TAR) is shown in (A). Trolox™ equivalents and area under the curve are shown in (B) and (C), respectively. Data are mean ± S.D. of 8–10 animals per group performed in triplicate.

increased mitochondrial electron transfer chain activity in the liver of the rats that received vitamin A supplementation for 28 days. Additionally, we have found increased lipid peroxidation levels in mitochondrial membranes. However, even in the presence of an ongoing lipid peroxidation process in hepatic submitochondrial particles, we did not observe any change in caspase-3 or caspase-8 activity. Also, the levels of a pro-inflammatory cytokine, tumor necrosis factor-α, was demonstrated to be unaltered in the present experimental model.

We have recently reported that vitamin A induces a several-fold increase of oxidative stress markers in adult rat hippocampus, striatum, *substantia nigra*, cerebral cortex, and cerebellum and altered rat behavior (De Oliveira and Moreira, 2007; De Oliveira et al., 2007a, b; De Oliveira et al., 2008a, b). Nevertheless, vitamin A did not induce similar effects on rat liver, which may be explained, at least in part, by the fact that liver is the main site of vitamin A storage and metabolism in the mammalian organism, as elegantly reviewed by Napoli (Napoli, 1999). Nevertheless, in some situations, vitamin A aggravates the manifestation of hepatotoxicity in rat, as demonstrated in a CCl_4-induced model of liver disease (Elsisi et al., 1993a, b; Badger et al., 1996). In addition, the differences observed in the studies mentioned above and the present one may be due to increased susceptibility of central nervous system to oxidative insult, since there is a high content of polyunsaturated fatty acids (PUFA) in brain, which are more sensitive to oxidative modifications than other lipid molecules (Halliwell and Chirico, 1993). Furthermore, central nervous system has a decreased activity of antioxidant enzymes activities than liver, which may favor the onset and maintenance of a pro-oxidative state (Halliwell, 2006).

Interestingly, we found increased 3-nitrotyrosine content, but not protein carbonylation in this experimental model regarding oxidative state of proteins (Table 1). Increased 3-nitrotyrosine content indicates increased peroxynitrite ($ONOO^-$) production, reactive specie originated from nitric oxide ($NO^\bullet$) and $O_2^{-\bullet}$ (Halliwell, 2006). Also, we found increased superoxide dismutase enzyme activity, but unaltered catalase enzyme activity in the present experimental model. However, it did not result in an imbalance in the superoxide dismutase/catalase ratio (Table 2). This is in agreement with the results showing unaltered lipid peroxidation (with the exception of the 9000 IU/kg

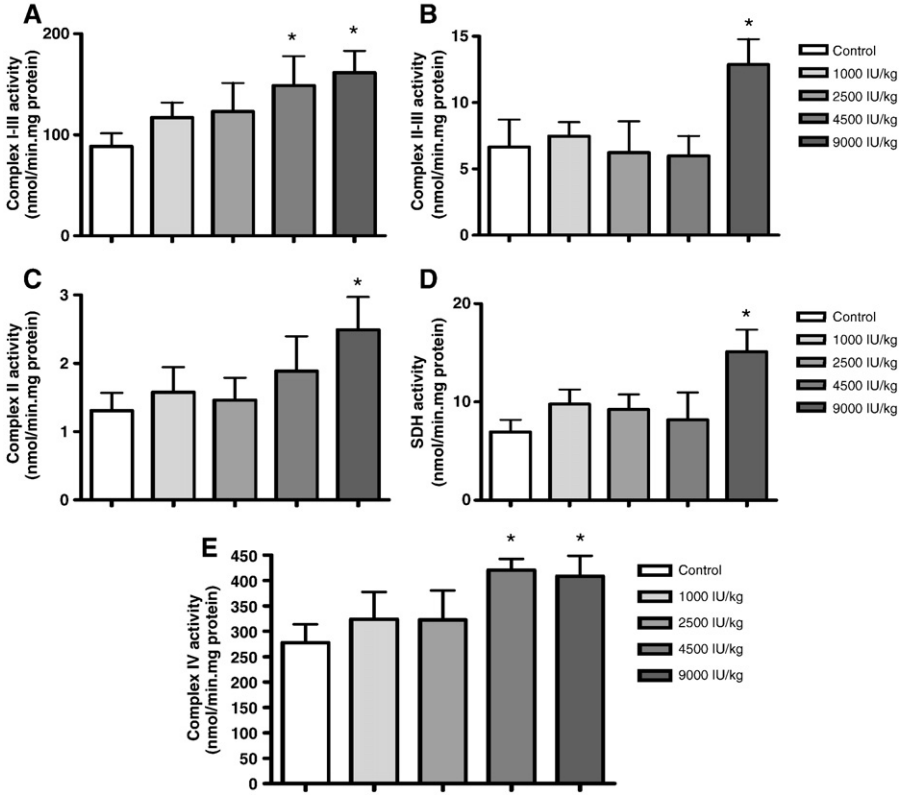

Fig. 2. Effects of vitamin A supplementation on complex I–III (A), complex II–III (B), complex II (C), succinate dehydrogenase (SDH) (D), and Complex IV (E) enzyme activities in the rat liver. Data are mean ± S.D. of 8–10 animals per group performed in triplicate. $^*P<0.05$ (one-way ANOVA followed by Tukey's test).

Table 4
The effects of vitamin A supplementation on caspase-3 and caspase-8 enzyme activities and TNF-α levels.

Groups	Caspase-3 (nmol AMC/min mg protein^{-1})	Caspase-8 (nmol/min mg protein^{-1})	TNF-α levels (pg/mg protein)
Control	0.61 ± 0.13	33.9 ± 9.3	3.3 ± 0.96
1000 IU/kg day^{-1}	0.46 ± 0.08	43.45 ± 20.8	3.93 ± 1.1
2500 IU/kg day^{-1}	1.08 ± 0.64	45.9 ± 1.1	3.8 ± 1.3
4500 IU/kg day^{-1}	0.99 ± 0.33	39.11 ± 7.4	4.2 ± 0.96
9000 IU/kg day^{-1}	0.92 ± 0.3	44.4 ± 7.6	3.9 ± 1.4

Values are means ± S.D. of 8–10 animals per group.

vitamin A dose), protein carbonylation, and protein thiol content, since an imbalance in the superoxide dismutase/catalase ratio is very likely to favor an increase in H_2O_2 production, a reactive oxygen specie that may give rise to more powerful oxidizing agents, as the hydroxyl ($^{\bullet}OH$) radical (Halliwell and Gutteridge, 1999). In regard to non-enzymatic antioxidant defenses, we did not find any change that would suggest that vitamin A modulates such parameter in rat liver (Fig. 1A–C).

The increased mitochondrial electron transfer chain activity observed in this work may represent a response of hepatic cells to the stressful condition imposed by high vitamin A intake, as postulated (Manoli et al., 2007). Indeed, we have seen that the same vitamin A doses that induced oxidative damage in liver samples also increased mitochondrial electron transfer chain activity (Fig. 2A–E). On the other hand, increased mitochondrial electron transfer chain activity may give rise to increased $O_2^{-\bullet}$ production due to electron leakage from the electron transfer chain (Halliwell and Gutteridge, 1999; Halliwell, 2006). Accordingly, we have found increased $O_2^{-\bullet}$ production in submitochondrial particles isolated from vitamin A-treated rats (Table 3).

In a recently published work, our group has demonstrated that retinol (vitamin A) induces apoptosis in cultured Sertoli cells through an oxidative stress-associated process (Klamt et al., 2008). Then, we have investigated here the effect of vitamin A supplementation on caspases activity in the liver of rats. Surprisingly, we did find any change neither in caspase-3 nor caspase-8 activity. Additionally, we did not observe any alteration in the levels of tumor necrosis factor-α cytokine (Table 4), suggesting that, in this experimental model, vitamin A supplementation at therapeutic doses for 28 days did not induce a pro-inflammatory state in rat liver.

Our results indicate a stressful event that is induced through vitamin A supplementation at clinical levels on rat liver. This becomes clearer to be seen when we analyze the activity of both mitochondrial electron transfer chain and glutathione-S-transferase enzyme. As mentioned above, it was previously reviewed that diverse cell types, under stressful conditions, modulate mitochondrial electron transfer chain activity and number of mitochondria in an attempt to produce more ATP as a response to the unfavorable environment (Manoli et al., 2007). Indeed, we have found increased both mitochondrial electron transfer chain and glutathione-S-transferase activities (Fig. 2 and Table 2, respectively).

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References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–126.
Allen, L.H., Haskell, M., 2002. Estimating the potential for vitamin A toxicity in women and young children. J. Nutr. 132, 2907S–2919S.
Badger, D.A., Sauer, J.-M., Hoglen, N.C., Jolley, C.S., Sipes, I.G., 1996. The role of inflammatory cells and cytochrome P450 in the potentiation of CCl_4-induced liver injury by a single dose of retinol. Toxicol. Appl. Pharmacol. 141, 507–519.
Borrás, E., Zaragozá, R., Morante, M., García, C., Gimeno, A., López-Rodas, G., Barber, T., Miralles, V.J., Viña, J.R., Torres, L., 2003. In vivo studies of altered expression patterns of p53 and proliferative control genes in chronic vitamin A deficiency and hypervitaminosis. Eur. J. Biochem. 270, 1493–1501.
Dal-Pizzol, F., Klamt, F., Benfato, M.S., Bernard, E.A., Moreira, J.C.F., 2001. Retinol supplementation induces oxidative stress and modulate antioxidant enzyme activities in rat Sertoli cells. Free Radic. Res. 34, 395–404.
De Oliveira, M.R., Moreira, J.C.F., 2007. Acute and chronic vitamin A supplementation at therapeutic doses induces oxidative stress to submitochondrial particles isolated from cerebral cortex and cerebellum of adult rats. Toxicol. Lett. 173, 145–150.
De Oliveira, M.R., Pasquali, M.A.B., Silvestrin, R.B., Mello e Souza, T., Moreira, J.C.F., 2007a. Vitamin A supplementation induces a prooxidative state in the striatum and impairs locomotory and exploratory activity of adult rats. Brain Res. 1169, 112–119.
De Oliveira, M.R., Silvestrin, R.B., Mello e Souza, T., Moreira, J.C.F., 2007b. 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 28, 1191–1199.
De Oliveira, M.R., Silvestrin, R.B., Mello e Souza, T., Moreira, J.C.F., 2008a. 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. 33, 378–383.
De Oliveira, M.R., Oliveira, M.W.S., Behr, G.A., Moreira, J.C.F., 2008b. Vitamin A supplementation at clinical doses induces a dysfunction in the redox and bioenergetics states, but did not change neither caspases activities nor TNF-alpha levels in the frontal cortex of adult Wistar rats. J. Psychiatr. Res. doi:10.1016/j.psychires.2008.10.002.
Draper, H.H., Hadley, M., 1990. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol. 186, 421–431.
Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77.
Elsisi, A.E.D., Hall, P., Sim, W.-Z.W., Earnest, D.L., Sipes, I.G., 1993a. Characterization of vitamin A potentiation of carbon tetrachloride-induced liver injury. Toxicol. Appl. Pharmacol. 119, 280–288.
Elsisi, A.E., Earnest, D.L., Sipes, I.G., 1993b. Vitamin A potentiation of carbon tetrachloride hepatotoxicity: enhanced lipid peroxidation without enhanced biotransformation. Toxicol. Appl. Pharmacol. 119, 289–294.
Esteronell, E., Tormo, J.R., Marin, P., Renau-Piqueras, J., Timoned, J., Barber, T., 2000. Effects of vitamin A deficiency on mitochondrial function in rat liver and heart. Br. J. Nutr. 84, 927–934.
Fenaux, P., Chomienne, C., Degos, L., 2001. Treatment of acute promyelocytic leukaemia. Best Pract. Res. Clin. Haematol. 14, 153–174.
Fischer, J.C., Ruitenbeek, W., Berden, J.Á., Trijbels, J.M., Veerkamp, J.H., Stadhouders, M., Sengers, R.C., Janssen, A.J., 1985. Differential investigation of the capacity of succinate oxidation in human skeletal muscle. Clin. Chim. Acta 153, 23–36.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Halliwell, B., 2006. Oxidative stress and neurodegeneration: where are we now? J. Neurochem. 97, 1634–1658.
Halliwell, B., Chirico, S., 1993. Lipid peroxidation: its mechanisms, measurement, and significance. Am. J. Clin. Nutr. 57, 715S–724S.
Halliwell, B., Gutteridge, J.M.C. (Eds.), 1999. Free Radicals Biology and Medicine. Oxford Science Publications, Oxford.
Klamt, F., De Oliveira, M.R., Moreira, J.C.F., 2005. Retinol induces permeability transition and cytochrome c release from rat liver mitochondria. Biochim. Biophys. Acta 1726, 14–20.
Klamt, F., Dal-Pizzol, F., Gelain, D.P., Dalmolin, R.S., Birnfeld de Oliveira, R., Bastiani, M., Horn, F., Fonseca Moreira, J.C., 2008. Vitamin a treatment induces apoptosis through an oxidant-dependent activation of the mitochondrial pathway. Cell Biol. Int. 32, 100–116.
Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.
Lowry, O.H., Rosebrough, A.L., Randal, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.
Mactier, H., Weaver, L.T., 2005. Vitamin A and preterm infants: what we know, what we don't know, and what we need to know. Arch. Dis. Child, Fetal Neonatal Ed. 90, 103–108.
Manoli, I., Alesci, S., Blackman, M.R., Su, Y.A., Rennert, O.M., Chrousos, G.P., 2007. Mitochondria as key components of the stress response. Trends Endocrinol. Metab. 18, 190–198.
Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.
Murata, M., Kawanishi, S., 2000. Oxidative DNA damage by vitamin A and its derivative via superoxide generation. J. Biol. Chem. 275, 2003–2008.
Myhre, A.M., Carlsen, M.H., Bohn, S.K., Wold, H.L., Laake, P., Blomhoff, R., 2003. Water-miscible, emulsified, and solid forms of retinol supplements are more toxic than oil-based preparations. Am. J. Clin. Nutr. 78, 1152–1159.
Napoli, J.L., 1999. Interactions of retinoids binding proteins and enzymes in retinoids metabolism. Biochim. Biophys. Acta 1440, 139–162.

Pasquali, M.A., Gelain, D.P., Zanotto-Filho, A., de Souza, L.F., de Oliveira, R.B., Klamt, F., Moreira, J.C., 2008. Retinol and retinoic acid modulate catalase activity in Sertoli cells by distinct and gene expression-independent mechanisms. Toxicol. In Vitro 22, 1177–1183.

Poderoso, J.J., Carreras, M.C., Lisdero, C., Riobo, N., Schopfer, F., Boveris, A., 1996. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. Arch. Biochem. Biophys. 328, 85–92.

Ross, A.C., Zolfaghari, R., 2004. Regulation of hepatic retinol metabolism: perspectives from studies of vitamin A status. J. Nutr. 134, 269S–275S.

Rustin, P., Chretien, D., Bourgeron, T., Gérard, B., Rötig, A., Saudubray, J.M., Munnich, A., 1994. Biochemical and molecular investigations in respiratory chain deficiencies. Clin. Chim. Acta 228, 35–51.

Seward, C.R., Vaughan, G., Hove, E.L., 1966. Effect of vitamin A deficiency or excess on the oxidative phosphorylation by rat liver mitochondria. J. Biol. Chem. 241, 1229–1232.

Shapira, A.H., Mann, V.M., Cooper, J.M., Dexter, D., Daniel, S.E., Jenner, P., Clark, J.B., Marsden, C.D., 1990. Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. J. Neurochem. 55, 2142–2145.

Wayner, D.D.M., Burton, G.W., Ingold, K.U., Locke, S., 1985. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. FEBS Lett. 187, 33–37.