

Total and Mitochondrial Nitrosative Stress, Decreased Brain-Derived Neurotrophic Factor (BDNF) Levels and Glutamate Uptake, and Evidence of Endoplasmic Reticulum Stress in the Hippocampus of Vitamin A-Treated Rats

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Abstract Vitamin A supplementation has caused concern among public health researchers due to its ability in decreasing life quality from acute toxicological effects to increasing mortality rates among vitamin supplement users. For example, it was described cognitive decline (i.e. irritability, anxiety, and depression) in patients subjected to long-term vitamin A therapy, as occurs in cancer treatment. However, the mechanism by which vitamin A affects mammalian cognition is not completely understood. Then, we performed the present work to investigate the effects of vitamin A supplementation at clinical doses (1,000–9,000 IU/kg day⁻¹) for 28 days on rat hippocampal nitrosative stress

levels (both total and mitochondrial), bioenergetics states, brain-derived neurotrophic factor (BDNF), alpha- and beta-synucleins, BiP and dopamine receptor 2 (D2 receptor) contents, and glutamate uptake. We observed mitochondrial impairment regarding respiratory chain function: increased complex I-III, but decreased complex IV enzyme activity. Also, decreased BDNF levels were observed in vitamin A-treated rats. The present data demonstrates, at least in part, that mitochondrial dysfunction and decreased BDNF and D2 receptors levels, as well as decreased glutamate uptake may take an important role in the mechanism behind the previously reported cognitive disturbances associated to vitamin A supplementation.

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Introduction

The importance of vitamin A to the well function of the mammalian central nervous system (CNS) is undeniable [1]. Additionally, either vitamin A or its derivatives—the retinoids—have been prescribed at moderate to high doses in the treatment of several diseases, from the dermatology to oncology fields [2–6]. For example, it was reported that infants and children have received retinol palmitate (a vitamin A-related medication) at doses exceeding 150,000 IU/day during treatment of some types of cancer [5]. Moreover, it was described that very-low-weight-birth preterm infants are treated with vitamin A at 8,500 IU/kg day⁻¹ during weight gain treatment (consider that some of these children were born weighting 0.8–1.1 kg) [7]. In spite of this, there is a crescent concern regarding vitamin A intake, even as part of a treatment, since it has

been described that vitamin A and/or retinoids are capable to negatively modulate CNS-dependent functions, for instance cognition, inducing mood disorders [6, 8]. Indeed, we have experimentally demonstrated that vitamin A (retinol palmitate) at clinical doses induced anxiety-related behavior in adult Wistar rats. Furthermore, we have observed decreased locomotory and exploratory activities performed by such animals in an open field apparatus [9–15]. Recently, it was reported decreased brain metabolism in patients subjected to daily retinoid therapy [16] and, more concerning, increased mortality rates among vitamin supplements users even when ingesting low daily vitamin A doses (mainly as retinol palmitate) [17].

The negative consequences of increased vitamin A intake are not restricted to alter cognition. Vitamin A is a redox-active molecule, i.e. it is either capable to protect biological systems against the potential toxicity exerted by pro-oxidative agents (for instance, hydrogen peroxide, superoxide anion radical) or to induce oxidant-dependent impairments, which may be achieved through high vitamin A intake therapeutically or inadvertently. Our research group recently reported that clinical vitamin A doses induced an imbalance in the adult rat hippocampal redox environment [9]. Furthermore, we have observed anxiety-like, but not depression-related behavior, in vitamin A-treated rats [15]. However, the exact mechanism behind this effect remains to be elucidated.

Based on the previously published data, and concerned with the lack of information regarding the consequences of increased vitamin A intake on mammalian hippocampus, we have analyzed here some parameters that may be involved in the previously seen effects of vitamin A in inducing neurotoxicity and behavioral disturbances in adult rats. In the present work, we aimed to investigate the levels of brain-derived neurotrophic factor (BDNF) (whose levels may vary during neurodegenerative events), as well as both total and mitochondrial nitrosative stress parameters and glutamate uptake in the hippocampus of vitamin A-treated rats. Monoamine oxidase (MAO) and mitochondrial superoxide dismutase (Mn-SOD) enzyme activities (as potential sources of hydrogen peroxide— H_2O_2) were also analyzed. Furthermore, we have studied mitochondrial electron transfer chain (METC) activity—as an index of hippocampal bioenergetics state and possible source of superoxide anion radical ($O_2^{\bullet-}$)—and α - and β -synucleins in such rat brain region. These neurochemical parameters were analyzed due to their role in impairing redox homeostasis in mammalian brain. Then, we have chosen to investigate the effects of chronically administrated (28 days) vitamin A at clinical doses (1,000–9,000 IU/kg day⁻¹), since it was reported the use of this vitamin in the treatment of some diseases, for instance dermatological disturbances, preterm infants weight gain, and cancer [5, 6].

Experimental Procedures

Animals

Adult male Wistar rats (90 days old; 270–300 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (7:00–19:00 h), at a temperature-controlled colony room ($23 \pm 1^\circ C$). 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.

Drugs and Reagents

Arovit[®] (retinol palmitate, a water-soluble form of vitamin A) was purchased from Roche, Sao Paulo, SP, Brazil. Polyclonal antibodies to BDNF, α - and β -synucleins, BiP, and D2 receptor were purchased from Chemicon International, USA. TNF- α assay kits were obtained from BD Biosciences, San Diego, CA, USA. RAGE polyclonal antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Caspase-8 activity assay kit was purchased from Biotium, Inc., Hayward, CA, USA. All other chemicals were purchased from Sigma, St. Louis, MO, USA. Vitamin A treatment was prepared daily and it occurred by protecting from light.

Treatment

The animals were treated once a day for 28 days with a gavage. The 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; $N = 10$ animals), 1,000 ($N = 10$), 2,500 ($N = 10$), 4,500 ($N = 10$), or 9,000 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.

Hippocampal BDNF Levels

BDNF levels were quantified in rat hippocampus with sandwich-ELISA, using a commercial kit according to the manufacturer's instructions. Briefly, microtiter plates

(96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in sample diluents and standard curve ranged from 7.8 to 500 pg of BDNF. Plates were then washed four times with wash buffer, monoclonal anti-BDNF rabbit antibody added (diluted 1:1,000 with sample diluents), and incubated for 3 h at room temperature. After washing, a second incubation with anti-rabbit antibody peroxidase conjugated (diluted 1:1,000) for 1 h at room temperature was carried out. After addition of streptavidin enzyme, substrate, and stop solution, the amount of BDNF was determined (absorbance set at 450 nm). The standard curve demonstrates a direct relationship between optical density (OD) and BDNF concentration. Total protein was measured according to [18].

Indirect ELISA to β -Amyloid Peptide_{1–40}, α - and β -Synucleins, D2 Receptor, RAGE, BiP, and 3-Nitrotyrosine

Indirect ELISA assay was performed to analyze changes in the content of β -amyloid peptide (1–40) by utilizing a polyclonal rabbit antibody to β -amyloid peptide (1–40) diluted 1:4,000 in phosphate-buffered saline (PBS) pH 7.4 with 5% albumin. The polyclonal rabbit antibodies to α - and β -synucleins were diluted 1:1,000 in PBS with 5% albumin. Dopamine D2R polyclonal antibody was diluted 1:4,000 in PBS with 5% albumin. RAGE polyclonal rabbit antibody was diluted 1:2,000 in PBS with 5% albumin. Polyclonal rabbit antibody to 3-nitrotyrosine was diluted 1:2,000 in PBS (pH 7.4) with 5% albumin according to the manufacturer's instructions. BiP polyclonal rabbit antibody was diluted 1:5,000 in PBS with 5% albumin. Briefly, microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:10 (this dilution may be optimized) 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 each plate for 2 h at room temperature. After washing (four times), a second incubation with anti-rabbit antibody peroxidase conjugated (diluted 1:1,000) 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.

Mitochondrial Electron Transfer Chain Activity

Mitochondria from fresh rat hippocampus were isolated as described elsewhere [27]. Briefly, hippocampus of Wistar rats was suspended in ice-cold isolation buffer A (220 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, and 0.5 mg/ml fatty-acid free bovine serum albumin) was gently homogenized with a glass-homogenizer

and centrifuged at 2,000g for 10 min at 4°C. Approximately three-quarters of the supernatant was further centrifuged at 10,000g for 10 min at 4°C in a new tube. The fluffy layer of the pellet was removed by gently shaking with buffer A and the firmly packed sediment was resuspended in the same buffer without EGTA and centrifuged at 10,000g for 10 min at 4°C. The mitochondrial pellet was resuspended in buffer B (210 mM mannitol, 70 mM sucrose, 10 mM HEPES–KOH (pH 7.4), 4.2 mM succinate, 0.5 mM KH_2PO_4 , and 4 $\mu\text{g}/\text{ml}$ rotenone). This procedure, which was designed to isolate intact mitochondria rather than to recover all of that present in the hippocampus, yielded about 7 mg of mitochondrial protein/g of hippocampus.

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 ($\epsilon = 19.1 \text{ mM}^{-1} \text{ cm}^{-1}$). 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 [19].

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 (DCPIP) at 600 nm with 700 nm as reference wavelength ($\epsilon = 19.1 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8.0 μM DCPIP 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 DCPIP and was monitored for 5 min at 30°C. Succinate dehydrogenase (SDH) activity was assessed by adding 1 mM phenazine methasulphate to the reaction mixture. Then, SDH activity was monitored for 5 min at 30°C at 600 nm with 700 nm as reference wavelength [20].

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 ($\epsilon = 21 \text{ mM}^{-1} \text{ cm}^{-1}$). 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 μ M rotenone were added and the reaction started by the addition of 0.6 μ g/ml cytochrome *c* and monitored for 5 min at 30°C [20].

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 ($\epsilon = 19.15 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction buffer contained 10 mM potassium phosphate, pH 7.0, 0.6 mM *n*-dodecyl- β -D-maltoside, 2–4 μ g supernatant protein and the reaction was started with addition of 0.7 μ g reduced cytochrome *c*. The activity of complex IV was measured at 25°C for 10 min [21].

Mitochondrial Superoxide Dismutase Enzyme Activity

Mitochondrial Superoxide Dismutase (Mn-SOD) enzyme activity was quantified in the presence of 2 mM KCN, which inhibits by 97–99% Cu/Zn-SOD enzyme, as previously described [22].

Oxidative Parameters in Submitochondrial Particles

Briefly, to obtain submitochondrial particles (SMP), rat hippocampus 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 SMP. The SMP solution was also washed (twice) with 140 mM KCl, 20 mM Tris-HCl (pH 7.4) to ensure Mn-SOD release from mitochondria. To quantify superoxide ($\text{O}_2^{\bullet-}$) production, SMP 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 μ M 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 [11, 23]. As a marker of lipid peroxidation, we measured the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, as previously described [24]. The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine (DNPH) as previously described above [25]. Protein thiol content in hippocampal SMP 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 (DTNB) in ethanol was added and the intense yellow color was developed and read in a spectrophotometer at 412 nm after 20 min [26]. To quantify 3-nitrotyrosine in

mitochondrial membranes, intact mitochondria were isolated from rat hippocampus as previously described, and the indirect ELISA protocol was applied as described above [27].

Monoamine Oxidase Enzyme Activity

Briefly, the oxidation of 0.06 mM kynuramine in PBS (pH 7.4) at 360 nm was recorded using a spectrophotometer at 37°C, as previously described [28].

Glutathione S-Transferase Enzyme Activity

Glutathione-S-transferase (GST) activity was determined spectrophotometrically according to the method described in [29]. GST activity was quantified in hippocampal 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.

Glutamate Uptake

The animals were killed by decapitation and the brains were immediately removed and submerged in Hank's balanced salt solution (HBSS), pH 7.2. Rat hippocampus was dissected and coronal slices (0.4 mm) were obtained using a McIlwain tissue chopper. Hippocampal slices were transferred to multiwell dishes and washed with 300 μ l HBSS followed by 280 μ l HBSS per well. Glutamate uptake was performed according to our previous reports [30]. The uptake assay was assessed by adding 0.33 μ Ci ml^{-1} D-[2,3- ^3H] glutamate with 100 μ M unlabeled glutamate in 20 μ l HBSS, at 37°C. Incubation was stopped after 7 min by two ice-cold washes with 1 ml HBSS immediately followed by addition of 0.5 N NaOH, which was kept overnight. Aliquots of lysates were taken for determination of intracellular content of L-[2,3- ^3H] glutamate by cintillation counting. Sodium-independent uptake ($0.027 \pm 0.003 \text{ nmol mg}^{-1} \text{ min}^{-1}$) was determined using N-methyl-D-glucamine instead of sodium chloride, which was subtracted from the total uptake to obtain the sodium-dependent uptake. Determination of protein was assessed using the method described in [18]. Experiments were performed in triplicate.

Caspase-3 Enzyme Activity

Caspase-3 activity was determined in the rat hippocampus through a fluorimetric commercial kit according

manufacturer's instructions. Briefly, the samples were homogenized in lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT), and centrifuged at $10,000\times g$ 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 7-amino-4-methylcoumarin (AMC) produced/min mg^{-1} protein.

Caspase-8 Enzyme Activity

Caspase-8 activity was determined through a colorimetric commercial kit according manufacturer's instructions. 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.

TNF- α Quantification

We have measured TNF- α through commercial kit for sandwich enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions. 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 supernatant was reserved for protein concentration measurement, and the remaining was stored at -80°C for posterior TNF- α levels quantification. The samples were read in a microplate spectrophotometer at 450 nm.

Statistical Analysis

Data are expressed as means \pm standard error of the mean (S.E.M.); 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.

Results

Brain-Derived Neurotrophic Factor and β -Amyloid Peptide (1–40 peptide long)

As depicted in Fig. 1a, vitamin A supplementation at any dose tested decreased the levels of BDNF in the hippocampus of vitamin A-treated rats ($P < 0.05$). However, β -amyloid peptide levels did not change in this experimental model (Fig. 1b).

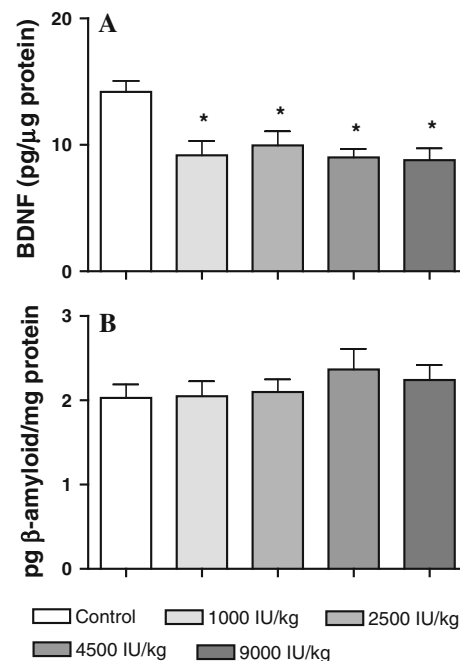


Fig. 1 Effects of vitamin A supplementation on hippocampal BDNF (a) and β -amyloid peptide (b) levels. Data are mean \pm S.E.M. ($N = 10$ rats per group) and the experiments were performed in triplicate. * $P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test)

Mitochondrial Electron Transfer Chain Activity

We observed increased complex I-III enzyme activity in the hippocampus of the rats that received vitamin A at 4,500 or 9,000 IU/kg day^{-1} ($P < 0.05$; Fig. 2a). However, complex II-III, succinate dehydrogenase (SDH), and complex II enzyme activity did not change in this work (Fig. 2b, c, and d, respectively). Interestingly, vitamin A supplementation at 4,500 or 9,000 IU/kg day^{-1} induced a decrease in complex IV enzyme activity in adult rat hippocampus ($P < 0.05$; Fig. 2e).

Superoxide Anion Radical ($\text{O}_2^{\bullet-}$) Production and Redox State of Mitochondrial Membranes

According with Fig. 3a, vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg day^{-1} induced an increase in the production of $\text{O}_2^{\bullet-}$ in hippocampal mitochondria ($P < 0.05$). However, lipid peroxidation levels were found increased only in mitochondrial membranes isolated from the hippocampus of the rats that received vitamin A supplementation at 9,000 IU/kg day^{-1} ($P < 0.05$; Fig. 3b). On the other hand, vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg day^{-1} increased the levels of protein carbonylation in hippocampal mitochondrial membranes ($P < 0.05$; Fig. 3c). Protein sulfhydryl content did not differ among the groups in this experimental model.

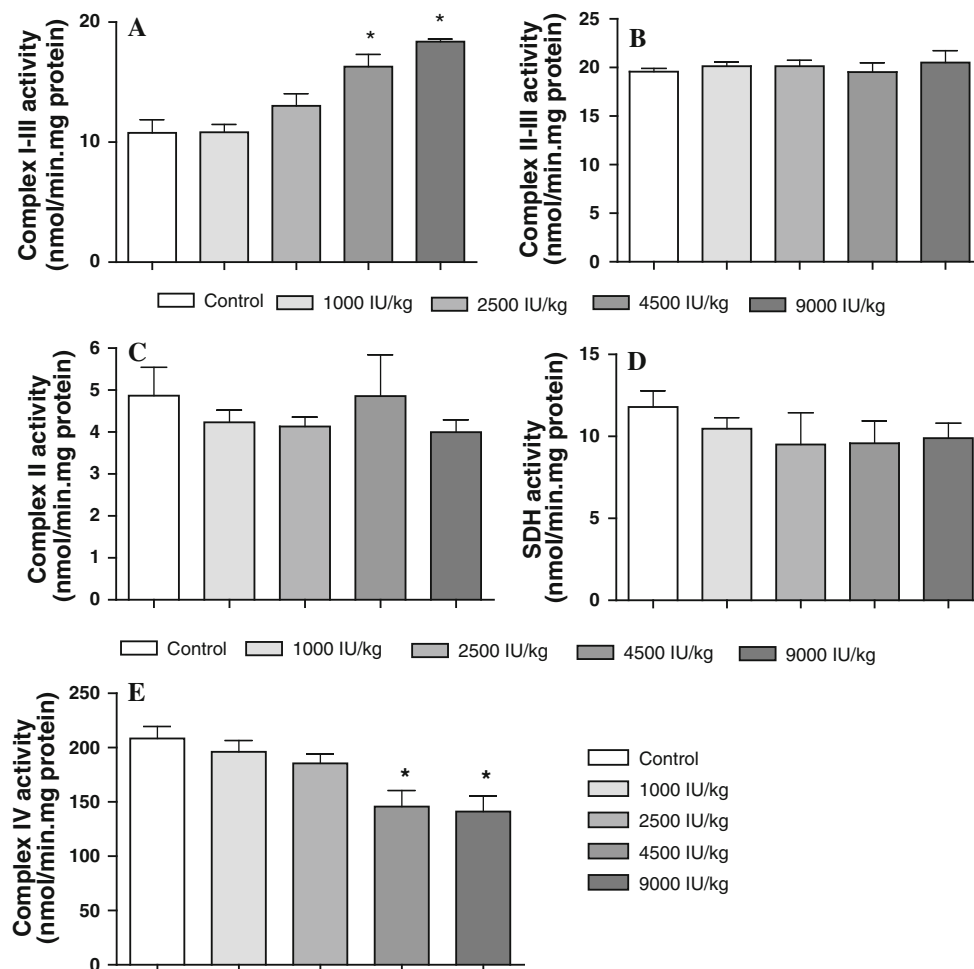


Fig. 2 Effects of vitamin A supplementation on complex I-III (a), complex II-III (b), complex II (c), succinate dehydrogenase (SDH) (d), and complex IV enzyme activities in rat hippocampus. Data are

(Fig. 3d). We observed increased 3-nitrotyrosine content in mitochondrial membranes isolated from the hippocampus of vitamin A-treated rats ($P < 0.05$; Fig. 3e). Vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg day⁻¹ increased Mn-SOD enzyme activity in hippocampal mitochondria ($P < 0.05$; Fig. 3f). Interestingly, MAO enzyme activity increased in the hippocampus of the rats that received vitamin A supplementation at 2,500, 4,500, or 9,000 IU/kg day⁻¹ ($P < 0.05$; Fig. 3g).

α - and β -Synucleins, D2 Receptor, RAGE, Total 3-Nitrotyrosine, and BiP Contents

We observed increased α -synuclein content in the hippocampus of the rats that were treated with vitamin A at 4,500 or 9,000 IU/kg day⁻¹ ($P < 0.05$; Fig. 4a). β -Synuclein content did not change in this experimental model (Fig. 4b). D2 receptor content was observed decreased in the hippocampus of the rats that received vitamin A supplementation at 4,500 or 9,000 IU/kg day⁻¹ ($P < 0.05$;

mean \pm S.E.M. ($N = 10$ rats per group) and the experiments were performed in triplicate. * $P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test)

Fig. 4c). Also, we quantified RAGE content, whose levels were found increased in the hippocampus of the rats that were treated with vitamin A at 4,500 or 9,000 IU/kg day⁻¹ ($P < 0.05$; Fig. 4d). Vitamin A at any dose tested increased total 3-nitrotyrosine contents in the adult rat hippocampus ($P < 0.05$; Fig. 4e). BiP protein content was increased in the hippocampus of the rats that received vitamin A at 4,500 or 9,000 IU/kg day⁻¹ ($P < 0.05$; Fig. 4f).

Glutathione S-Transferase Enzyme Activity

Vitamin A supplementation at 4,500 or 9,000 IU/kg day⁻¹ induced an increase in GST enzyme activity ($P < 0.05$; Fig. 5).

Glutamate Uptake

As is shown in Fig. 6, vitamin A supplementation at 4,500 or 9,000 IU/kg day⁻¹ decreased glutamate uptake in the rat hippocampus ($P < 0.05$).

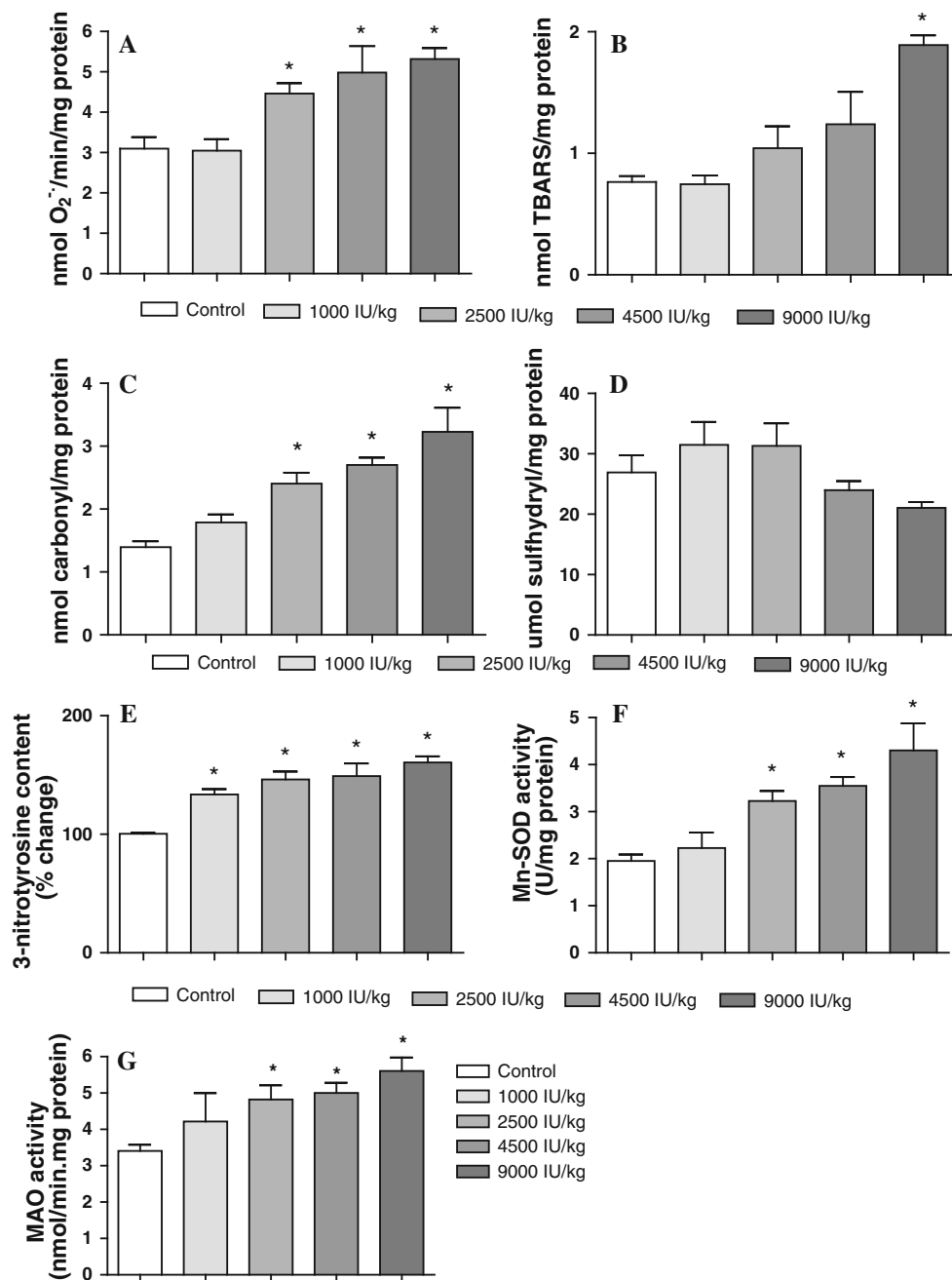


Fig. 3 Effects of vitamin A supplementation on superoxide anion radical production (a) in mitochondria isolated from adult rat hippocampus. Lipid peroxidation (b), protein carbonylation (c), protein thiol (d), and 3-nitrotyrosine levels were quantified in mitochondrial membranes isolated from the hippocampus of vitamin A-treated rats. Manganese superoxide dismutase (Mn-SOD—the

mitochondrial SOD enzyme) and monoamine oxidase (MAO) enzyme activities are shown in (f) and (g), respectively. Data are mean \pm S.E.M. ($N = 10$ rats per group) and the experiments were performed in triplicate. $*P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test)

Caspases Enzyme Activity and TNF- α Levels

Vitamin A supplementation at clinical doses did alter neither caspases enzyme activities nor TNF- α levels in this work (Fig. 7).

Discussion

In the present work, we investigated the consequences of daily vitamin A treatment at clinical doses on some hippocampal parameters that have been demonstrated to take

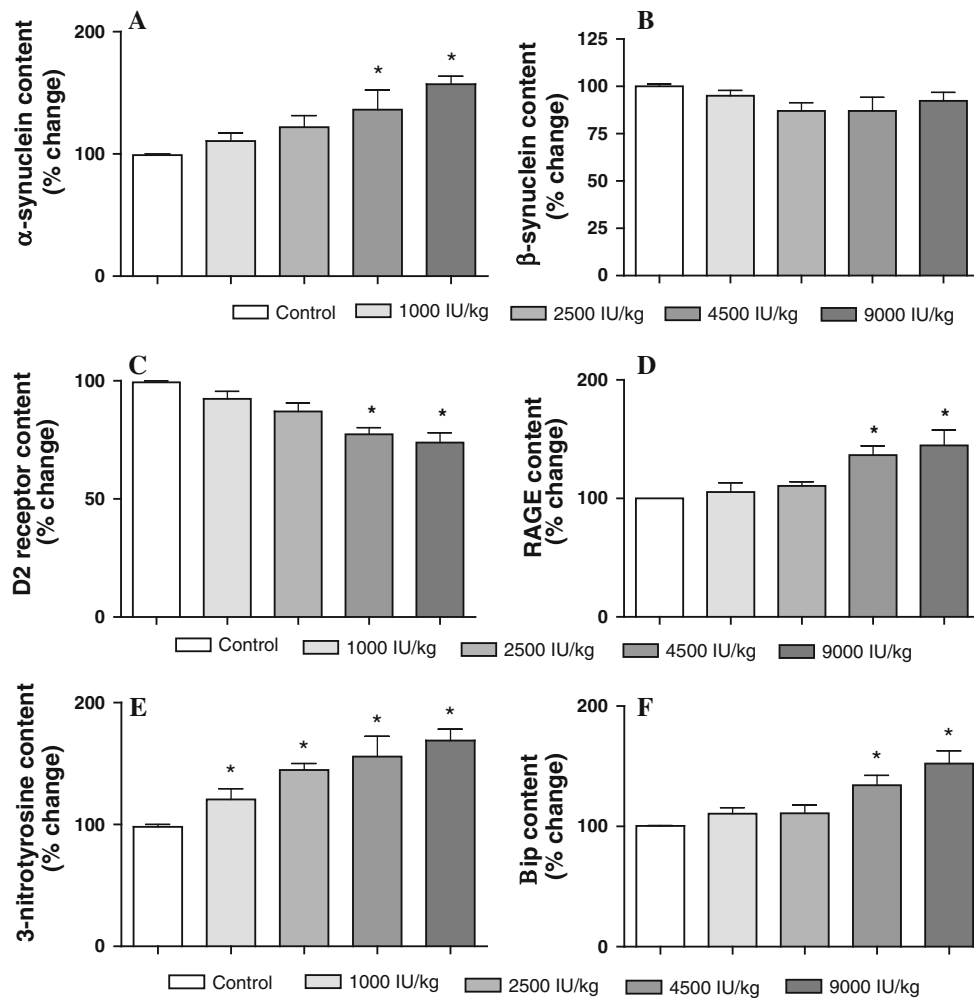


Fig. 4 Effects of vitamin A supplementation on hippocampal α - and β -synucleins contents (a and b, respectively). In (c), D2 receptor content is shown. RAGE and total 3-nitrotyrosine contents are demonstrated in (d) and (e), respectively. In (f) BiP content is shown.

Data are mean \pm S.E.M. ($N = 10$ rats per group) and the experiments were performed in triplicate. * $P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test)

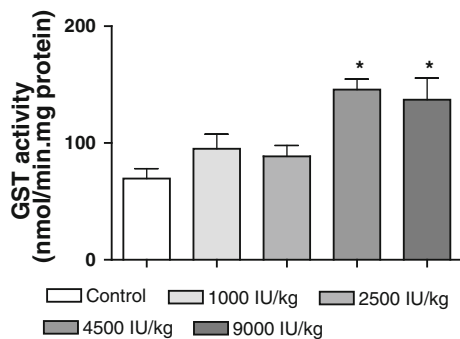


Fig. 5 Effect of vitamin A supplementation on hippocampal glutathione S-transferase (GST) enzyme activity. Data are mean \pm S.E.M. ($N = 10$ rats per group) and the experiments were performed in triplicate. * $P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test)

an important role in neurodegenerative and neurotoxic events in mammals. We observed decreased levels of BDNF, mitochondrial impairment, decreased glutamate uptake and altered levels of α -synuclein, D2 receptor, 3-nitrotyrosine, and BiP protein in the rat hippocampus. β -Amyloid peptide and caspases enzyme activities, as well as TNF- α levels did not change in this experimental model. It is important to note that neither food intake nor weight gain changed among the groups in this work (data not shown), indicating that the effects seen here are not dependent on an indirect effect of vitamin A in causing alterations in rat metabolism, but it is very likely to be triggered by the central action of vitamin A directly, whose ingestion levels were increased through supplementation.

BDNF is the main neurotrophin found in the mammalian brain, and is not only responsible for inducing neuronal

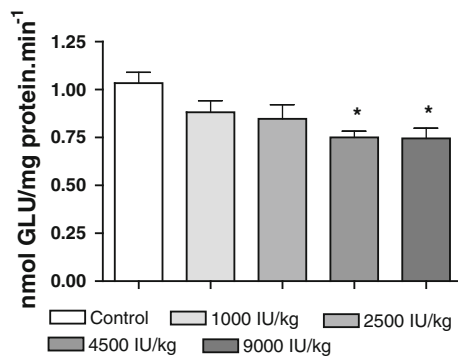


Fig. 6 Effect of vitamin A supplementation on glutamate uptake in adult rat hippocampus slices. Data are mean \pm S.E.M. ($N = 10$ rats per group) and the experiments were performed in triplicate. * $P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test)

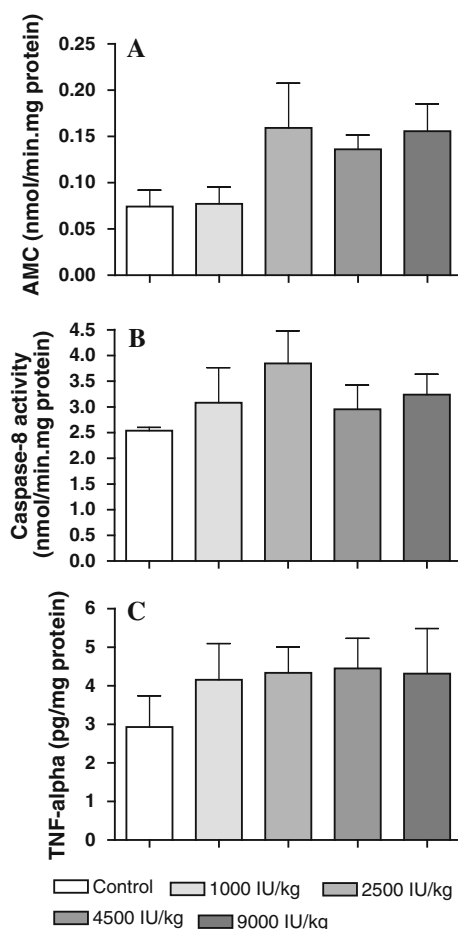


Fig. 7 Effects of vitamin A supplementation on hippocampal caspase-3 (a) and caspase-8 (b) enzyme activities. In (c), TNF- α levels are shown. Data are mean \pm S.E.M. ($N = 10$ rats per group) and the experiments were performed in triplicate. * $P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test)

proliferation, but also in maintaining nervous cells survival [31]. It has been described that BDNF, through an intricate pathway, induces mitochondrial biogenesis in several

cellular types, including neurons [32, 33]. Then, BDNF favors ATP homeostasis in central cells, which consume large amounts of ATP. Interestingly, we observed here mitochondrial impairment in the hippocampus of vitamin A-treated rats (Figs. 2 and 3). It was found increased complex I-III enzyme activity, but decreased complex IV enzyme activity (Fig. 2). Moreover, we observed increased superoxide anion radical ($O_2^{\bullet-}$) production and levels of oxidative stress markers in mitochondrial membranes (Fig. 3). These data suggest that mitochondrial impairment may lead to increased formation of $O_2^{\bullet-}$ due to partial reduction of oxygen at complex IV, leading to mitochondrial oxidative damage. Even though we observed decreased BDNF levels, and that this neurotrophin regulates mitochondrial biogenesis, it remains to be investigated whether exists a causal link between alterations in BDNF levels and mitochondrial impairment in the hippocampus of vitamin A-treated rats.

Hydrogen peroxide (H_2O_2) is a reactive oxygen specie (ROS) and, by reacting with either Fe^{2+} or Cu^{2+} (transition metals) through Fenton chemistry, it may originate hydroxyl radical ($\cdot OH$) [39]. Among the potential sources of H_2O_2 physiologically, Mn-SOD and MAO enzymes present a central role regarding redox environment maintenance. Mn-SOD produces H_2O_2 by dismutating $O_2^{\bullet-}$ inside mitochondrial matrix. MAO originates H_2O_2 when deaminates monoamines (for example, dopamine and serotonin). Then, such enzymes contribute, even physiologically, to increase H_2O_2 availability. We observed here increased Mn-SOD and MAO enzyme activities in the hippocampus of vitamin A-treated rats (Fig. 3f and g, respectively). It is possible that, by increasing H_2O_2 production both at mitochondrial matrix and cytosol, vitamin A impairs redox homeostasis throughout the cell. In addition, H_2O_2 is a diffusible molecule, which may cross biomembrane, spreading the prooxidant effect to other environments inside the cells [34, 35].

As depicted in Figs. 3e and 4e, vitamin A supplementation increased the content of both mitochondrial and total 3-nitrotyrosine contents in rat hippocampus, respectively. 3-Nitrotyrosine is a consequence of increased production of $O_2^{\bullet-}$ and nitric oxide (NO), which produce peroxynitrite ($ONOO^-$), an inductor of nitrosative stress [36]. Additionally, $ONOO^-$, after giving rise to peroxynitrous acid ($ONOOH$) under physiological conditions, generates products of increased reactivities, for instance nityl cation (NO_2^+), nitrogen dioxide radical (NO_2^{\bullet}), and hydroxyl radical ($\cdot OH$) through homolytic fission [37, 38]. Nitrated molecules, including lipids and proteins, have been demonstrated to exert deleterious effects in central pathologies [39]. Furthermore, 3-nitrotyrosine per se is able to alter protein conformation and, consequently, its function, as occurs with α -tubulin [40] and α -synuclein [41, 42], which

could lead to loss of function and protein aggregation, respectively. Additionally, NO^{*} is a neurotransmitter, and its levels may be maintained within the physiological range to avoid abnormal communication between neurons [38].

We also demonstrated here that daily vitamin A supplementation at 4,500 or 9,000 IU/kg day⁻¹ increased the hippocampal contents of α -synuclein and receptor for advanced glycation end products (RAGE), and decreased the content of D2 receptor in such brain area (Fig. 4). α -Synuclein is a pre-synaptic protein whose physiological function is still on debate; however, the accumulation of α -synuclein is important in Parkinson's disease, where it has been demonstrated to possess a neurotoxic role during neurodegeneration processes [39, 43]. Also, increased levels of α -synuclein indicate a pro-oxidant environment, since oxidized molecules may aggregate with α -synuclein [44] in a vicious cycle where α -synuclein aggregates interfere with membrane stability (for instance mitochondrial membranes) maintaining the production of reactive molecules at high rates [45, 46]. In this experimental model, β -synuclein content did not change (Fig. 4). β -Synuclein is thought to acts like a buffer to α -synuclein, inhibiting the interaction of α -synuclein with membranes and other cellular systems [39, 43]. Then, we may suggest that the neurotoxicity of vitamin A may account, at least in part, with this imbalance between these two synucleins, favoring the perpetuation of a pro-oxidant state.

Decreased D2 receptor content was observed in the hippocampus of the animals that received vitamin A supplementation at 4,500 or 9,000 IU/kg day⁻¹ (Fig. 4c). It is very likely that such effect may also favor the maintenance of a prooxidant state in rat hippocampus under vitamin A treatment, since decreased D2 receptor levels may lead to a weak negative feedback signaling inhibiting dopamine release, which could lead to increased dopamine content in the synaptic cleft [47]. Dopamine is a reactive molecule since it may give rise to semi-quinones in alkali systems, which may react, for example, with thiol protein groups, altering its conformation and, consequently, leading to decreased activity [48–50]. Also, increased dopamine release may lead to increased dopamine deamination through MAO enzyme, generating more H₂O₂, as discussed above.

Moreover, we observed increased RAGE content in rat hippocampus (Fig. 4d). It has been postulated that RAGE activation maintains redox impairment in several experimental models [51, 52]. Additionally, increased RAGE content may indicate ongoing inflammatory events; however, we did find any change neither in TNF- α levels nor in caspases enzyme activities in this work, suggesting that vitamin A supplementation did not induce neuroinflammation in this work (Fig. 7).

Interestingly, vitamin A supplementation seems to induce endoplasmic reticulum (ER) stress in this protocol,

as assessed through BiP protein content quantification, demonstrating that, at a subcellular level, the negatives effects of such treatment are not restricted to mitochondria. BiP is a molecular chaperone and has been demonstrated as a marker of reticular stress, whose levels increase during chemical or physical ER stress [53, 54]. More studies are necessary to better investigate by which mechanism vitamin A impaired ER homeostasis.

In Fig. 5, it is shown increased glutathione S-transferase (GST) enzyme activity, which is a phase II detoxifying enzyme that by utilizing reduced glutathione (GSH) increases the solubility of apolar molecules in aqueous environments in order to excrete such potential toxic molecules. In this role, GST consumes GSH, which is responsible, at least in part, for maintaining the non-enzymatic antioxidant defense in a wide range of mammalian cells [55].

Glutamate uptake was observed decreased in the hippocampus of vitamin A-treated rats (Fig. 6). It was reported that increased lipid peroxidation alters glutamate uptake by astroglial cells in different experimental models [39]. Indeed, we have recently published increased levels of lipid peroxidation in the hippocampus of vitamin A-treated rats [9]. Then, it is possible that vitamin A impairs glutamate uptake by a redox mechanism, as reviewed [56, 57].

Overall, the data showed here indicate, at least in part, a possible mechanism by which vitamin A supplementation for 28 days induced cognitive decline in previously reported works from our group and others [9, 10]: decreased BDNF levels, impaired mitochondrial function, nitrosative stress, and ER stress. Oxidative stress has been linked to cognitive decline, and vitamin A may take a pivotal role in facilitating redox imbalance that may favor neurodegenerative processes. Then, we recommend caution when applying vitamin A therapy in infants and children, or even adult patients suffering from mental disorders, since vitamin A may aggravate such symptoms by a mechanism which accounts with decreased hippocampal neurotrophin levels and mitochondrial impairment. Additionally, the treatment of vitamin A intoxication leads to increased costs with the management of public health, which is especially significant to developing countries.

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