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ORIGINAL ARTICLE

Omega-3 fatty acid supplementation can prevent changes in mitochondrial energy metabolism and oxidative stress caused by chronic administration of L-tyrosine in the brain of rats

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Abstract

Deficiency of hepatic enzyme tyrosine aminotransferase characterizes the innate error of autosomal recessive disease Tyrosinemia Type II. Patients may develop neurological and developmental difficulties due to high levels of the amino acid tyrosine in the body. Mechanisms underlying the neurological dysfunction in patients are poorly known. Importantly, Tyrosinemia patients have deficient Omega-3 fatty acids (n-3 PUFA). Here, we investigated the possible neuroprotective effect of the treatment with n-3 PUFA in the alterations caused by chronic administration of L-tyrosine on important parameters of energetic metabolism and oxidative stress in the hippocampus, striatum and cerebral cortex of developing rats. Chronic administration of L-tyrosine causes a decrease in the citrate synthase (CS) activity in the hippocampus and cerebral cortex, as well as in the succinate dehydrogenase (SDH) and isocitrate dehydrogenase (IDH) activities, and an increase in the α-ketoglutarate dehydrogenase activity in the hippocampus. Moreover, in the striatum, L-tyrosine administration caused a decrease in the activities of CS, SDH, creatine kinase, and complexes I, II-III and IV of the mitochondrial respiratory chain. We also observed that the high levels of L-tyrosine are related to oxidative stress in the brain. Notably, supplementation of n-3 PUFA prevented the majority of the modifications caused by the chronic administration of L-tyrosine in the cerebral enzyme activities, as well as ameliorated the oxidative stress in the brain regions of rats. These results indicate a possible neuroprotective and antioxidant role for n-3 PUFA and may represent a new therapeutic approach and potential adjuvant therapy to Tyrosinemia Type II individuals.

Keywords Tyrosinemia type II · L-tyrosine · Omega-3 fatty acids · Mitochondrial energy metabolism · Oxidative stress

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Introduction

Deficiency of hepatic enzyme tyrosine aminotransferase (TAT; EC 2.6.1.5) which is involved in the degradation pathway of the amino acid tyrosine, characterizes the Tyrosinemia Type II (OMIM 276600), an inborn error of metabolism (IEM). Tyrosinemia Type II is also known as Richner-Hanhart Syndrome or Oculocutaneous Tyrosinemia (Mitchell et al. 2013). At this moment, 17 different mutations have been found in the coding region of the *TAT* gene, including partial mutations and complete deletion of both alleles (Gokay et al. 2016; Mitchell et al. 2013). Thus, with reduced TAT enzyme activity, patients have high levels of tyrosine (plasma values range from 370 to 3300 μmol/l) and tyrosine metabolites in tissues, cerebrospinal fluid, blood and urine. On the other hand, these individuals present normal levels of phenylalanine in the blood (Soares et al. 2017; Zribi et al. 2016;

Mitchell et al. 2013; Held 2006; Valikhani et al. 2006; Macsai et al. 2001).

Clinical symptoms characteristic of Tyrosinemia Type II include skin lesions (painful palmoplantar hyperkeratotic lesions), ocular symptoms (bilateral pseudodendritic keratitis) and neurological complications -intellectual disability, microcephaly, tremor, ataxia, self-mutilating behavior, fine motor coordination disturbances, language deficits, and convulsions (Peña-Quintana et al. 2017; Zribi et al. 2016; Gokay et al. 2016; Held 2006; Mitchell et al. 1995, 2001; Goldsmith 1983). Patients may present isolated symptoms or even the combination of all symptoms, and may appear right after birth, or after years of the patient's life (Peña-Quintana et al. 2017; Zribi et al. 2016).

The mechanisms of brain damage in Tyrosinemia Type II patients are still poorly understood. In this regard, we and other authors experimentally demonstrated that the metabolites that accumulate (mainly tyrosine) in this IEM cause oxidative stress (Streck et al. 2017; Macêdo et al. 2013; De Andrade et al. 2011a, b, 2012; Sgaravatti et al. 2008, 2009), DNA damage (Carvalho-Silva et al. 2017; Streck et al. 2017; De Prá et al. 2014), alternating levels of neurotrophins (Ferreira et al. 2013a, 2014), induces an increase in acetylcholinesterase activity (Ferreira et al. 2012), and affect mitochondrial energy metabolism in rat brain (Teodorak et al. 2017; Ferreira et al. 2013b, 2015; Ramos et al. 2013; De Andrade et al. 2011a, 2011b, 2012). Specifically, in the study of Ferreira et al. (2015) we reported that repeated administrations of L-tyrosine lead to inhibition on Krebs cycle enzymes and mitochondrial respiratory complexes in brain structures of rats. Moreover, recent results from our laboratory have demonstrated that the administration of antioxidants (N-acetylcysteine and deferoxamine) prevents changes caused by the accumulation of amino acid L-tyrosine on biochemical parameters, i.e., DNA damage, oxidative stress and mitochondrial energetic metabolism (Streck et al. 2017; Teodorak et al. 2017). In addition, supplementation of Omega-3 fatty acids (n-3 PUFA) also showed a neuroprotective effect, preventing oxidative DNA damage caused by hypertyrosinemia in the rat's brain (Carvalho-Silva et al. 2017).

The n-3 PUFA, mainly eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6), demonstrate beneficial effects on the cardiovascular system, brain development and vision, as well as anti-inflammatory, antiplatelet, antihyperlipemic, antioxidant and neuroprotective actions (Carvalho-Silva et al. 2017; Gomes et al. 2017; Salberg et al. 2017; Siscovick et al. 2017; Solberg et al. 2017; Wiest et al. 2017; Schmidt et al. 2012; Schuchardt et al. 2010). Notably, studies have shown that n-3 PUFA supplementation prevent the progression of neurological impairment or improve neural function in patients with IEM, including Tyrosinemia (Peña-Quintana et al. 2017; Dercksen et al. 2016; Jans et al. 2013; Gil-Campos and Sanjurjo Crespo 2012; Mazer et al. 2010; Paker et al. 2010; Vlaardingerbroek et al. 2006).

EPA and DHA can be provided by diet through the ingestion of fish (salmon, sardines, cod and tuna) and some vegetables (flaxseed, canola and soybean), or synthesized from the essential polyunsaturated fatty acid alpha-linolenic acid (ALA; 18:3) (Kaur et al. 2014; Wu et al. 2008). Because of the dietary restriction imposed as treatment for this IEM, patients are prone to n-3 PUFA deficiency (Peña-Quintana et al. 2017; Dercksen et al. 2016; Jans et al. 2013; Gil-Campos and Sanjurjo Crespo 2012; Mazer et al. 2010; Paker et al. 2010; Vlaardingerbroek et al. 2006). In this context, the present study aimed to evaluate the in vivo influence of supplementation with n-3 PUFA in a chemically-induced chronic model of Tyrosinemia Type II on important parameters of mitochondrial energy metabolism and oxidative damage in the hippocampus, striatum and cerebral cortex of developmental rats.

Materials and methods

Animals

Male Wistar rats of 7 days old were obtained from the Central Animal House of the Universidade do Extremo Sul Catarinense (UNESC). The rats were left with their dams until weaning (day 21 of life). All rats were caged in groups of 5 with free access to food and water and were maintained on a 12-h light-dark cycle (lights on 7:00 am) at a temperature of 23 ± 1 °C. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Ethics Committee of the UNESC (protocols numbers 74/2014–01 and 14/2016–01).

Chronic administration of the L-tyrosine and omega-3 fatty acids

The animals were divided into four groups (n = 6–5 animals per group): 1) control (tween 2% + water), 2) n-3 PUFA (tween 2% + n-3 PUFA), 3) hypertyrosinemia (induced by the L-tyrosine + water), and 4) hypertyrosinemia supplemented with n-3 PUFA. The animals received intraperitoneally (i.p.) administrations twice a day (at 12-h intervals) of the L-tyrosine or tween 2% for 21 days starting at postnatal day (PD) 7 (last injection at PD 27). L-Tyrosine was

dissolved in tween 2% solution (pH was adjusted to 7.4) in 500 mg/kg body weight (Sgaravatti et al. 2009). It is important mentioning that one hour after administration of L-tyrosine is obtained a concentration plasmatic, measured by Ultra performance liquid chromatography (UPLC-MS) analysis, about ten times the normal value, which are similar to the concentrations observed in patients affected by hereditary Tyrosinemia Type II (Bongiovanni et al. 2003; Mitchell et al. 2001; Morre et al. 1980). In addition, the levels of tyrosine in brain regions also increase after administration of this amino acid (Morre et al. 1980). After the first administration of L-tyrosine the animals were supplemented by the administration via orogastric of n-3 PUFA (100 mg/kg) (El-Ansary et al. 2011) or water, once a day (at 24-h intervals) for a total of 21 days. The n-3 PUFA used is acquired via commercial fish oil capsule, in which each 1 mL of fish oil contains 188 mg EPA and 125 mg DHA. Twelve hours after the last injection, the animals were sacrificed by decapitation, the brain was quickly removed, and the hippocampus, striatum and cerebral cortex were collected. Tissues were weighed and then homogenized (1:10, *w*/*v*) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin). The homogenates were centrifuged at 800×g for 10 min, at 4 °C and the supernatants kept at −80 °C until being used to determine the activity of the Krebs cycle enzymes, creatine kinase and mitochondrial respiratory chain complexes. In addition, tissues were weighed and then homogenized in buffers specific for each parameter analysis of oxidative stress. Protein content was determined by the method described by Lowry et al. (1951) using bovine serum albumin as a standard.

Activity of Krebs cycle enzymes

Citrate synthase activity Citrate synthase (CS; EC 2.3.3.1) activity was assayed according to the method described by Srere (1969). The reaction mixture contained 100 mM Tris, pH 8.0, 0.1 mM acetyl CoA, 0.1 mM 5,5′-di-thiobis-(2-nitrobenzoic acid), 0.1% triton X-100, and 2–4 μg supernatant protein, and it was initiated with 0.2 μM oxaloacetate and monitored at 412 nm for 3 min at 25 °C. The results are expressed as nmol. min-1. mg protein-1.

Aconitase activity The activity of the enzyme aconitase (EC 4.2.1.3) was measured according to Morrison (1954). The reaction mixture consisted of 36 mM Tris-HCl, pH 7.4, 20% triton X-100, 10.5 mM citrate, 1.3 mM MnCl2, 120 mM FeSO4, 12 mM L-Cysteine, 27 mM $NADP^+$, 7 U/μL Isocitrate dehydrogenase, 15 μL of tissue homogenate. The reduction of $NADP^+$ was followed at wavelengths of excitation and emission of 340 and 466 nm for 15 min at 30 °C, respectively. The results are expressed as nmol. min-1. mg protein-1.

Isocitrate dehydrogenase activity Isocitrate dehydrogenase (IDH; EC 1.1.1.41) activity was determined in 33 mM Tris buffer, pH 7.4, containing 33 mM Tris-HCl, 10 μM rotenone, 1.2 mM MnCl2, 0.67 mM ADP, 0.1% Triton X-100, 0.3 mM NAD, homogenates (75 μg protein), and 5 mM isocitrate (Plaut 1969). NAD reduction at λ = 340–400 nm was followed (at 25 °C for 3 min) and the results are expressed as nmol. min-1. mg protein-1.

Alpha-ketoglutarate dehydrogenase activity The activity of α-ketoglutarate dehydrogenase complex (α-KGDH; EC 1.2.4.2) was determined according to the method of Lai and Cooper (1986) and Tretter and Adam-Vizi (2004). α-KGDH complex activity was assayed in a buffer containing 35 mM potassium phosphate, pH 7.4, 0.5 mM NAD+, 0.2 mM thiamine pyrophosphate, 0.04 mM coenzyme A, and 2 mM α-ketoglutarate. α-KGDH activity was determined by following NAD+ reduction at 340 nm at 37 °C. The results are expressed as nmol. min-1. mg protein-1.

Succinate dehydrogenase activity Succinate dehydrogenase (SDH; EC 1.3.99.1) activity was determined according to the method of Fischer et al. (1985), measured by following the decrease in absorbance because of the reduction of 2,6-dichloro-indophenol (2,6-DCIP) at 600 nm with 700 nm as the reference wavelength (e = 19.1 mM-1 cm-1) in the presence of phenazine methosulphate (PMS). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate and 8 mM 2,6-DCIP was preincubated with 40–80 μg homogenate protein at 30 °C for 20 min. Subsequently, 4 mM sodium azide, 7 μM rotenone and 40 μM 2,6-DCIP were added, and the reaction was initiated by an addition of 1 mM PMS and was monitored for 5 min. The results are expressed as nmol. min-1. mg protein-1.

Fumarase activity Fumarase (EC 4.2.1.2) activity was assayed in 100 mM sodium phosphate buffer, pH 7.3, containing 50 mM L-malate. The activity was determined by measuring the increase of absorbance at λ = 250 nm at 37 °C for 10 min (O'Hare and Doonan 1985). The results are expressed as nmol. min-1. mg protein-1.

Malate dehydrogenase activity Malate dehydrogenase (MDH; EC 1.1.1.37) was measured as described by Kitto (1969). Aliquots (20 mg protein) were transferred into a

medium containing 10 mM rotenone, 0.2% Triton X-100, 0.15 mM NADH, and 100 mM potassium phosphate buffer, pH 7.4, at 37 °C. The reaction was started by addition of 0.33 mM oxaloacetate. Absorbance was monitored at 340 nm for 3 min at 25 °C. The results are expressed as nmol. min-1. mg protein-1.

Activity of mitochondrial respiratory chain enzymes

Complex I activity NADH dehydrogenase (complex I; EC 1.6.5.3) was evaluated according to Cassina and Radi (1996) by determining the rate of NADH-dependent ferricyanide reduction at 420 nm during 3 min at 25 °C. The results are expressed as nmol. min-1. mg protein-1.

Complex II activity The activity of succinate-2,6-dichloroindophenol (DCIP) oxidoreductase (complex II; EC 1.3.5.1) was determined using the method described by Fischer et al. (1985). The reagents were preincubated for 20 min at 30 °C and was used 20–80 μg homogenate protein. Complex II activity was measured by following the decrease in absorbance due to the reduction of 2,6-DCIP at 600 nm during 5 min at 25 °C. The results are expressed as nmol. min-1. mg protein-1.

Complex II–III activity The activity of succinate: cytochrome c oxidoreductase (complex III; EC 1.3.99.1) was determined using the method described by Fischer et al. (1985). 10 μL of homogenates were preincubated during 30 min at 30 °C with reagents. Complex II–III activity was measured by cytochrome c reduction using succinate as substrate at 550 nm during 5 min at 25 °C. The results are expressed as nmol. min-1. mg protein-1.

Complex IV activity The activity of cytochrome c oxidase (complex IV; EC 1.9.3.1) was assayed according to the method described by Rustin et al. (1994) and measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome c (prepared by reduction of cytochrome with NaBH4 and HCl) at 550 nm with 580 nm as the reference wavelength during 10 min at 25 °C. The activities of the mitochondrial respiratory chain complexes were calculated as nmol. min-1. mg protein-1.

Activity of Creatine kinase

The total creatine kinase (EC 2.7.3.2) activity was measured in brain homogenates pretreated with 0.625 mM lauryl maltoside. The reaction mixture consisted of 60 mM Tris–HCl, pH 7.5, containing 7 mM phosphocreatine, 9 mM $MgSO_4$ and approximately 0.4–1.2 μg protein in a final volume of 100 μL. After 15 min of pre-incubation at 37 °C, the reaction was started by the addition of 3.2 mmol of ADP. The reaction was stopped after 10 min by the addition of 1 μmol of p-hydroxymercuribenzoic acid. The formed creatine was estimated according to the colorimetric method of Hughes (1962). The colour was developed by the addition of 100 μL 2% α-naphthol and 100 μL 0.05% diacetyl in a final volume of 1 mL; after 20 min, the colour was read spectrophotometrically at 540 nm. The results were calculated as nmol. min-1. mg protein-1.

Parameters of oxidative damage

Reactive species production determination Reactive species production was assessed according to LeBel et al. (1992) by using 2′,7′-dihydrodichlorofluorescein diacetate (DCF-DA). Dichlorofluorescin diacetate (DCFH-DA) was prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, and incubated with homogenates during 30 min at 37 °C. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. A calibration curve was performed with standard DCF (0.25–10 mM), and the levels of reactive species were calculated as pmol. mg protein-1.

Nitrate and nitrite determination Nitrate and nitrite levels were determined according to Miranda et al. (2001), by using Griess reagent (sulphanilamide 2% in HCl 5% and N-1-(naphtyl)ethylenediamine 0.1% in H_2O). A calibration curve was established using sodium nitrate and each curve point was subjected to the same treatment as supernatants. The absorbance was monitored spectrophotometrically at 540 nm. The concentrations were calculated as μmol. mg $protein^{-1}$.

Thiobarbituric acid-reactive species (TBA-RS) levels TBA-RS levels, a parameter of lipid peroxidation, were determined according to Esterbauer and Cheeseman (1990). The samples were mixed with 1 ml of trichloroacetic acid 10% and 1 ml of thiobarbituric acid 0.67%, and then heated in a boiling water bath for 30 min. A calibration curve was established using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as supernatants. The absorbance was monitored spectrophotometrically at 532 nm. TBA-RS values were calculated as nmol.mg $protein^{-1}$.

Protein carbonyl content Protein carbonyl content, a marker of protein oxidation, was measured spectrophotometrically according to previously (Reznick and Packer 1994). Proteins were precipitated by the addition of 20% trichloroacetic acid and were redissolved in DNPH. The absorbance was monitored spectrophotometrically at 370 nm. Results were calculated as nmol. mg $protein^{-1}$, using 22,000. m^{-1} .cm^{-1} as the extinction coefficient for aliphatic hydrazones.

Statistical analysis

The results are presented as mean ± standard deviation of the mean. All variables were tested for Gaussian distribution by the Kolmogorov-Smirnov normality test. Differences among between experimental groups were analyzed by one-way analysis of variance followed by Tukey HSD post hoc tests. Differences between the groups were considered significant at $P \leq 0.05$. All analyses were carried out on an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Results

Effects of n-3 PUFA treatment on Tyrosinemia-induced alterations in brain mitochondrial energy metabolism

We first investigated the effects of chronic L-tyrosine administration and co-administration of n-3 PUFA on Krebs cycle enzymes activities in the hippocampus, striatum and cerebral cortex of rats. It was demonstrated that chronic administration of L-tyrosine significantly decreased the CS activity in the hippocampus, striatum and cerebral cortex of the animals compared to controls. Notably, n-3 PUFA supplementation prevented the decrease in CS activity in the hippocampus and cerebral cortex (Fig. 1a). In addition, the chronic administration of L-tyrosine led to further significant decreases in the enzymatic activity of the IDH, but only in the hippocampus of rats. In this case, the supplementation with n-3 PUFA was not able to ameliorate the activity of this enzyme (Fig. 1c). In contrast, the activity of α-KGDH was increased in the hippocampus after the L-tyrosine administration (Fig. 1d). Moreover, the supplementation of n-3 PUFA prevented the increase in the activity of α-KGDH induced for L-tyrosine. Chronic administration of L-tyrosine decreases the activity of the SDH enzyme in the hippocampus and striatum, when compared to the control group, while the exposition to n-3 PUFA prevented this impairment in the hippocampus and striatum enzymes (Fig. 1e). Aconitase, fumarase, and MDH activities were not altered by L-tyrosine administration and supplementation (Fig. 1b, f and g, respectively).

In the sequence, we analyzed the effects of L-tyrosine administration on the activity of CK enzyme brain areas of rats. As we can see in the Fig. 2, CK activity was decreased in the striatum after the chronic administration of L-tyrosine compared to the control group. Importantly, n-3 PUFA co-administration was able to prevent the inhibition in the CK activity in the striatum induced by the chronic exposition to L-tyrosine injection (Fig. 2).

High levels of tyrosine significantly reduced activity of the complexes I, II-III and IV in striatum of animals (Fig. 3a, c and d respectively). The supplementation with n-3 PUFA partially prevented the inhibition of complex I activity in striatum (Fig. 3a). Moreover, co-administration of n-3 PUFA completely prevented alterations in the complexes II-III and IV (Fig. 3c and d). The activity of complex II (Fig. 3b) did not present significant alterations in the hippocampus, striatum and cerebral cortex.

Effects of n-3 PUFA treatment on brain oxidative damage induced by L-tyrosine chronic administration

The results of oxidation of DCFH and measurement of nitrites and nitrates are demonstrated in Fig. 4. Our results showed that chronic administration of L-tyrosine causes an increase in oxidation of DCFH only in the cerebral cortex, while the co-administration of n-3 PUFA was able to prevent DCFH oxidation in the cerebral cortex (Fig. 4a). Furthermore, we found a significant increase in the nitrites and nitrates levels in the hippocampus and striatum after chronic administration of L-tyrosine. The n-3 PUFA co-administration was not able to prevent these alterations observed in the brain area (Fig. 4b).

To characterize oxidative damage in the brains of rats submitted to a chronic administration of L-tyrosine and co-administration of n-3 PUFA, TBA-RS levels and carbonyl content were assessed. We found a significant increase in TBA-RS levels only in the cerebral cortex after chronic administration of L-tyrosine. Additionally, the co-administration of n-3 PUFA was able to prevent the increase of TBA-RS induced by L-tyrosine in the cerebral cortex of rats (Fig. 5a). In contrast, the analysis of carbonyl content showed no significant alterations in the hippocampus, striatum and cerebral cortex after chronic administration of L-tyrosine (Fig. 5b).

Discussion

Tyrosinemia Type II is an IEM of autosomal recessive inheritance. In Tyrosinemia Type II, patients present high levels of the amino acid L-tyrosine in tissues and physiological fluids. Studies have shown that the increase of this amino acid is responsible for causing neurological sequelae in patients (Gokay et al. 2016; Mitchell et al. 2013; Held 2006). In order to better understand the mechanisms that cause brain dysfunction in this IEM, recent studies have shown that the increase of L-tyrosine in the brain of developing rats alters parameters of mitochondrial energy metabolism, which was associated to oxidative stress (Teodorak et al. 2017; Ferreira et al. 2013b, 2015; Ramos et al. 2013; De Andrade et al. 2011a, 2011b, 2012). Moreover, patients may present low levels of EPA and DHA, and supplementation of it can prevent the progression of neurological impairment or neural improvement (Peña-Quintana et al. 2017; Dercksen et al. 2016; Jans et al.

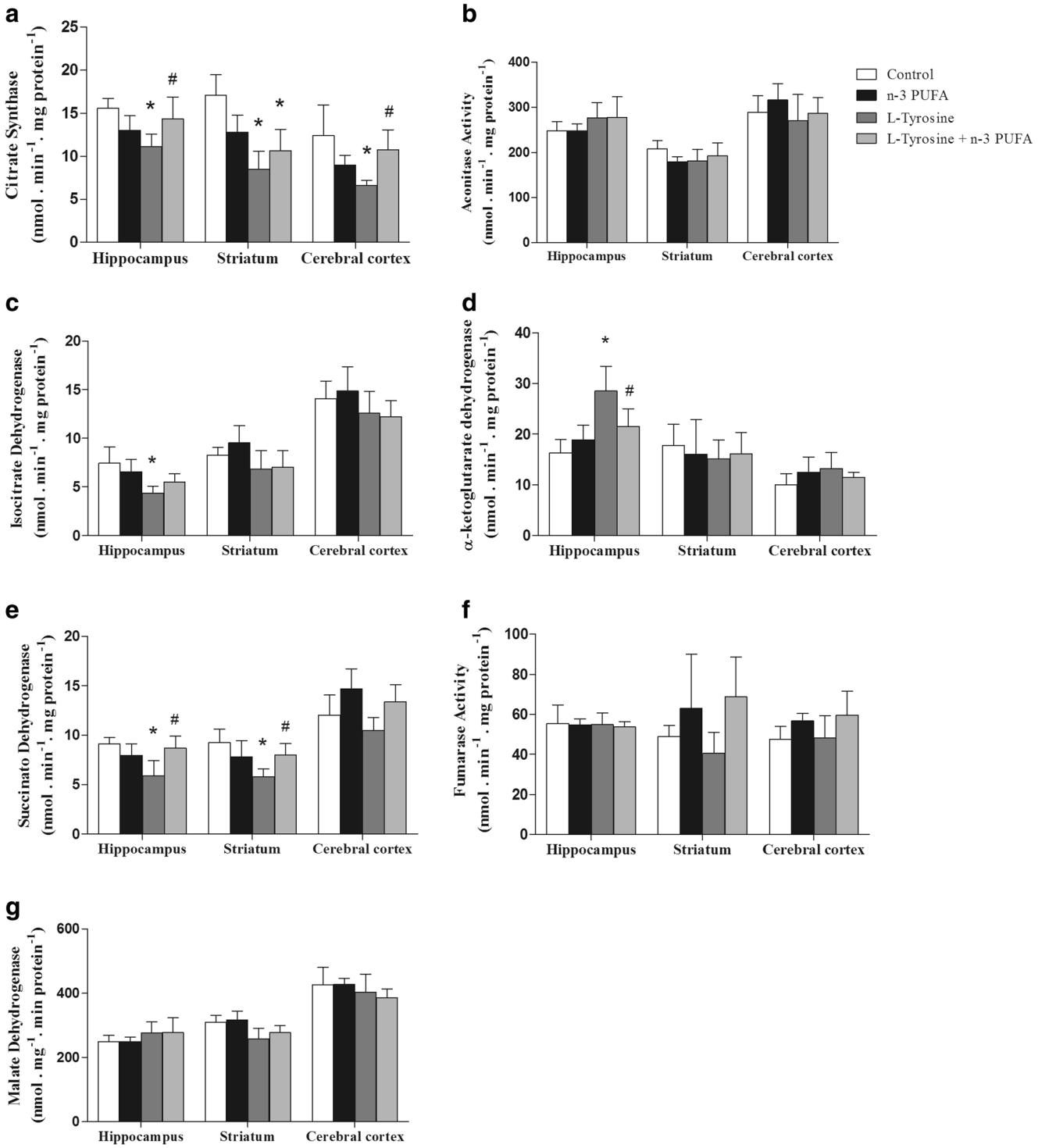

Fig. 1 Effect of chronic administration of the L-tyrosine, with or without n-3 PUFA supplementation, on the activity of the enzymes citrate synthase (a), aconitase (b), isocitrate dehydrogenase (c), α-ketoglutarate dehydrogenase (d), succinate dehydrogenase (e), fumarase (f) and malate dehydrogenase (g) in the hippocampus, striatum and cerebral cortex of rats. The data is expressed as mean ± standard deviation (mean ± SD of 6–5 animals per group) for independent experiments performed in duplicate. *Compared to control group, $P < 0.05$; #Compared with the L-tyrosine group, $P < 0.05$ (Tukey's HSD post hoc test). [n-3 PUFA = omega-3 fatty acids]

2013; Gil-Campos and Sanjurjo Crespo 2012; Mazer et al. 2010; Paker et al. 2010; Vlaardingerbroek et al. 2006).

Cell respiration is formed by three pathways, glycolysis, the mitochondrial Krebs cycle (or also named tricarboxylic

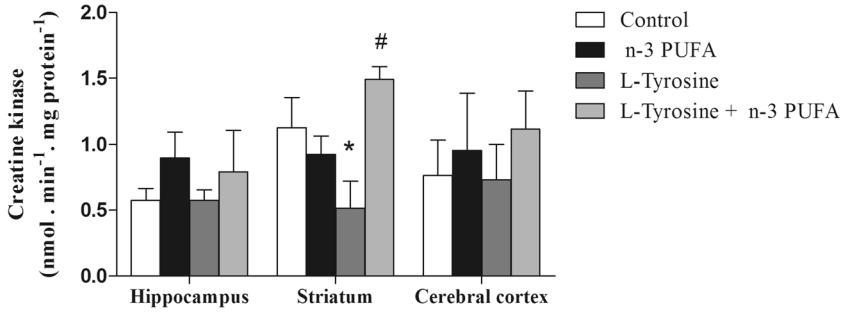

Fig. 2 Effect of chronic administration of the L-tyrosine, with or without n-3 PUFA supplementation, on creatine kinase activity in the hippocampus, striatum and cerebral cortex of young rats. The data is expressed as mean ± standard deviation (mean ± SD of 6–5 animals per group) for independent experiments performed in duplicate. *Compared to control group, $P < 0.05$; #Compared with the L-tyrosine group, $P < 0.05$ (Tukey's HSD post hoc test). [n-3 PUFA = omega-3 fatty acids]

acid cycle) and mitochondrial electron transport chain (Fernie et al. 2004). The Krebs cycle is a series of enzymatic reactions that catalyze the aerobic metabolism of nutrients to carbon dioxide (CO_2) and water, thereby generating reducing equivalents NADH and $FADH_2$. These reduced coenzymes deliver their electrons to the electron transport chain that ultimately utilizes oxygen as the final acceptor, promoting energy for the production of adenosine triphosphate (ATP) molecules (Sawa et al. 2017). Complementing the previous studies using acute and chronic injection of L-tyrosine in rats (Ferreira et al. 2013a, b, 2014; Ramos et al. 2013), our results demonstrate that the chronic administration of L-tyrosine leads to a decrease in CS activity in the hippocampus, striatum and cerebral cortex. The enzyme CS is considered a marker of mitochondrial metabolism, since it initiates the Krebs cycle by the irreversible condensation of oxaloacetate to the acetyl group of acetyl-CoA (Chepelev et al. 2009). In addition, we observed the decrease in the activity of the enzyme IDH and the increase in α-KGDH, only in the hippocampus of rats after the L-tyrosine chronic exposition. The enzyme SDH

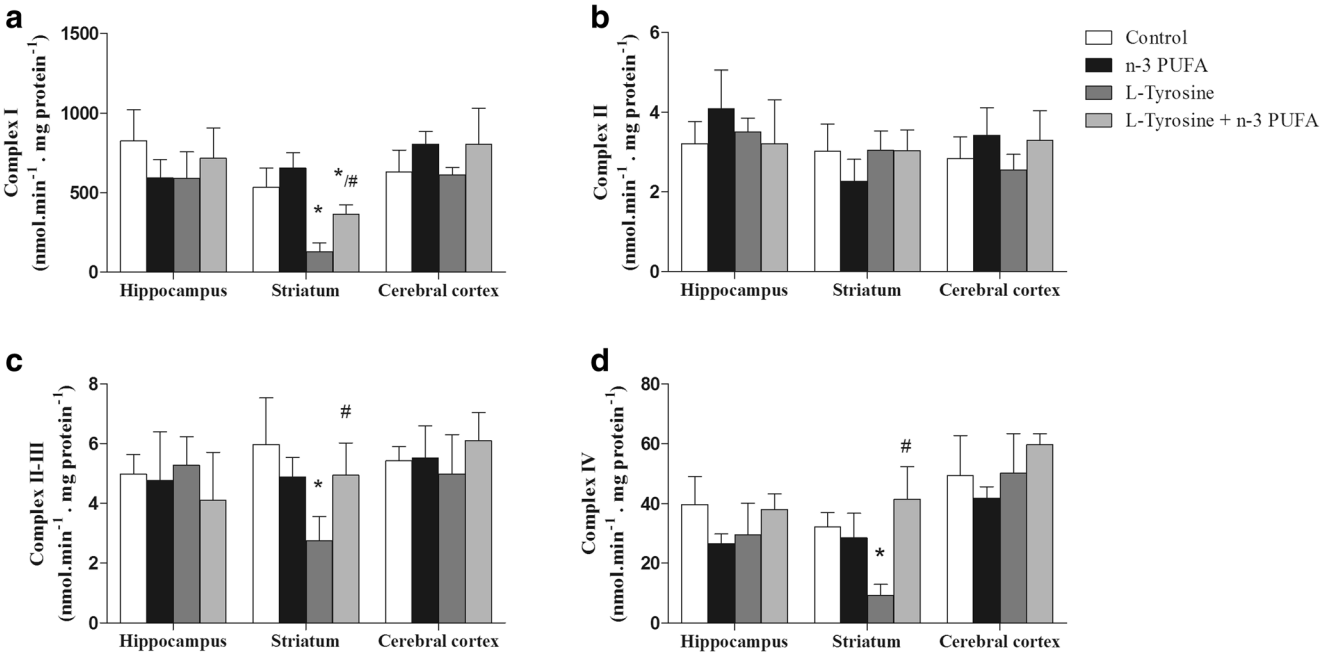

Fig. 3 Effect of chronic administration of the L-tyrosine, with or without n-3 PUFA supplementation, on complex I (a), complex II (b), complex II-III (c) and complex IV (d) activities of mitochondrial respiratory chain in the hippocampus, striatum and cerebral cortex of young rats. The data is expressed as mean ± standard deviation (mean ± SD of 6–5 animals per group) for independent experiments performed in duplicate. *Compared to control group, $P < 0.05$; #Compared with the L-tyrosine group, $P < 0.05$ (Tukey's HSD post hoc test). [n-3 PUFA = omega-3 fatty acids]

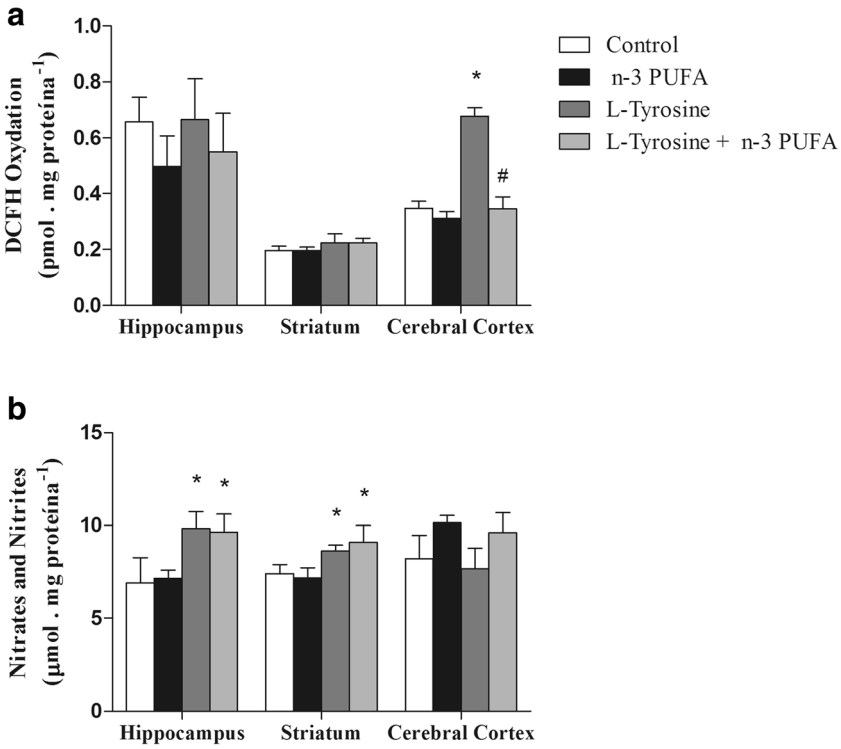

Fig. 4 Effect of chronic administration of the L-tyrosine, with or without n-3 PUFA supplementation, on the quantification of reactive species (a) as well as nitrates and nitrites (b) production in hippocampus, striatum and cerebral cortex of young rats. The data is expressed as mean ± standard deviation (mean ± SD of 6–5 animals per group) for independent experiments performed in duplicate. *Compared to control group, $P < 0.05$; #Compared with the L-tyrosine group, $P < 0.05$ (Tukey's HSD post hoc test). [n-3 PUFA = omega-3 fatty acids]

demonstrated reduced activity in the hippocampus and striatum; fumarase and MDH showed no significant changes. It is suggested that fumarase and MDH are in normal activity because the amino acid tyrosine is metabolized to fumarate, which is a substrate for the reaction of the fumarase enzyme to malate, and then oxidized to oxaloacetate. In the striatum, we also observed a reduction in CK activity.

Changes in Krebs cycle, CK and mitochondrial respiratory chain complexes may lead to decreased ATP synthesis, and trigger mitochondrial injury and cell death (Horn and

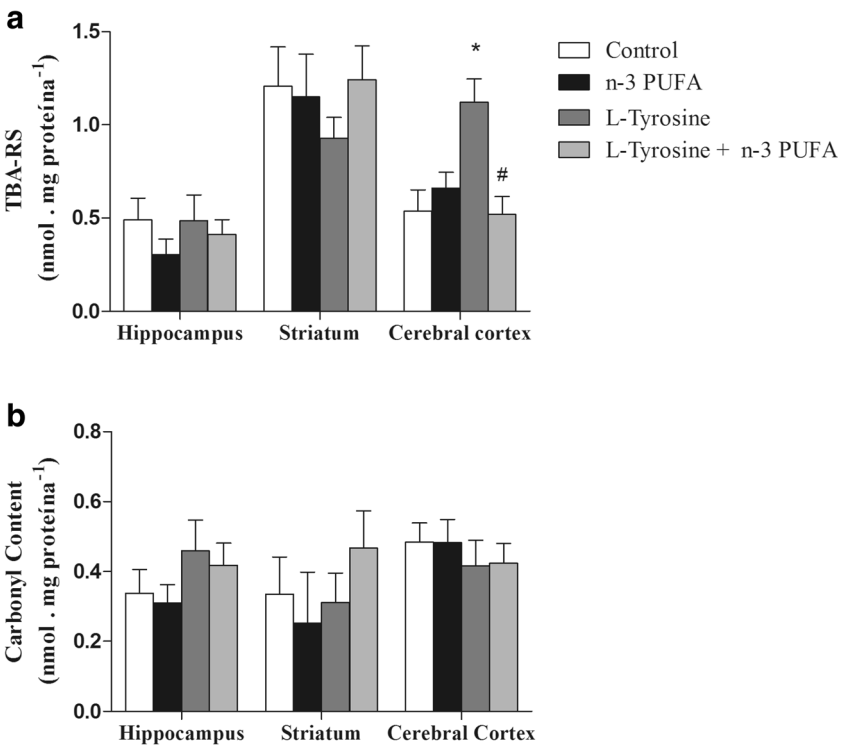

Fig. 5 Effect of chronic administration of the L-tyrosine, with or without n-3 PUFA supplementation, on the quantification of TBA-RS levels (a) and carbonyl content (b) in hippocampus, striatum and cerebral cortex of young rats. The data is expressed as mean ± standard deviation (mean ± SD of 6–5 animals per group) for independent experiments performed in duplicate. *Compared to control group, $P < 0.05$; #Compared with the L-tyrosine group, $P < 0.05$ (Tukey's HSD post hoc test). [n-3 PUFA = omega-3 fatty acids]

Barrientos 2008; Schurr 2002). In fact, changes in energy metabolism are present in the pathophysiology of some diseases that affect the brain, such as Bipolar Disorder, Depression, Schizophrenia, Alzheimer's disease and in several IEM (Maple Syrup Urine Disease, Homocystinuria and Tyrosinemia Type I) (Cancelier et al. 2016; Kolling et al. 2012; Rezin et al. 2009; Ribeiro et al. 2008; Atamna and Frey 2007; Rigante et al. 2005). Disorders in ATP availability can contribute to the onset of neurological problems (Garcia-Cazorla et al. 2008). In this regard, we also investigated the activity of mitochondrial respiratory complexes. Importantly, the chronic exposition to L-tyrosine causes an impairment in the complexes I, II and IV only in the striatum of rats exposed to L-tyrosine for 21 days.

The mitochondrial respiratory chain is a major source of reactive species, such as oxygen reactive species (ROS) in the cells. Physiologically, the mitochondrial ROS production presents redox-signaling function. However, when the mitochondria is dysfunctional this organelle could become a source of oxidative stress, i.e. uncontrolled ROS production. On the other hand, ROS could induce impairment in the function of mitochondrial respiratory chain. Furthermore, the excess of ROS can lead to DNA, protein and lipids oxidation and damage (Murphy 2009; Liemburg-Apers et al. 2015). Herein, we observed that the impairment in the function of mitochondrial enzymes induced experimentally by Tyrosinemia, is associated with enhanced reactive species production in the cerebral cortex and high levels of nitrates and nitrites in the hippocampus and striatum. We also observed that the high production of reactive species is accompanied by increased lipid peroxidation in the cerebral cortex.

Of note, we observed that the tyrosine toxicity affected, in some way, all the brain structures evaluated. Markedly, it is important to highlight the evidence for striatal susceptibility to energy metabolism impairment, i.e. inhibition in the Krebs cycle enzymes and mitochondrial complexes activities, induced by chronic tyrosine injection in rats, which corroborate findings from our previous works (Ferreira et al. 2015; Ramos et al. 2013; Ferreira et al. 2013a, b). In addition, Ferreira et al. (2014) demonstrated that the acute and chronic exposition to tyrosine induced a decrease in the BDNF levels in striatum of rats. On the other hand, taking together our data from the present study and previous (Streck et al. 2017), we can speculate that, in rats, the cerebral cortex, compared to other brain structures, seems more prone to be affected by tirosyne-induced oxidative damage. In fact, the L-tyrosine uptake is different across the brain areas (Bongiovanni et al. 2003).

In the present study, we also evaluated the effect of n-3 PUFA supplementation on the chemically induced Tyrosinemia Type II model. Notably, we found that supplementation of n-3 PUFA prevented some of the changes in the activity of metabolism enzymes and decreased oxidative stress caused by the chronic administration of L-tyrosine in rats. Experimental studies demonstrate that, in rats, n-3 PUFA supplementation increment brain levels of DHA and EPA (Chung et al. 2008; Avramovic et al. 2012; Cutuli et al. 2016). Importantly, brain phospholipids contain high concentrations of n-3 PUFA, and the EPA and DHA fatty acids are fundamental for the formation, development and functioning of the brain and, particularly, the visual system (Cheatham et al. 2006; Smith 1992; Wurtman 2008). The neuroprotective effect observed in our study might possibly be attributed to the antioxidant effect of the n-3 PUFA, DHA and EPA (Carvalho-Silva et al. 2017; Schmidt et al. 2012; Zhang et al. 2011; McAnulty et al. 2010). Recently, we demonstrated that n-3 PUFA prevented increased DNA damage, evaluated through alkaline comet assay and 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels, in the striatum, hippocampus and cerebral cortex in the rats exposed chronically to L-tyrosine. Markedly, in the hippocampus and striatum the n-3 PUFA completely prevented the oxidation of DNA induced by tyrosine toxicity (Carvalho-Silva et al. 2017). Indeed, n-3 PUFA adhere to the different lipid membranes, and due to their unsaturations in the hydrocarbon chain, they are vulnerable to the attack of oxygen and nitrogen reactive species, and, as a shield, they protect the cell against oxidative stress. In addition, n-3 PUFA were able to improve fluidity, flexibility and permeability of cell membranes (Wall et al. 2010; Wu et al. 2008; Godwin and Prabhu 2006). An important point is that the supplementation was not able to prevent the inhibition of CS in the striatum of the animals exposed to tyrosine. We can speculate that, at least in part, the energy metabolism remained impaired in the striatum of these animals.

Previously, we also worked with other neuroprotective strategies to ameliorate the brain alterations in the animal model of Tyrosinemia (Gomes et al. 2018; Teodorak et al. 2017; Streck et al. 2017). For instance, we investigated the effects of N-acetylcysteine (NAC), antioxidant donor of thiol groups, in the energy metabolism impairments and oxidative damage induced by chronic administration of tyrosine in infant rats (Teodorak et al. 2017; Streck et al. 2017). The positive outcomes of n-3 PUFA supplementation in Krebs cycle enzymes activity and function of respiratory complexes were superior to those observed with NAC treatment, in Tyrosinemia. In addition, we also showed that both treatments, n-3 PUFA and NAC, result in similiar beneficial effects in the oxidative stress induced by chronic administration of tyrosine in rat's cerebral structures. It is important to observe that NAC was administrated in association with deferoxamine (DFX), an iron chelator, which could be pointed as a disadvantage. In fact, the isolated use of NAC could be limited by its pro-oxidant effects, probably through its interaction with iron, since oxidative metabolism of NAC can produce thiyl free radicals (Barbosa et al. 2010; Ritter et al. 2004). Since the mechanisms of NAC and n-3 PUFA diverge, we can propose

that a combination of these compounds would be beneficial in the Tyrosinemia Type II.

Besides the antioxidant role, El-Ansary et al. (2011) suggest that supplementation of n-3 PUFA 100 mg/kg, as used in this study, may work by normalizing the levels of the neurotransmitters dopamine, gamma-aminobutyric acid (GABA) and serotonin in the brains of rats submitted to intoxication by propionic acid. In addition, Decker et al. (2016) suggested that n-3 PUFA supplementation confers neuroprotection against hypoxia-induced dopaminergic dysfunction in newborn rats. Thus, another possible hypothesis to justify the protective effects of DHA and EPA supplementation in the chemically induced animal model of Tyrosinemia Type II would be through the regulation of neurotransmitters; however, studies are needed to strengthen this hypothesis.

Another important point is that inborn errors, e.g. aminoacidopathies, seems to be associated with deficiency in n-3 PUFA. Gil-Campos and Sanjurjo Crespo (2012) pointed out, in their systematic review, some studies that suggested n-3 PUFA supplementation over long periods as a strategy to prevent the development of cognitive impairment in children, and inhibits the onset of neuronal function loss. In fact, the beneficial effects of fats in children were evidenced early in the 1960s (Gil-Campos and Sanjurjo Crespo 2012).

To conclude, the results of the study reinforce the hypothesis that high levels of L-tyrosine, in a chronic way, cause changes in Krebs cycle enzymes, CK and mitochondrial respiratory chain complexes, and consequently oxidative damage (Teodorak et al. 2017; Streck et al. 2017; Ferreira et al. 2015). In addition, we demonstrated that the supplementation of n-3 PUFA presented beneficial effects on the changes caused by L-tyrosine, acting as a neuroprotector, suggesting that n-3 PUFA may be used in the clinic as adjunctive treatment for Tyrosinemia Type II.

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