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Fish oil and treadmill exercise have age-dependent effects on episodic memory and oxidative state of the hippocampus

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Abstract

There is a growing interest to better understand how lifestyle choices can improve memory functions. Treadmill exercise (Ex) and long-chain n-3 polyunsaturated fatty acids (PUFAs) found in fish oil (Fo) are able to stimulate hippocampal antioxidant defenses and improve memory. The aim was to test whether (Fo) and (Ex) can improve rat’s performance on memory tasks and optimize hippocampal antioxidant state in an age-dependent manner. Therefore, young and adult rats were exercised and received (Fo) during four weeks. The (Ex) was performed during 30min/day, with the speed gradually increasing from the first to the last week. Afterwards, episodic memory was measured by the recognition of object identity and spatial location. Hippocampal oxidative state was investigated with the levels of malondialdehyde (MDA), carbonyls content, antioxidant enzymatic activity (superoxide dismutase (SOD), catalase (CAT)), and antioxidant non enzymatic activity (reduced glutathione, sulphydryl content). The (Fo&Ex) adult rats were able to recognize object’s shape and placement, however (Fo&Ex) young rats had impaired spatial recognition (p<0.05). (Fo&Ex) young rats did not have reduced MDA or carbonyl content, though either (Fo) or (Ex) reduced MDA (p<0.05) and carbonyl levels (p<0.01). (Ex) increased SOD (p<0.001) and CAT activities (p<0.05), and (Fo) enhanced SOD activity (p<0.05) in young rats. At adulthood, (Ex) increased MDA levels (p<0.05), and (Fo&Ex) reduced MDA (p<0.001). Lastly, (Ex) and (Fo) improved non-enzymatic antioxidant defense (p<0.05) only in adult rats. Results support age-dependent effects of the (Fo) and (Ex) on memory and oxidative state of the hippocampus during either neurodevelopment or adulthood.

Keywords: fish oil, EPA, DHA, treadmill exercise, object recognition, hippocampal oxidative status.
1.0 Introduction

Memory disorders are associated with the malfunctioning of neuroanatomical structures that are related to the storage, retention and recollection of memories. The prevalence and complications of memory disorders such as Dementia and Alzheimer’s disease represent a worldwide problem (Qiu et al. 2007). Despite the ongoing research, the use of preventive lifestyle choices and the underlying mechanisms that are related to the incidence of these neurodegenerative diseases are still unclear.

Since lifestyle choices can improve cognition and memory, there is a growing interest to understand how these choices can benefit neurodevelopment and possibly counteract either the incidence or the complications of neurodegenerative diseases (Murphy et al. 2014, Reijs et al. 2015). For instance, the task of object recognition is a tool to study memory and it investigates different memory processes such as acquisition, consolidation and retrieval (Prickaerts et al. 2005).

Studies in humans (Churchill et al. 2002, Murphy et al. 2014, Sachdeva et al. 2015) and animals (Radak et al. 2001 and Murphy et al. 2014) suggest that the effects of long chain omega-3 (n-3) fatty acids or physical exercise can act on cognition, as well as improve brain function. Since the consumption of (n-3) PUFA and exercise (Ex) can be simultaneously present in daily life, the association between them could indicate that their effects on the brain are complementary (Wu et al. 2008).

From fetal life to adulthood, adequate nutrition is an essential requirement for morphogenesis of the brain. During this initial period, PUFAs play an important role (Gharami et al. 2015). The n-3 consumption can benefits brain health, and these benefits have been investigated by experimental and epidemiologic studies. Specific features caused by n-3 consumption are associated to hippocampal BDNF elevation, stimulation of mitochondrial function and reduced oxidative stress (Gomez-Pinilla 2008).
The supplementation with n-3 polyunsaturated fatty acids enhances hippocampal functionality in aged mice (Cutuli et al. 2014), as well as prevents brain oxidative damage in the ketamine model of schizophrenia in young rats (Zugno et al. 2014). Furthermore, beneficial properties of fish oil (Fo) can be extended to a better performance of memory tests in animal models of Alzheimer’s disease (Hashimoto et al. 2011) and brain injury (Wang et al. 2013).

On the other hand, whether or not (Ex) can improve cognition seems to depend on the animal life stage (Churchill et al. 2002, Radak et al. 2001). The (Ex) demonstrated beneficial effects, because it prevents oxidative stress in brain regions of adolescent (Chalimoniuk et al. 2015) and aging rats (Flores et al. 2014).

It is also known that the association of (Fo) supplementation and (Ex) are characterized by enhanced antioxidant defenses (da Silva Pedroza et al. 2015). However, data show that the effects of (Fo) supplementation (Pusceddu et al. 2015) or (Ex) (Batista-de-Oliveira et al. 2012) depend on the animal age.

Fish oil and exercise can ameliorate oxidative stress and benefit memory; although there is still lack of information about the underlying mechanisms. It is also unclear if there is a timing-related association between the oxidative state of the hippocampus and behavioral findings. Therefore, we aimed to investigate whether the daily supplementation with (Fo) coupled with moderate treadmill (Ex) in both young and adult rats would influence memory and hippocampal oxidative stress.

2. Materials and methods

2.1 Animals and experimental design

Wistar rats were used from the colony of the Nutrition Department at the Federal University of Pernambuco (Brazil). The experimental design followed the recommendations of the Ethics
Committee for Research on Animals (23076.027072/2014-20). These recommendations were in accordance to the "Principles of Laboratory Animal Care" (National Institutes of Health, Bethesda, USA). Every effort has been made to minimize animal suffering and to reduce the number of animals per group.

After mating and gestation, the pregnant rats delivered 7–12 pups per litter. The pups from 6 to 8 litters were first joined in a big pool. The animals were randomly distributed, 24 hours after birth, in 9 rats per litter. They were kept in a room with a temperature of 23 ± 1 °C and raised in a 12h light / dark cycle (lights on from 7:00 am to 7:00 pm). After weaning, 21 days-old animals were divided into cages with 3-4 rats with free access to water and food - standard diet of pellets laboratory (Presence of Brazil Ltd, Present, São Paulo, Brazil) according to the American Institute of Nutrition (Reeves et al. 1993).

Either 15 or 90 days-old rats were randomly assigned into 4 different experimental groups: (1) supplemented with fish oil and exercised (Fo&Ex, n=12 (young) and n = 11 (adult rats)); (2) supplemented with fish oil and sedentary (Fo&S, n= 12 (young) and n = 11 (adult rats)); (3) supplemented with vehicle solution and exercised (V&Ex, n= 12 (young) and n = 11 (adult rats)) (4) supplemented with vehicle solution and sedentary (V&S, n=12 (young) and n = 11 (adult rats)). The trial period was 4 weeks.

2.2 Supplementation with fish oil

The daily supplementation was performed by gavage. Rats received a single daily dose of fish oil (Fo, 85mg/kg/d and 1ml/250g/d) or vehicle solution (V, 1 ml/250g/d) as adapted from (Rachetti et al. 2013, da Silva Pedroza et al. 2015). The capsules of fish oil (Sundown®) containing polyunsaturated fatty acids [docoisahexaenoico (DHA; 85mg/1g) and ecosapentaenoico (EPA; 128mg/1g)] were dissolved in Cremophor (Sigma ®) 0.009%, then in distilled water and were
administered via gavage. To perform the control group, a vehicle solution (V) was given with the same amount of Cremophor and distilled water that was used to prepare the solution provided to supplement the fish oil.

2.3 Treadmill exercise

All exercised rats were placed on the treadmill (Insight EP-131, 0° inclination) following parameters of moderate exercise that were adapted from previous studies (Batista-de-Oliveira et al. 2012). Briefly, animals were placed 30min/day on the treadmill. The speed was increased as follows: 5m/min (first week); 10 m/min (second week) and 15m/min (third and fourth weeks). The rats in the sedentary group (S) were placed at same time in the treadmill, but the apparatus remained off.

Treadmill exercise lasted for 4 weeks, and was performed at the same time frame of the supplementation with fish oil. Therefore, either from 15 to 45 or 90 to 120 days old, rats were submitted to the treadmill and supplementation to perform the experimental groups with young and adult animals, respectively.

2.4 Behavioral analysis

All the behavioral tests were assessed individually at either 46 or 121 days old to perform the young and adult groups, respectively. Detailed protocols and the rationale for the test choices provided are discussed elsewhere (Ennaceur and Delacour 1988, Dere et al. 2005, Viana et al. 2013).

In the present study, two experiments were designed to test the novelty recognition paradigm, regarding the object’s novel shape or location. These experiments consisted of three days. In the **first day**, the rats were placed during five minutes in the open field apparatus to explore and
familiarize itself with the experiment’s environment. The apparatus consisted of a circular arena with brown walls and an opened top. The floor of the arena was divided into 17 fields which are separated by black lines. In this arena, there are three concentric circles, and it was located in a sound-attenuated room, with reduced lighting.

24 and 48 hours later, in the second and third days, each rat was returned to the circular arena, which then contained two equal objects, made of clear glass. These objects were then explored by each rat during five minute intervals, and this constituted the trial session. After a fifty-minute interval the animal returned to the arena to perform a five-minute test session. The test session assessed the rat’s capability to recognize novel object’s shape, or novel object’s location (dislocated object). This was tested in the second and third day, respectively. Each experiment was recorded by a camera installed in the ceiling. The files were analyzed with the ANY-Maze Software (version 4.99 m) by two previously trained observers who were “blinded” regarding the previous treatment of the animal. The videos were analyzed to assess the time spent by the rat to explore each object. The criteria to define the time spent by the rats were based on the “active exploration”, defined as touching the objects with the vibrissae, snout or forepaws, as previous published (Dere et al. 2005, Viana et al. 2013). In the trial session, preference ratio was calculated for each animal as time spent by the rat in exploring each object/total exploration time. The trial sessions ensured that rats employed equal time in exploring each of the two objects, and therefore did not exhibit a preference for one single item. Rats that presented in the trial session a ratio near 0.5, indicating equal exploration of the two objects, were eligible to realize the test session; otherwise they were discarded.

In each test session, the rats were expected to recognize the familiar object or the familiar (stationary) position previously presented (in the trial session). The preference ratios for the familiar, the novel shape or the object position (dislocated) were also calculated for these animals.
The animals would be expected to spend more time exploring the objects that represented a novel shape or a dislocated position in the arena.

The performance was represented by a *discrimination index* (%) which consisted of the exploration time for each analyzed criteria (familiar *versus* novel shape and stationary *versus* dislocated position), expressed as a percentage of the total time of exploration. This paradigm does not involve the learning of any rule, since it is entirely based upon the spontaneous exploratory behavior of rats towards objects (Ennaceur and Delacour 1988). The objects were devoid of any ethological meaning, as they had never been paired as a reinforce and were heavy enough to prevent being moved by the animal. Because the objects were made with the same material, the rats could not distinguish them by olfactory cues during the trial session. After each session, the objects and the apparatus were thoroughly cleaned with 70% ethanol solution.

2.5 Drugs and reagents

All drugs and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) (Sinc Pernambuco, Brazil).

2.6 Oxidative Stress Biomarkers

2.6.1 Sample preparation for oxidative stress and antioxidant analyses

Hippocampus of young and adult rats were homogenized in 50 mM-TRIS and 1mM-EDTA (pH 7.4), with the addition of 1 mM-sodium orthovanadate and 200\(\mu\)g/mL phenylmethanesulfonyl fluoride. Homogenates were centrifuged at 1180g for 10 min at 4°C and the protein supernatant used for biochemical analyses after the protein oxidation had been quantified by the *Bradford* method (Bradford 1976).
2.6.2 Evaluation of Lipid Peroxidation

Lipid peroxidation was evaluated using malondialdehyde (MDA) levels as previously published (Buege and Aust 1978). Three hundred µg of protein was sequentially mixed to 30% (w/v) trichloroacetic acid and 10 mM-TRIS buffer (pH 7.4). This mixture was centrifuged at 2500g for 10 min and the supernatant was boiled for 15 min with 0.73% (w/v) thiobarbituric acid. The pink pigment yielded then was measured at 535 nm absorption at RT and expressed as mmol/mg protein.

2.6.3 Evaluation of Protein Oxidation

The protein oxidation was assessed using the procedures highlighted by Reznick and Packer (1994). With the samples on ice, 30% (w/v) TCA was added to the sample (300 µg of protein) and then centrifuged for 14 min at 1180g. The pellet was suspended in 10 mM 2,4dinitrophenylhydrazine and immediately incubated in a dark room for 1h with shake turned on each 15min. Then the samples were washed and centrifuged three times in an ethyl/acetate buffer and then final pellet was suspended in 6M guanidine hydrochloride, incubated for 30 min at 37°C and the absorbance read at 370nm. The results were expressed as µM/mg protein.

2.6.4 Measurement of superoxide dismutase (SOD) activity

SOD determination was performed in accordance with the protocol developed by Misra and Fridovich (1972). In brief, 300 µg of protein were added to 0.05 M-Carbonate buffer with 0.1 mM-EDTA (pH 10.2). The reaction was started by the addition of 150mM-epinephrine and the SOD activity was determined by adrenaline auto-oxidation inhibition at 30°C. The decrease in absorbance was followed for 1.5 min at 480 nm and the results expressed as U/mg protein (Misra
and Fridovich 1972). The 1U of SOD is the amount of SOD necessary to decompose 1.0 µM of anion superoxide per minute.

2.6.5 Measurement of Catalase (CAT) activity

The CAT activity was performed as previously described (Aebi 1984). Briefly, 0.3 M-hydrogen peroxide and 300 µg of protein were added to a 50 mM-phosphate buffer (pH 7.0) at 20° C and the absorption decay was monitored for 3 min at 240nm with the results expressed as U/mg protein (Aebi 1984). The 1U of CAT is the amount of catalase necessary to decompose 1.0 µM of hydrogen peroxide per minute.

2.6.6 Measurement of Glutathione-S-transferase (GST) activity

GST activity was measured as described previously by Habig et al. (1974). Two hundred µg of protein was incubated in a 0.1 M-phosphate buffer (pH 6.5) containing 1mM-EDTA at 30° C and had the assay started with the addition of 1 mM 1-chloro-2.4-dinitrobenzene and 1 mM-GSH. The formation of 2.4-dinitrophenyl-S-glutathione was monitored through the absorbance at 340 nm. One unit of enzymatic activity was defined as the amount of protein required to catalyze the formation of 1 µmol-2.4-dinitrophenyl-S-glutathione (Habig et al. 1974). The results of GST are expressed in unit/mg of protein.

2.6.7 Measurement of reduced glutathione (GSH)

To assess GSH levels, the samples (300 µg of protein) were first diluted in a 0.1 M phosphate buffer containing 5 mM-EDTA (pH 8.0). Then, an aliquot from the diluted sample was incubated with o-Phthalaldialdehyde at room temperature for exactly 15 min. Fluorescence intensities measured at 420 nm and excitation at 350 nm were compared with a standard curve of known concentrations.
of GSH (0.5-10 mM) as previously described (Hissin and Hilf 1976). The results of GSH are expressed in micromol/mg of protein.

2.6.8 Sulphydryl content

To evaluate the levels of sulphydryl, we based the assay on the reduction of 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) that absorbs at 412 nm in spectrophotometry (Aksenov and Markesbery 2001). Briefly, at TRIS buffer, pH7.4, 450 µg of protein was added in addition to 1 mM DTNB. This mix was incubated 30 min at room temperature in a dark room. Absorption was measured at 412 nm. The sulphydryl content was inversely correlated to oxidative damage to proteins. Results were reported as mmol TNB/mg protein.

2.7 Statistical analysis

Results are expressed as means ± the standard error of the mean (SEM). Two-way ANOVA test was performed to assess significant differences between the groups. The tests of significance were recommended based on the results of the tests of normality. Kolmogorov-Smirnov test and Tukey test were used to evaluate the oxidative state and behavioral data, respectively. Data were considered as statistically significant for p<0.05. All data were plotted and the statistical analysis performed using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA).

3.0 Results

3.1 Behavioral assays

Discrimination indexes (MEAN±SEM) regarding the performance of young and adult rats on the tests of object identity and placement recognition are shown in Figure 1. Two way ANOVA demonstrated that young animals were able to recognize object identity [F (1, 80) = 195.6, ****p <
Tukey post-hoc test showed the following significant differences (novel versus familiar object): Fo&Ex, 63.3±2.5 versus 36.7±2.5; Fo&S, 71.3±3.8 versus 28.7±3.8; V&Ex, 66.0±4.0 versus 34.0±4.0, and V&S, 68.7±3.5 versus 31.3±3.5), p < 0.0001. On the other hand, when the placement recognition was evaluated, young rats showed a treatment-dependent impairment. Two-way ANOVA demonstrated significant differences related to the treatment-task interaction [F (3, 62) = 19.3, p< 0.0001] and task of object placement [F (1, 62) = 5.6, p = 0.02] alone. The effect of the treatment-task interaction was observed among V&S versus Fo&S, and V&S versus Fo&Ex (**p< 0.05). The treatment- dependent impairment regarding the placement task was observed by the lower discrimination indexes for the dislocated versus stationary object (Fo&Ex, 39.6±2.7 versus 60.4±2.7, p< 0.005; Fo&S, 39.3±3.1 versus 60.7±3.1, p<0.01). The impairment was not observed in young rats from the respective control groups (V&Ex, 48.0±3.8 versus 52.0±3.8, p>0.05; V&S, 62.2±2.6 versus 37.8±2.6, p< 0.01). These results are described in Figure 1 A and C.

At adulthood, two-way ANOVA showed that rats had the ability to recognize the novel [F (1, 66) = 400.4, ****p < 0.0001] and dislocated [F (1, 60) = 133.3, ****p < 0.0001] objects as shown by the higher discrimination indexes for the recognition of object identity (novel versus familiar) and object placement (dislocated versus stationary). In regards of the object identity recognition, Tukey post-hoc test showed that adult rats presented higher values of the discrimination indexes (MEAN±SEM) for the novel when compared to the familiar object (Fo&Ex, 69.3±2.9 versus 27.8±2.9, p< 0.001; Fo&S, 72.9±2.9 versus 27.0±2.9, p<0.001; V&Ex, 76.0±3.6 versus 24.0±3.6, p< 0.001 and V&S, 75.5±4.26 versus 24.5±4.26, p<0.001). Similar results were observed for the object placement tasks as judged by the increased discrimination indexes ((MEAN±SEM) Fo&Ex, 61.8±2.7 versus 38.2±2.7, p< 0.01; Fo&S, 64.4±3.1 versus 35.6±3.1, p<0.01; V&Ex, 63.2±3.6 versus 36.8±3.6, p< 0.01 and V&S, 63.9±3.7 versus 36.1±3.7, p<0.01)). Figure 1 B and D describe these results.
3.2 Oxidative state

In addition to previous data, we observe that in young rats the exercise induces decrease in lipid peroxidation evaluated by malondialdehyde (MDA) levels (V&S: 37.7 ± 8.0 nmol/mg prot, n=6; V&Ex: 16.1 ± 0.6 nmol/mg prot, n=6, p<0.05) and protein oxidation evaluated by carbonyls content (V&S: 19.4 ± 1.7 µmol/mg prot, n=6; V&Ex: 14.4 ± 0.9 µmol/mg prot, n=7, p<0.01). Moreover, fish oil supplementation decreases lipid peroxidation (V&S: 37.7 ± 8.0 nmol/mg prot, n=6; Fo&S: 17.0 ± 2.7 nmol/mg prot, n=6, p<0.01) and protein oxidation (V&S: 19.4 ± 1.7 µmol/mg prot, n=6; Fo&S: 10.5 ± 0.6 µmol/mg prot, n=7, p<0.001); however the association between exercise and fish oil supplementation did not induce a significant difference in the young rats. In adult rats, exercise induces a different response; it causes a significant increase in MDA levels (V&S: 11.3 ± 2.4 nmol/mg prot, n=6; V&Ex: 55.1 ± 8.0 nmol/mg prot, n=6, p<0.001), on the other hand there was no differences in carbonyl content (V&S: 1.6 ± 0.2 µmol/mg prot, n=5; V&Ex: 1.4 ± 0.2 µmol/mg prot, n=5, p<0.05). However, when exercise is associated to fish oil supplementation, we observed a significant decrease in MDA levels (Fo&S: 15.9 ± 2.8 nmol/mg prot, n=6; Fo&Ex: 1.7 ± 0.4 nmol/mg prot, n=6, p<0.001), and in regards of protein oxidation (Fo&S: 1.7 ± 0.1 µmol/mg prot, n=5; Fo&Ex: 1.1 ± 0.1 µmol/mg prot, n=5; p<0.05). These results are described in figure 2.

To better understand effects of the association between exercise and fish oil supplementation, we evaluated antioxidant defenses, such as enzymatic and non-enzymatic mechanisms. In young rats, the exercise increases SOD activity (V&S: 31.5 ± 2.9 U/mg prot, n=8; V&Ex: 46.8 ± 1.7 U/mg prot, n=8, p<0.001); in addition, in supplemented rats, exercise also induces increase in SOD activity (Fo&S: 46.4 ± 1.7 U/mg prot, n=8; Fo&Ex: 55.9 ± 1.3 U/mg prot, n=8, p<0.05). It was interesting to note that fish oil supplementation per se increases SOD activity in sedentary as well
as in exercised rats (V&S: 31.5 ± 2.9 U/mg prot, n=8; Fo&S: 46.4 ± 1.7 U/mg prot, n=8, p<0.001 and V&Ex: 46.8 ± 1.7 U/mg prot, n=8; Fo&Ex: 55.9 ± 1.3 U/mg prot, n=8, p<0.05).

Furthermore, in terms of the capacity to convert $H_2O_2$ in $H_2O$ by catalase, we observed that exercise is the only variable that can induce a significant increase. This increase does not depend on the supplementation with or without fish oil (V&S: 1.0 ± 0.1 U/mg prot, n=6; V&Ex: 1.7 ± 0.1 U/mg prot, n=7, p<0.01, and Fo&S: 0.9 ± 0.1 U/mg prot, n=6; Fo&Ex: 1.6 ± 0.3 U/mg prot, n=6, p<0.05). However, in regards to glutathione-S-Transferase, neither exercise nor fish oil supplementation induced differences in GST activity. In adult rats, the antioxidant defense had a different response than observed in young rats, neither exercise nor fish oil supplementation were able to induces differences in SOD, CAT or GST activity. All the data of SOD, CAT and GST are described in figure 3.

Since the antioxidant defense is constituted by enzymatic and non-enzymatic mechanisms, we also investigated non-enzymatic mechanisms. GSH is an important non-enzymatic antioxidant that can be found in mammalian cells. In the reduced state, the thiol group of cysteine present in GSH is able to donate a reducing equivalent to these molecules, such as reactive oxygen species, that can decrease the toxicity of the unstable molecules. Young rats did not present any significant differences among the groups. Moreover, when evaluating the sulfhydryl content in samples from young rats we did not observe any difference.

On the other hand, in adult rats we observed that fish oil supplementation associated to exercise can increase GSH levels in a significant manner (Fo&S: 4.5 ± 0.5 µM/mg prot, n=5; Fo&Ex: 8.5 ± 1.1 µM/mg prot, n=6, p<0.05). The same effect was observed in the Sulfhydryl content (Fo&S: 0.1 ± 0.01 mM/mg prot, n=6; Fo&Ex: 0.26 ± 0.04 mM/mg prot, n=5, p<0.05). Similar results (figure 4) were also observed when we compared exercised supplemented with fish oil to exercised animals that received vehicle (GSH levels V&Ex: 4.1 ± 0.2 µM/mg prot, n=5;
4.0 Discussion

Our data suggest that fish oil supplementation changes the rat performance on the task of placement recognition in an age-dependent manner. Among the animals, only young rats that received fish oil during the critical brain development period displayed reduced ability to recognize the novel object placement as compared to the performance of the respective controls. Since the effects of fish oil supplementation can depend on the life stage, the present data highlight the need to better understand the relationship between: (1) the dose and volume of fish oil supplementation, (2) the combination of (Fo) and moderate exercise, (3) and an appropriate timing for this intervention.

Similarly to the present data, previous findings show that PUFAs and exercise have been implicated in enhancing cognition and memory functions in adult rodents (Murphy et al. 2014). These authors reviewed a variety of data demonstrating a strong and influential relationship between dietary intervention with PUFAs, exercise, and brain function. Besides these previous findings in adult rodents, there are published data showing that the perinatal supplementation with essential fatty acids improved hippocampal development in the offspring, only when the dam received the enriched diet also during pregnancy (Niculescu et al. 2011). Herein, we observed an impairment to recognize the novel object placement when young rats received fish oil during suckling period. Present and previous data highlight the importance of the optimal period for this lifestyle intervention to maximize results on memory functions (Niculescu et al. 2011, Murphy et al. 2014).
Fish oil is a reliable source of (LC-PUFAs). Theses lipids have an important role in one’s health state; they are considered essential because mammals are not able to produce them, therefore LC-PUFAs have to be obtained by dietetic sources (Yehuda et al. 2005). On the other hand, there are challenges to reach the daily standard LC-PUFA recommendations through dietetic sources, and to achieve beneficial effects on health care. Thus, the supplementation with fish oil as well as with other dietetic sources of LC-PUFA has grown exponentially; either prescribed by several physicians and nutritionists, or by the use from the general population without an acknowledgement from a health care professional. In fact, the use of LC-PUFA has been pointed out in a variety of acute and chronic inflammatory settings (Calder 2006). However, experimental and clinical data about LC-PUFA underlying mechanisms and clinical efficacy are still weak in some settings (Murphy et al. 2014).

Experimental data show that the effects of LC-PUFA and treadmill exercise on heart metabolism are related to enhancement of antioxidant defenses (da Silva Pedroza et al. 2015). On the other hand, clinical trials describe LC-PUFA benefits by reducing the time to fatigue, increased grip strength, and decreased use of nonsteroidal anti-inflammatory drugs (Calder 2006).

Similarly to the age-dependent effects of fish oil and exercise on the rat behavior, we observed that this intervention affected hippocampal oxidative status, depending on the rat life stage. The present data demonstrated that fish oil supplementation or exercise reduced lipid peroxidation and carbonyl content as judged by the amount of MDA and carbonyl content in young rats. On the other hand, if fish oil was associated to exercise, there was a significant reduction in MDA and protein oxidation in adult rats. In regards of the antioxidant defenses, while exercise increased SOD and CAT activity in young rats, fish oil supplementation only increased SOD activity. Data show similar results since the dietary supplementation of n-3 fatty acids decreased lipid peroxidation and increased activity of SOD (Avramovic et al. 2012).
Furthermore, our results with adult rats demonstrated that fish oil and exercise improved non-enzymatic antioxidant defenses because of the increased activity of GSH and sulfhydryl content. On the other hand, the literature shows that swimming training did not influence non-enzymatic status in the rat brain, but attenuated oxidative damage and increased enzymatic antioxidant status (Nonato et al. 2016). Therefore, it is important to notice that data is conflicting, and there is little evidence addressing the influence of exercise associated or not to fish oil consumption. The optimal effects might depend on the choice of training.

In conclusion, we present relevant findings with the effects of fish oil supplementation and treadmill exercise on memory function and hippocampal oxidative state. These findings are highlighting a timing-related effect of this low-cost and suitable lifestyle intervention.

5.0 Conflict of interest

The authors report no conflict of interest associated with this manuscript.

6.0 Acknowledgements

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Figure captions

Figure 1. Discrimination indexes with the performance of young and adult rats are presented in the left (A and C) and right (B and D) panels, respectively. In the left panel (young) there is a significant effect between the discrimination indexes for the recognition of object identity (A), young rats were able to recognize the novel object \[F (1, 80) = 195.6, ****p < 0.0001\]. In regards of the placement recognition (C), young rats presented a significant difference for the treatment-task interaction \[F (3, 62) = 19.3, p< 0.0001\] and task of object placement \[F (1, 62) = 5.6, p = 0.02\] alone. There is a significant difference among V&S versus Fo&S, and V&S versus Fo&Ex (**p< 0.05). In the right panel (adult), there is also a significant effect between the discrimination indexes for the recognition of object identity (B), adult rats were able to identify the novel object \[F (1, 66) = 400.4, ****p < 0.0001\]. Similar difference was observed for the placement recognition (D), since adult rats distinguished the dislocated position \[F (1, 60) = 133.3, ****p < 0.0001\] as compared to the respective stationary. All the results above were evaluated with two-way ANOVA followed by Tukey post-hoc test. V&S: sedentary supplemented with vehicle solution; V&Ex: exercised supplemented with vehicle solution; Fo&S: sedentary supplemented with fish oil; Fo&Ex: exercised supplemented with fish oil.

Figure 2. Oxidative stress biomarkers. Evaluation of lipid peroxidation and protein oxidation in hippocampus of young (left) and adult (right) rats under different treatment (fish oil and/or exercise) and control. MDA levels (A & B); Carbonyls content (C & D). Values are presented as mean ± SEM. Asterisks indicate significant difference (two-way ANOVA, *p <0.05; **p<0.01; ***p<0.001).

Figure 3. Evaluation of enzymatic defense in hippocampus of young (left) and adult (right) rats obtained from the four different experimental conditions. SOD activity (A & B); Catalase
activity (C&D); GST activity (E&F). Values are presented as mean ± SEM. Asterisks indicate significant differences (two-way ANOVA, *p <0.05; **p<0.01; ***p<0.001).

**Figure 4.** The analysis of non-enzymatic antioxidant defense in the hippocampus of young (left) and adult (right) rats obtained from the four different experimental conditions. GSH concentration (A & B); Sulfhydryl content (C & D). Values are presented as mean ± SEM. Asterisks indicate significant differences (two-way ANOVA, *p <0.05; **p<0.01).
**SOD activity (U/mg protein)**

- **A)**
  - Vehicle: Black bars
  - Supplemented: Gray bars
  - Significance: * (p < 0.05), ** (p < 0.01), *** (p < 0.001)

**Catalase activity (U/mg protein)**

- **C)**
  - Vehicle: Black bars
  - Supplemented: Gray bars
  - Significance: * (p < 0.05), ** (p < 0.01)

**GST activity (U/mg protein)**

- **E)**
  - Vehicle: Black bars
  - Supplemented: Gray bars

**B)**

- Vehicle: Black bars
- Supplemented: Gray bars

**D)**

- Vehicle: Black bars
- Supplemented: Gray bars

**F)**

- Vehicle: Black bars
- Supplemented: Gray bars