RESEARCH PAPER

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Physical exercise and flaxseed oil supplementation influence the glial plasticity in the rat hippocampus

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Brain benefits from physical exercise associated with antioxidant supplements such as flaxseed oil. This low cost and simple association may improve hippocampal plasticity, which may work as a preventive and effective therapy in neuroprotection and neuroplasticity processes. This work evaluated the effects of physical exercise with flaxseed oil supplementation (*Linum usitatissimum L.*) in the hippocampus of Wistar rats. We separated male Wistar rats into four experimental groups: control group (sedentary), a sedentary group with a supplemental diet of flaxseed oil, a group under exercise program with flaxseed oil supplementation, and a group exclusively under exercise program. The swimming exercise consisted of a progressive 28-day protocol followed by behavioral assessment, brain perfusion, microtomy, immunohistochemistry for glial fibrillary acidic protein (GFAP), cellular morphology, and optical density analysis. We used the ANOVA test with Tukey's post-test for behavioral analysis. The exercise program with flaxseed oil supplementation was able to alter the GFAP expression in astrocytes in the CA1, CA3 and dentate gyrus regions of the hippocampus and modulate the behavioral aspects of memory and anxiety.

Key words: antioxidants, behavior, GFAP, glial plasticity, swimming, flaxseed oil

INTRODUCTION

Physical activity is defined as any bodily movement produced by muscle contraction that causes energy expenditure (Howley, 2001). When performed regularly is called physical exercise. The body adapts to the stimulus through morphofunctional changes that lead to better physical performance and numerous health benefits (Zaryski and Smith, 2005). A positive relationship between physical exercise and the central nervous system (CNS) metabolism is well-established (Myers and Olson, 2012), where physical effort increases the synthesis of neurotransmitters and neuroplasticity promotion (McMorris et al., 2011; El-Sayes et al., 2019). The practice of physical exercises promotes cellular and molecular cascades activation followed by cell plasticity maintenance, potential neuroprotective effect by reducing pro-inflammatory microglial response, synaptogenesis and neurogenesis in the hippocampus, increase in vascularization and brain metabolism, anxiety decrease, and morphological alteration of hippocampal astrocytes which are shown to be linked with neuronal plasticity. Forced swimming as a moderate-intensity physical exercise improves cognitive aspects, antioxidant activity, and neurotrophin expression in the hippocampus (Oliveira et al., 2021).

Neuroprotective interventions, such as physical exercise or dietary supplementation, are relevant since the brain is susceptible to oxidative damage caused by free radicals, mainly due to its high metabolic rate and

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high levels of energy expenditure (Uttara et al., 2009). Antioxidant compounds have already demonstrated a neuroprotective and plasticity- inducing effect from those flaxseed oil stands out for its beneficial effects on the nervous tissue, such as increased brain-derived neurotrophic factor (BDNF) and reduced depression (Blondeau et al., 2009; Poorbaferani et al., 2020).

Flaxseed (*Linum usitatissimum. L*) is a functional food rich in long-chain omega 3 fatty acids, nutrients, fiber, and antioxidant compounds (Murray et al., 2003). The oil obtained from its seed acts on cognitive processes, increasing the cellular plasticity in the hippocampus, and reducing oxidative damage caused by free radicals in the brain, in addition to promoting neuroprotection and reducing inflammatory processes in astrocytes (Sarsimaz et al., 2003).

In this context, plasticity processes are crucial tools for adjusting the nervous system to the environment, optimizing synapses, cognition and producing new cells based on the individual's lifestyle habits (Gage, 2004; Mora, 2013). The hippocampus is an investigation target of neuroplastic processes. An interesting aspect of hippocampal plasticity is the assessment of glial proliferation causing astrocytes proliferation followed by an increase in the glial fibrillary acid protein (GFAP) expression. The circumstantial increase in this molecule is considered a biochemical indication of the transformation of normal cells into reactive cells (Torre et al., 1993).

The interaction between regular physical exercise and the antioxidant flaxseed oil is not extensively explored. Thus, due to the great importance of the hippocampus to cognitive and memory processes and the practice of physical exercise being a necessary element for CNS conditioning and metabolism in general, this work aimed to investigate the impact of a regular physical exercise program associated with flaxseed oil supplementation in neuroplastic processes in the hippocampus, specifically the effects in the GFAP expression in hippocampal astrocytes and evaluation of hippocampus-dependent behaviors.

Table 1. Fatty acid percent composition of the flaxseed oil*.

Fatty acid	Percentage (%)
Palmitic Acid (16:0)	6.8%
Palmitoleic acid (C16:1)	0.1%
Stearic Acid (C18:0)	5.3%
Oleic Acid (C18:1, 9)	18.5%
Vaccenic Acid (C18:1)	0.8%
Linoleic Acid (C18:2)	16%
Linolenic Acid (C18:3)	58.4%
Arachidic Acid (C20:0)	0.4%

*The fatty acid composition was obtained from the supplier Fagron.

METHODS

Animals and supplemental diet

Twenty-four Wistar Rattus norvegicus, males, aged three months old and weight 250-350 g were randomly placed into four study groups (n=6): 1. sedentary control group (without supplementation) with oral gavage of saline solution (SEDENTARY); 2. group with flaxseed oil supplementation (OLSE); 3. group with flaxseed oil (Fragon, Brazil) supplementation and exercise (OLEX); 4. group submitted to exercise only (EXERCISE). The flaxseed oil (500 mg/kg), at a weekly dose of 1% v/c, was used and administered 30 minutes before swimming by oral gavage. The fatty acid composition of the flaxseed oil is shown in Table 1. We used distilled water with 1% Tween 80 as a dilution vehicle for the flaxseed oil. The animals were maintained in a controlled environment with standard rodent chow (Nuvilab Cr-1®, Nuvital Nutrientes S/A, Brazil) and water *ad libitum*, at a 12:12 h dark-light cycle. The fatty acid composition of the standard chow is shown in Table 2. This study was performed under license from the Ethics Committee on Animal Experimentation (CEEA) of

Table 2. Fatty acid percent composition of the standard chow diet*.

Fatty acid	Percentage (%)**
Palmitic Acid (16:0)	11 ± 0.3
Palmitoleic acid (C16:1)	0
Stearic Acid (C18:0)	3 ± 0.2
Oleic Acid (C18:1)	37 ± 0.4
Linoleic Acid (C18:2)	49 ± 0.4
Arachidonic Acid (20:4)	0
EPA (20:5)	0
DHA (22:6)	0

* Ingredients: ground whole maize, soya bean meal, wheat bran, calcium carbonate, dicalcium phosphate, sodium chloride, vitamins A, D3, E, K2, B1, B6 and B12, niacin, calcium pantothenate, folic acid, biotin, chloride choline, iron sulphate, manganese monoxide, zinc oxide, calcium sulphate, sodium selenite, cobalt sulphate, lysine, methionine and butylatedhydroxytoluene. The ingredient composition was obtained from the chow label for Nuvilab Cr-1 (Nuvital Nutrientes S/A).
** The fatty acid composition was obtained from Naliwaiko, 2009 (unpublished results).

the State University of Rio Grande do Norte (UERN), with opinion number 001/17 and under the ethical principles adopted by the Brazilian Society of Laboratory Animal Science and according to law number 11,794, the Arouca law, of the Ministry of Science, Technology, and Innovation.

Physical exercise program

The rats underwent a training protocol of swimming exercise, based on the protocol by Dos-Santos for humans (Dos-Santos and de Mello, 2010). For 28 consecutive days, the animals were placed in opaque plastic cylinders (30 cm diameter; 60 cm height) containing warm water (30±1°C) to reduce additional stress to the exercise (Drugan et al., 2005), and up to 45 cm to avoid any tail support in the bottom. The program lasted four weeks with progressive intensity increasing swimming cycles (Fig. 1). Each cycle consisted of a 4 min exercise stage and 1 min and 30 s of rest. From the second week, a load based on their body weight (b.w.) was attached in their backs: 2.5 % b.w. in the second, 3.5 % b.w. in the third and fourth weeks. The rats were weighed weekly to update the loads in their backs.

At the end of each experiment, the rodents were dried and returned to their cage.

Behavioral analysis

Elevated plus maze (EPM) test

The EPM is arranged in a cross shape composed of 50 cm long by 10 cm wide two open and closed arms, containing a central square of 12 × 12 cm, elevated 45 cm from the floor. The closed arms were arranged perpendicular to the open ones and surrounded by 15 cm high walls. The wooden cross-shaped maze was used, each arm measuring 50 cm in length by 10 cm in width, containing a central square of 12 × 12 cm and four arms arranged in a cross shape, at 45 cm from the floor. Two of the arms were open arms. The other two were arranged perpendicular to the open ones and surrounded by 15 cm high walls and called closed arms. The rats were placed individually in the central area with the head facing one of the open arms and observed for 8 minutes. We evaluated the entry frequency and permanence duration into the closed and open arms. We considered "Entry" when the animal placed all four paws inside one of the arms. After each session, we cleaned the apparatus using 10% alcohol (Handley and Mithani, 1984; Pellow et al., 1985).

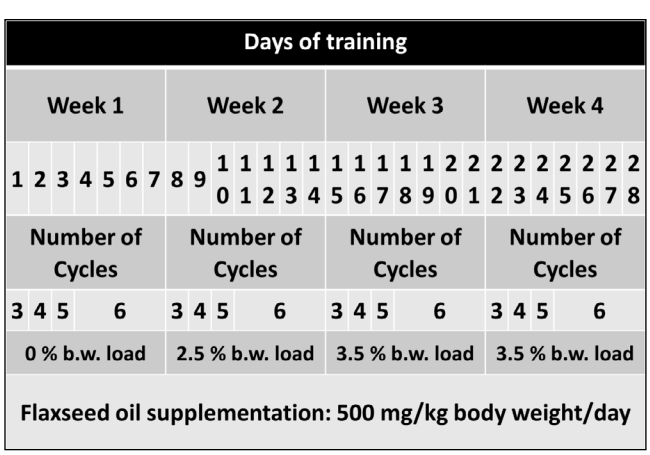

Fig. 1. Swimming exercise protocol.

Y-maze test

The Y-maze (Dellu et al., 1992) consisted of 3 wooden arms (16 cm high, 5 cm wide, and 40 cm long) surrounded by 15 cm walls and connected in an equilateral triangular central area. Each animal was placed in the center of the maze and allowed to explore for 8 minutes. We numbered the arms for memory assessment. Rats have a strong tendency to alternate entry into different environment. The animal was placed into arm 1, and each arm number that the animal entered was registered in sequence for 8 min. We considered a favorable outcome (triads) every time the animal entered three different arms without repetition. After each session, the maze was cleaned with 10% alcohol and dried with paper towels. The result was expressed as a percentage and obtained through the following mathematical formula:

$$\text{Spontaneous alternations (\%)} = (\text{Number of triads}) / (\text{Total number of entries} - 2) \times 100$$

Hole-board test

The test evaluated the anxious behavior by quantifying the number of immersions of the rat's head in the maze holes (headips). The hole-board apparatus is a table-like structure measuring 50 × 50 cm elevated 45 cm from the floor. It has 16 holes, each 3 cm in diameter, evenly spaced over the surface and 3.5 cm from the edge (Crawley, 1985; Saitoh et al., 2006). The number of headips was counted for 5 minutes for each animal.

Immunohistochemistry and morphological analysis

All animals underwent anesthesia administered intraperitoneally with ketamine (90 mg/kg) and xylazine (15 mg/kg). After complete sedation detection, perfusions were performed by placing a cannula inside the left ventricle, connected to a peristaltic pump. After the incision in the right atrium, we infused 200 mL of 0.9% saline (NaCl) solution in 0.1 M phosphate buffer, pH 7.4 (PBS), and heparin for approximately six minutes. Then, we administered 450 ml of fixative solution (4% paraformaldehyde in PBS, pH 7.4). After perfusion, the brains were subjected to microtomy using a cryostat. Slice thickness was standardized at 30 µm and then underwent immunohistochemistry for GFAP. They were incubated in a mouse anti-GFAP monoclonal primary antibody (Sigma-Aldrich) at a 1:500 dilution in PBS for 12 h. At the end of this period, the slices were washed using PBS in an orbital shaker and then placed in contact with the secondary anti-mouse antibody obtained from donkey (Jackson) diluted to 1:1000 using the same vehicle as before, for 2 h. After more washings, they were placed in the avidin-biotin-peroxidase complex solution at a 1:100 dilution in 0.4% Triton X-100, containing NaCl, for 2 h. The sections were placed in a medium containing H2O2 as substrate and 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich) as the chromogen to visualize the reaction. The sections were mounted on silanized slides. They were immersed in a 0.05% osmium tetroxide solution after drying. After the dehydration steps, in batteries of alcohol, the coverslips were placed using DPX. The slides were examined using an optical microscope (Leica PM6 B) in a bright field. For the morphological analysis, three digital images were obtained from each section. Overall, five sections from each animal were analyzed. Thus, fifteen images were analyzed in each animal.

Optical density

For the optical density (OD) analysis, the ImageJ must be calibrated beforehand to distinguish the different shades of gray. The information for the calibration is available at: https://imagej.nih.gov/ij/docs/examples/calibration/. The ImageJ free-hand tool was also used to delimit the cells of interest. To minimize the effects of within-group variability, we adopted a normalized scale based on regions that do not show immunoreactivity for the proteins of interest. Therefore, based on Freire et al. (2007), equations were applied to find the contrast index (CI) of both studied cores. CI = (immunoreactive cell + non-reactive area) / (immunoreactive cell – non-reactive area).

Statistical analysis

For the behavioral tests and the optical density analyses, the Kolmogorov-Smirnov and Shapiro-Wilk normality tests were used, and then unilateral ANOVA was performed followed by Tukey's post-test for multiple comparisons. The level of significance adopted in all tests was defined as $P<0.05$.

RESULTS

EPM test

Assessing the number of entries and the time of permanence in the open arms, the analysis of variance showed no statistically significant difference concerning the number of entries between groups, $F_{(3,18)}=0.5033$, $P=0.68$ (Fig. 2A). When evaluating the permanence time in the open arms of the maze, the analysis of variance indicated a significant difference between groups, $F_{(3,14)}=1.351$, $P<0.001$. Tukey's *post hoc* analyses indicated that the OLSE group had a longer permanence time in the open arms when compared to the sedentary ($P=0.005$), exercise ($P<0.001$), and OLEX groups ($P<0.001$), thus presenting a greater anxiolytic effect on the exploration of open environments (Fig. 2B). As for the number of entries into the closed arms the analysis of variance indicated a significant difference between groups, $F_{(3,14)}= 9.94$, $p<0.001$. Tukey's *post hoc* analyses indicated that the animals in the OLSE group showed increased exploration of the closed arms compared to the sedentary ($P<0.001$), exercise ($p<0.001$), and OLEX groups ($P<0.001$). Regarding the permanence time, for all groups, the analysis of variance showed no significant difference in the exploration time in the closed arms of the EPM, $F_{(3,18)}=1.23$, $P<0.32$ (Fig. 2C, D).

Y-maze test

The analysis of variance showed a significant difference among groups $F_{(3,17)}=5.55$, $P=0.007$. Tukey's *post hoc* analysis indicated an improvement in the correct answers to the maze sequences by the OLSE group compared to the sedentary group ($P=0.004$). The animals in the OLSE group aggregated better memory performance when compared to all other groups, with the best working memory (Fig. 3A).

Hole-board test

The analysis of variance showed a significant difference among groups $F_{(3,17)}$=29.7, P=0.007. Tukey's *post hoc* analyses indicated differences between the animals in the OLEX group, the OLSE group, and the animals submitted exclusively to physical exercise showed a higher number of headips compared to the sedentary group (P<0.001), indicating a reduction in the anxiety levels of the supplemented and exercised animals regarding the exploration of the holes in the maze (Fig. 3B).

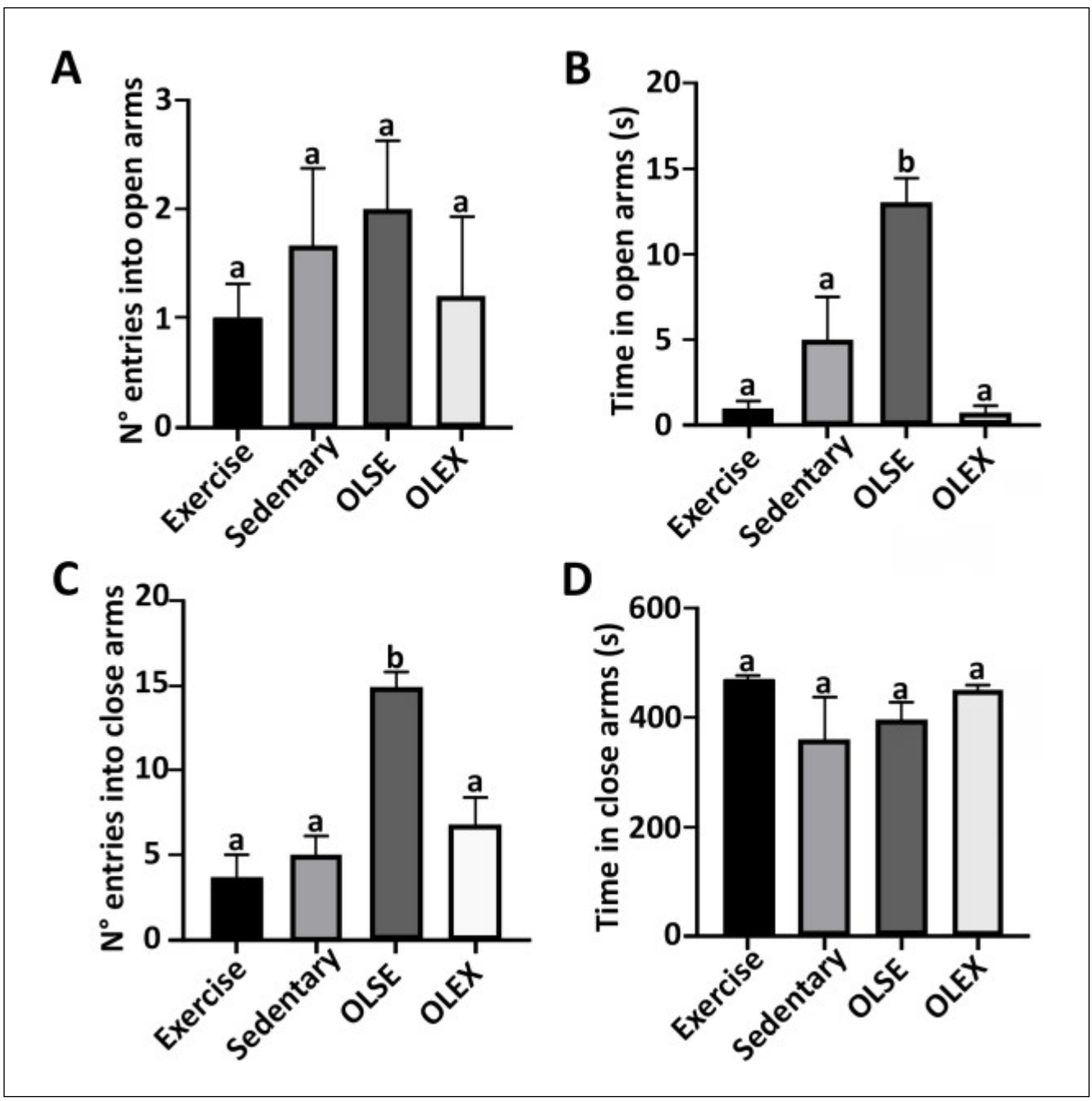

Fig. 2. Effect of flaxseed oil supplementation and exercise in rats on EPM test. Results were expressed using the statistical test for comparison of means ANOVA, with Tukey's post-test (n=6/group), with mean ± SEM of the number of observations. Different letters represent statistical differences. Significance: (B) exercise *vs.* OLSE (P<0.001), OLSE *vs.* OLEX (P=0.005), sedentary *vs.* OLSE (P=0.005); (C) exercise *vs.* OLSE (P<0.001), sedentary *vs.* OLSE (P<.001); OLSE *vs.* OLEX (P<0.001).

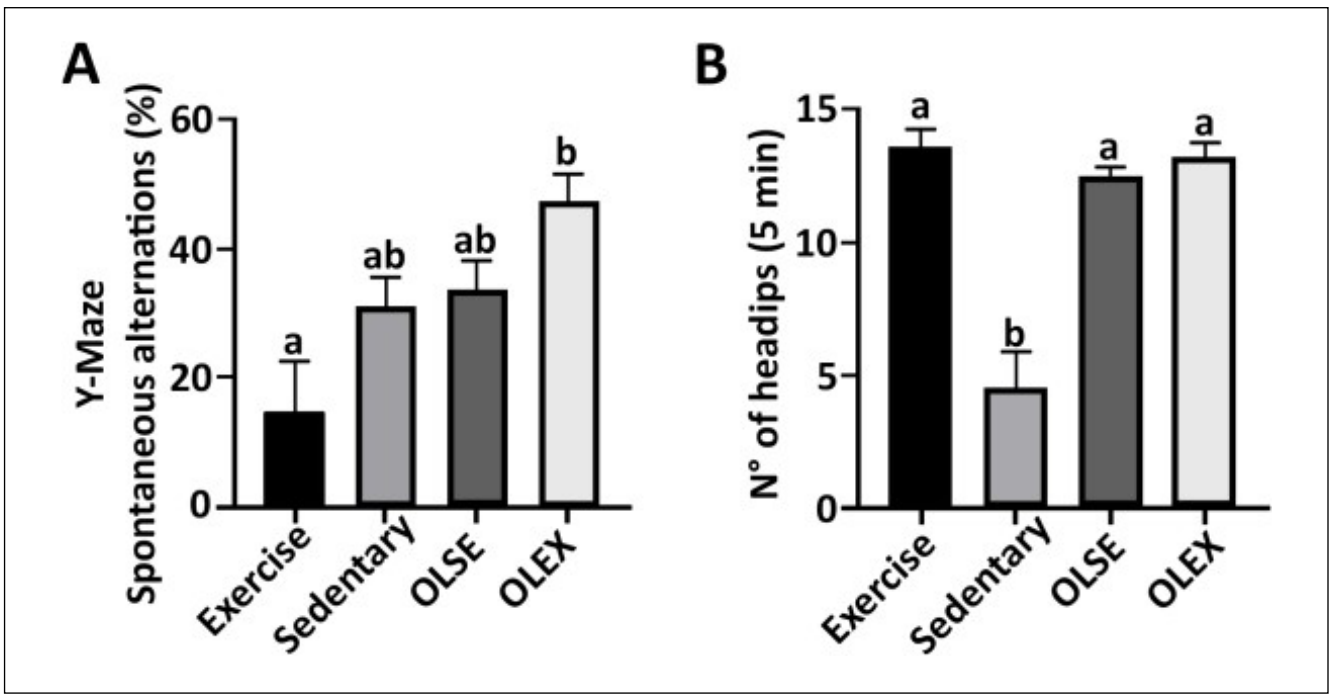

Fig. 3. Effect of flaxseed oil supplementation and exercise on rats in the Y-maze and hole-board tests. The results were expressed using the statistical test for comparison of means ANOVA, with Tukey's post-test (n=6/group), with mean ± SEM of the number of observations. Different letters represent statistical difference. Significance: (A) sedentary *vs.* OLSE (P=0.004); (B) exercise *vs.* sedentary, sedentary *vs.* OLSE, sedentary *vs.* OLEX (P<0.001).

Analysis of the astrocyte immunoreactivity pattern for GFAP in the hippocampal regions CA1, CA3, and dentate gyrus

In this study, we performed immunohistochemistry for GFAP to assess the plasticity of astrocytes in the hippocampus. The regions with the most functional impact of the hippocampus were considered for analysis: the CA3, CA1, and dentate gyrus regions, where the synaptic circuits related to cell plasticity are located (Perea et al., 2009) (Fig. 4).

The photomicrographs of coronal sections demonstrate a divergent marking pattern in the GFAP reactive regions in the hippocampus, where it is possible to in-

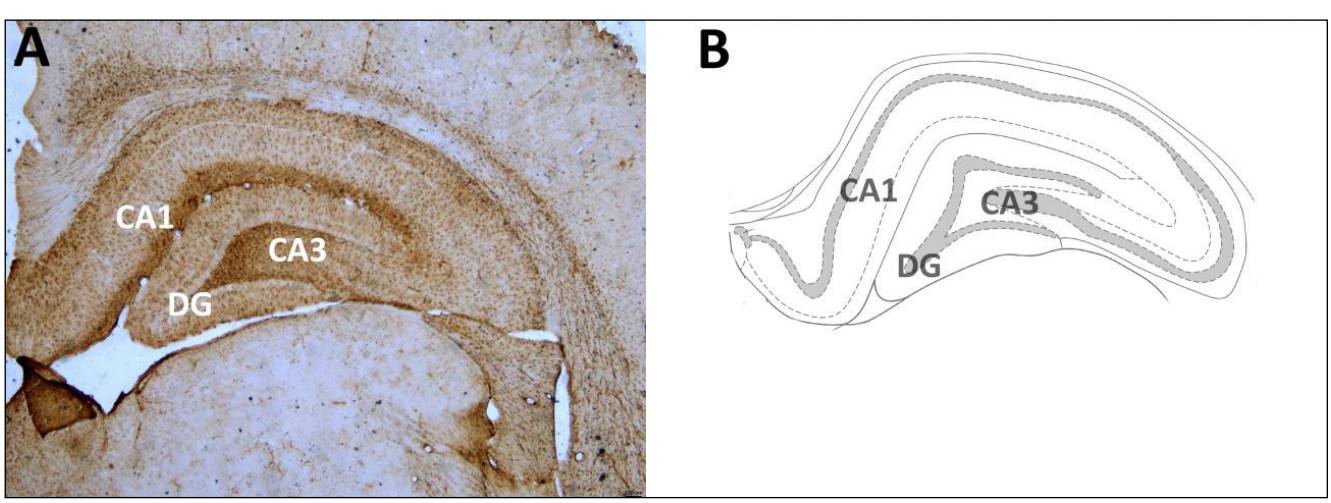

Fig. 4. Coronal section of the rat hippocampus labeled for GFAP. (A) Coronal section of the rat hippocampus labeled for GFAP showing the hippocampus and its regions CA1, CA3 and dentate gyrus; (B) corresponding regions demarcated in the anatomical brain atlas by Paxinos and Watson (Paxinos and Watson, 2006).

fer a marking with greater intensity in the supplemented groups, OLEX and OLSE, compared to the sedentary and exercise groups (Fig. 5).

GFAP immunoreactivity also revealed a more intense staining pattern in the OLEX and OLSE groups when compared to the sedentary and exercise groups (Fig. 5), and an increase in the plastic effect in the OLEX and OLSE groups compared to the sedentary and exercises groups in the regions of CA1, CA3, and dentate gyrus (Fig. 6).

Optical density

The analysis of variance showed a significant difference between groups for optical density values of GFAP immunostaining in the CA1 area, $F_{(3,49)}$=128.6, p<0.0001; in the CA3 area, $F_{(3,65)}$=60.3, *P*<0.0001; and in the dentate gyrus area, $F_{(3,54)}$=39.74, *P*<0.0001 (Fig. 7).

In the CA1 area, Tukey's *post hoc* analyses indicated higher values for the animals in the OLSE group when compared to the OLEX group (*P*<0.0001), the exercise group (*P*<0.0001), and the sedentary group (*P*<0.0001). Also, the OLEX group had higher values when compared to the exercise group (*P*<0.0001) and to the sedentary group (*P*<0.0001) (Fig. 7).

In the CA3 area, Tukey's *post hoc* analyses indicated higher values for the animals in the OLSE group when compared to the exercise group (*P*<0.0001) and to the sedentary group (*P*<0.0001); higher values for the animals in the OLEX group when compared to the exercise group (*P*<0.0001) and to the sedentary group (*P*<0.0001); and higher values for the animals in the sedentary group when compared to the exercise one (*P*<0.0001) (Fig. 7).

In the dentate gyrus area, Tukey's *post hoc* analyses indicated higher values for the animals in the OLSE group when compared to the OLEX group (*P*<0.0001), the exercise group (*P*<0.0001), and the sedentary group (*P*<0.0001). Also, higher values for the animals in the OLEX group when compared to the exercise group (*P*<0.0001) and to the sedentary group (*P*<0.0001) (Fig. 7).

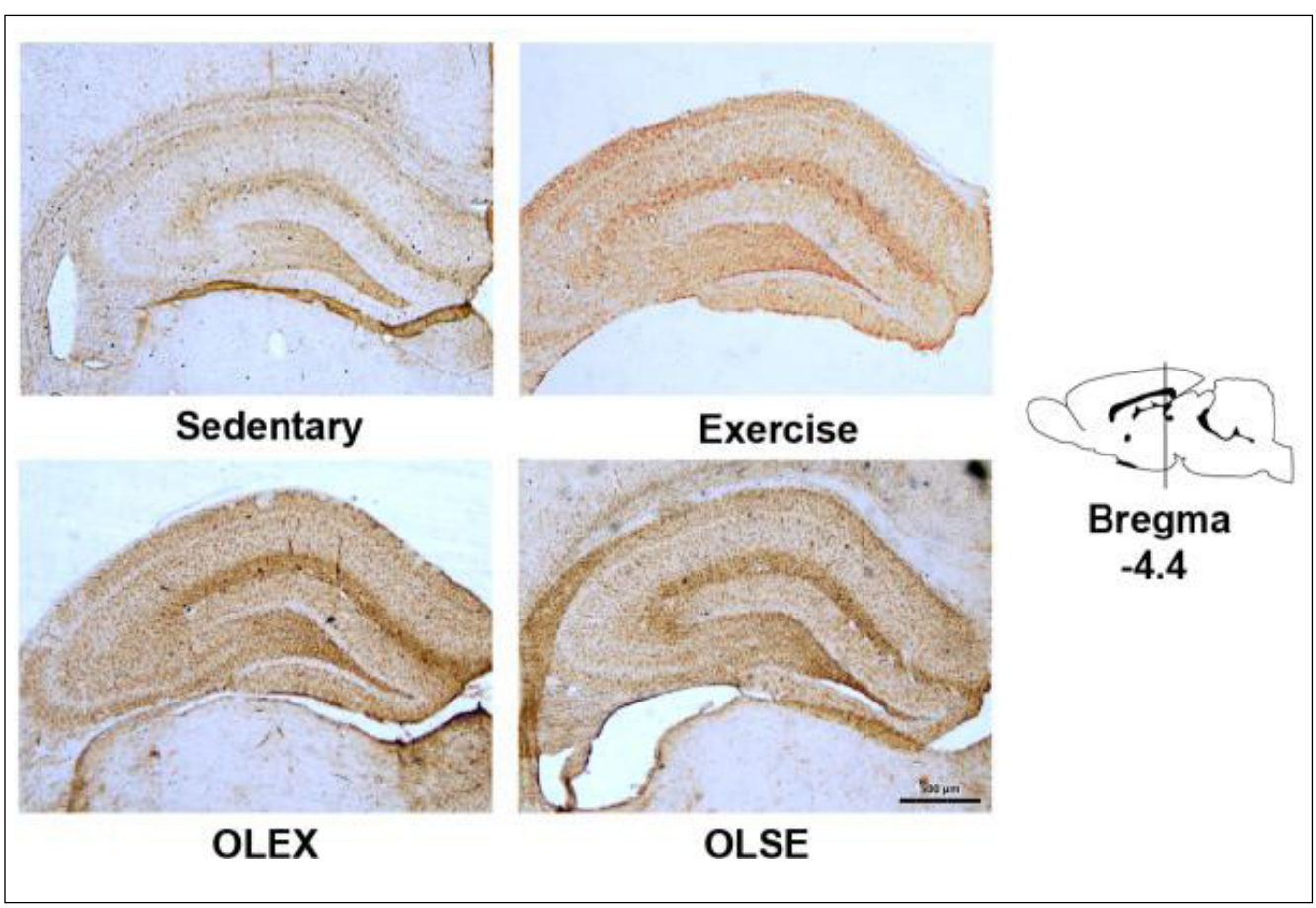

Fig. 5. Brightfield photomicrographs of coronal sections of middle hippocampal regions from rats subjected to immunohistochemistry for GFAP. Scale bar: 100 µm.

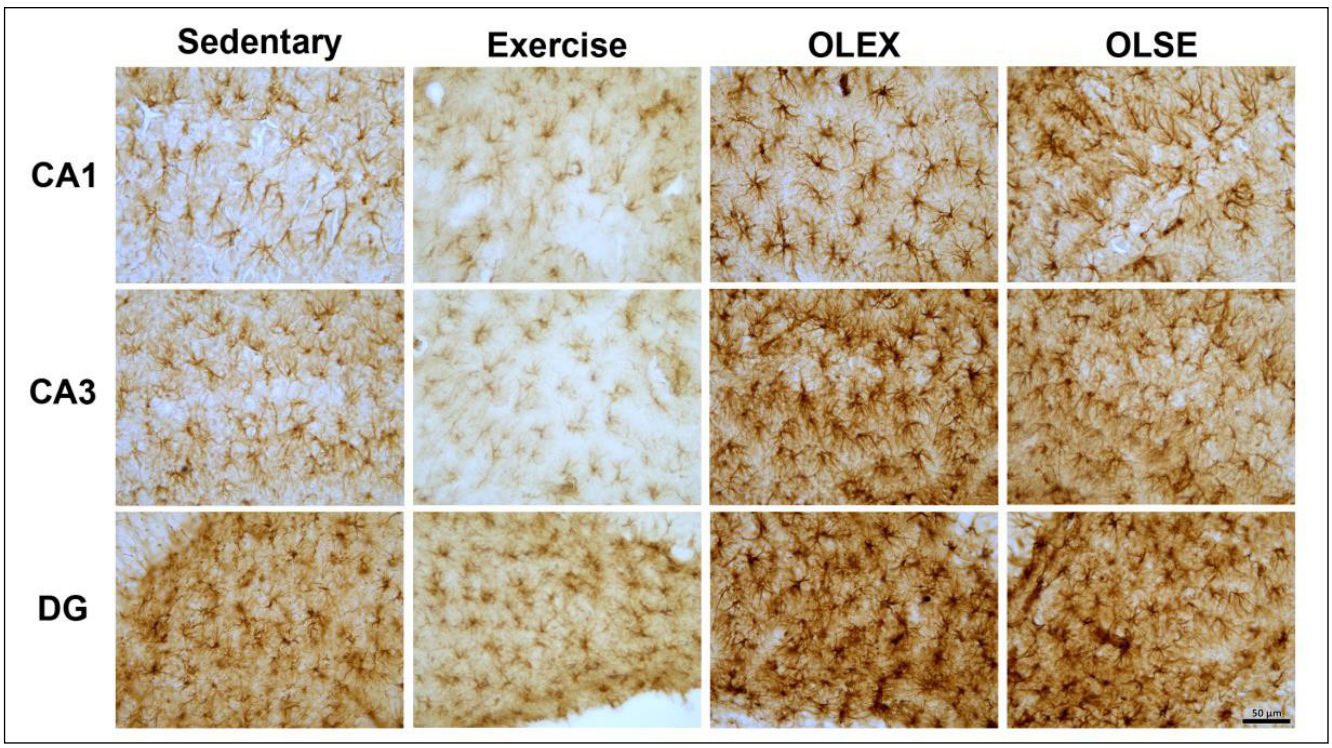

Fig. 6. GFAP immunoreactivity in the CA1, CA3 and Dentate Gyrus (GD) regions in the rat hippocampus. Scale bar: 50 µm.

DISCUSSION

Behavioral aspects of anxiety and memory

In the present study, animals were submitted to an aerobic exercise program of swimming and flaxseed oil supplementation to assess the behavioral divergences among groups and the molecular behavior of astrocytes in the hippocampus from the specific interaction with the plasticity marker GFAP (Fig. 8). Male rats were used because the hormonal components are more suitable for evaluation in the study. Estrogen present in females has the ability to have neuroplastic and/or neuroprotective actions in the hippocampus and is shown to interfere with molecular aspects such as potential antioxidant action, which may compromise the results from flaxseed oil and exercise (Prange-Kiel and Rune, 2006). The differences in the GFAP reactivity patterns among all groups demonstrate the direct influence of physical exercise with supplementation and DHA on the plasticity of glial cells in the rat hippocampus. As previously reported, the increase in astrocyte projections can significantly influence the neural synapse, enhancing cognitive processing and memory consolidation in the hippocampus (Henneberger et al., 2010; Sampedro-Piquero et al., 2014).

We investigated the influence of physical exercise and flaxseed oil supplementation on cognitive aspects of memory and anxiety in rats. The behavioral and morphological aspects evaluated can contribute to the attenuation of neurodegenerative diseases and treatment of mental illnesses related to the hippocampus, such as major depressive disorder (MDD), Alzheimer's disease, and anxiety. The less anxious-like behavior presented by the group of sedentary animals submitted to flaxseed oil supplementation (OLSE) corroborates the positive influence of omega 3 substances and their derivatives on the brain (Niculescu et al., 2011; Harauma et al., 2017; Mongan et al., 2021). The animals in the OLSE group showed less anxious-like performance in the EPM test regarding the time of permanence and exploration of the open arms compared to the sedentary group, inferring a direct influence of flaxseed oil supplementation on the anxiety levels of these animals.

In the EPM test occurs the behavioral event called "approach-avoidance conflict" in which the animal is evaluated in front of an unknown environment added to the natural tendency to avoid open and potentially dangerous spaces, associated with the mechanism of thigmotaxis (Handley and Mithani, 1984; Lister, 1987). This event generates observable behavior resulting from unconditioned responses, as observed in this experiment.

Animals submitted exclusively to physical exercise presented exploratory behavior decreased in the EPM test compared to the sedentary and OLSE groups. These results may be associated with stress resulting from the swimming protocol since exhaustive and stress- generating activities compromise the behavioral response, which may be related to the levels of glucocorticoids released during practice (Drogos et al., 2019).

Anxious behavior induced by forced exercise practices such as cold-water swimming (Linthorst et al., 2008) correlates with elevated extracellular serotonin levels in the prosencephalon and swimming stress. Christianson et al. (2013) concluded that forced swimming altered the behavioral response of rats by inducing anxious-like behavior and reducing social exploration in these animals in anxiety tests. Furthermore, swim-

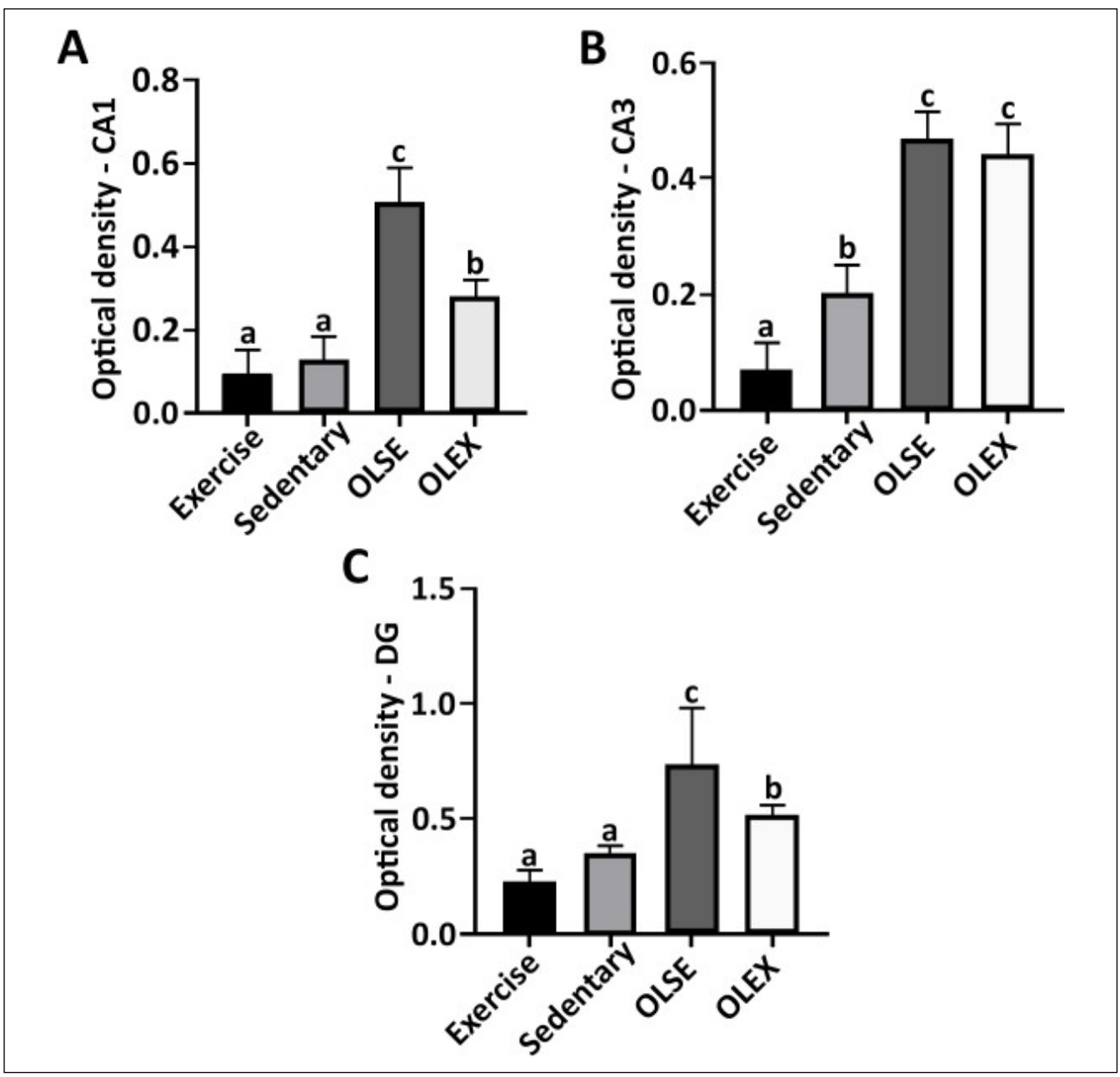

Fig. 7. Effect of flaxseed oil supplementation and exercise in rats on optical density analysis of GFAP immunostaining. Results were expressed using the statistical test for comparison of means ANOVA, with Tukey's post-test (n=6/group), with mean ± SEM of the number of observations. Significance: (A) OLSE *vs.* OLEX, OLSE *vs.* sedentary, OLSE *vs.* exercise, OLEX *vs.* sedentary, OLEX *vs.* exercise ($P<0.0001$); (B) OLSE *vs.* sedentary, OLSE *vs.* exercise, OLEX *vs.* sedentary, OLEX *vs.* exercise, sedentary *vs.* exercise ($P<0.0001$); C) OLSE *vs.* OLEX, OLSE *vs.* sedentary, OLSE *vs.* exercise, OLEX *vs.* sedentary, OLEX *vs.* exercise ($P<0.0001$).

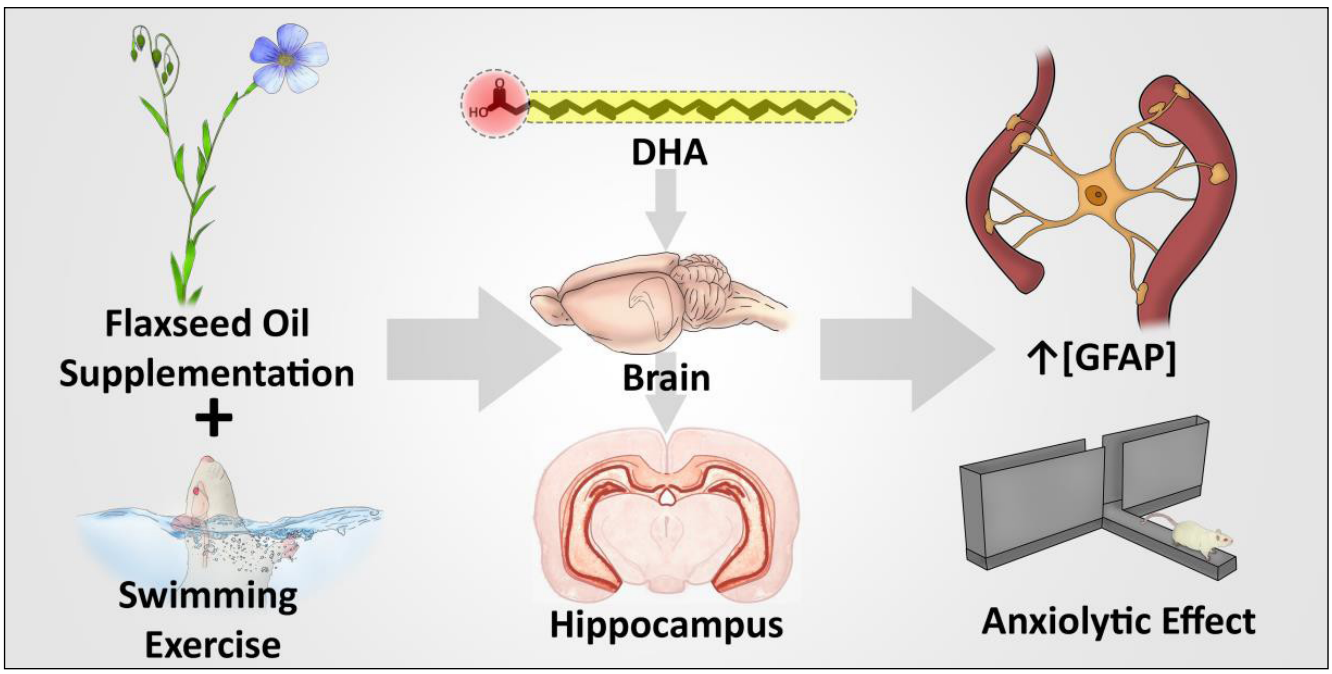

Fig. 8. General aspects of the effects of flaxseed oil supplementation and forced swimming exercise on the rat hippocampus. Flaxseed oil supplementation and forced swimming exercise influence cells and behavior in rats. Docosahexaenoic acid (DHA) is metabolized from the oil and penetrates the brain. Its combination with swimming causes hippocampal astrocytes to increase their Glial Fibrillary Acid Protein (GFAP) immunoreactivity and in behavioral tests to induce anxiolytic effects.

ming can be used as a model of traumatic stress with potential uses in understanding developmental vulnerability stress throughout the animal's life (Moore et al., 2012). Thus, it is predictable that the group submitted to forced swimming exercise, considered anxiogenic, presented more anxious behavior due to the traumatic environment. Therefore, it is necessary to evaluate means of reducing anxiogenic traumatic stress, as reported in the present study that investigated the administration of flaxseed oil.

The result suggests that supplementation with flaxseed oil minimized the anxiolytic effects from open environments exploration at a physiological level compared to the sedentary group. The results observed in the exercise groups show that the execution of forced swimming as a physical practice provoked anxiogenic-like behavior in the animals regardless of supplementation. Thus, swimming is considered a stressor and inducer of anxious-like behavior. However, the test also aimed to evaluate the anxiolytic property of flaxseed oil in this model and its implications in the forced exercise relationship.

Constant exposure to acute stressors leads to stress responses in humans and animals (de Abreu et al., 2021). In humans, chronic stressors can produce maladaptive responses that result in psychological disorders such as anxiety and MDD (Blanchard et al., 1993; Kozicz et al., 2008). Similar to the type of exercise used, the forced swim test induces stress in healthy rats. Reduction in brain-derived neurotrophic factor (BDNF), anxiolytic neurotransmitters, and low social interaction have been reported (Badowska-Szalewska et al., 2010).

The hippocampus is a structure commonly associated with cognitive processes such as memory and learning and with stress response, since it is activated by different stress mediators and participates in the information processing in response to threats (Riedel and Micheau, 2001). There are several glucocorticoid receptors on this structure that can inhibit the activity of the hypothalamus-pituitary-adrenal (HPA) axis, which limits the defense against the deleterious effects caused by exercise stress (Joca et al., 2003). This effect has been shown to induce negative remodeling in hippocampal and apopstosis (Kim et al., 2013).

In humans, physical exercise has been shown to improve cognition associated with increased volume in the prefrontal and hippocampal cortex, which represents a larger size of this structure, providing more efficient synaptic connections between cells (Vogiatzis et al., 2011; Maass et al., 2015; Mandolesi et al., 2018). Thus, the increase in cognition and memory evidenced from the behavioral tests in this study must also be associated with the increase in the apparent increase in labeling against GFAP, indicating greater astrocytic activity in the hippocampus.

The GFAP is a specific astrocyte marker related to glial proliferation associated with inflammatory and plastic mechanisms of the CNS used as a biological tracer. Astrocytes employ networks of intermediate filaments containing GFAP as a signaling and structural basis to coordinate appropriate responses to physiological or pathological changes in the brain, as demonstrated by physical exercise studies (Li et al., 2005; Hol and Pekny, 2015).

As can be seen in the results of the hole-board test, animals submitted to exercise, supplemented or not, showed a positive performance in terms of exploration through headips. This result denotes the importance of physical exercise and antioxidant supplementation in reducing the anxious behavior of healthy individuals and the evaluation of these parameters for experimental protocols using an easy-to-perform anxiety test validation.

As for the cognitive behavior related to memory demonstrated in the Y-maze test, the best memory performance presented by the OLSE group regarding working memory relates to the presence of DHA metabolized from antioxidant supplementation and its influence on hippocampus structure. Flaxseed reduces brain mass loss in rodents, improving the spatial memory of these animals, in addition to preventing depression and increasing the concentration of DHA in this structure, where DHA is directly related to the reduction of anxiety in individuals treated with omega 3 substances (Mucci et al., 2015).

These data suggest a protocol of prolonged supplementation with flaxseed oil to optimize the behavioral results obtained in this experiment, increasing brain levels of DHA. DHA acts by potentiating synaptic connections in the hippocampus, due to its role in the formation of the myelin sheaths present in nerve fibers, since its presence allows for better conduction of electrical impulses maintaining normal conditions of neuronal functioning (Cohen and Ward, 2005), thus improving memory processing.

Morphophysiological changes in the hippocampus

The immunohistochemistry technique was used to compare the distribution and OD of GFAP expression in astrocytes in the different functional structures of the hippocampus of animals performing physical swimming exercise and/or supplementation with flaxseed oil (Fig. 6 and 7). An increase in the immunoreactivity labeling pattern was observed in the OLSE and OLEX groups and quantitatively confirmed from the OD, demonstrating the glial plastic effect in these regions compared to the control groups.

The tripartite synapse in the hippocampus demonstrates that information flows beyond the connection of a traditional pre and postsynaptic neuron, where astrocytes directly influence synaptic activity, also having sensitivity to glutamate, reflecting cooperation between neurons and glial cells, such as astrocytes during a nervous impulse, mainly in the CA1 and CA3 regions of the hippocampus, these regions concentrate the functional units of synaptic activity (Perea et al., 2009).

The OLEX group demonstrated a greater morphological change in GFAP-positive hippocampal astrocytes in the CA3, CA1 and dentate gyrus regions. This result corroborates previous studies that used GFAP as a marker of morphological changes generated by exercise in different regions of the hippocampus (Bernardi et al., 2013; Cobb et al., 2013; Kumar et al., 2018). The GFAP expression in glial cells is regulated by epigenetic mechanisms. During the formation of astrocytes, the demethylation of the GFAP gene mediates the activation of the genetic transcription that expresses this molecule (Kumar et al., 2018).

Animals from the sedentary group showed a plastic increase in the CA3 region seen in the increased GFAP staining. The CA3 has dynamic dendritic spines that may have a divergent regulation pattern. Its projection to the CA1 region in the hippocampus facilitates synapses, promotes plasticity, and increases episodic memory (Attardo et al., 2015). The environmental enrichment during the performance of stressful practices, such as forced swimming exercise, preserves the synaptic maintenance and plasticity of this region (Artola et al., 2006). The morphological changes of the glial CA1 region in our experiment reinforce the observed plastic property since during the practice of exercising may be a greater energy demand and a consequent increase in the functional and structural activity of astrocytes (Bernardi et al., 2013).

The animals submitted exclusively to physical exercise presented markings with lower reactivity compared to the other groups, which may be associated with a weak interaction with the antibody during the immunohistochemistry assay. We observed a strong influence of supplementation to maintain plastic effects, where flaxseed oil allowed an increased GFAP staining of glial cells regardless of exercise performance, but it can act in synergy, improving behavioral aspects related to hippocampal functions. A neuroimaging study found a smaller hippocampal volume in patients with MDD (Schmaal et al., 2015) which suggests a decrease in the cells' volume of this structure because of the depressive disorder, with impairment of the emotional state and cognitive aspects of memory. On the other hand, physical exercise has been shown to positively contribute to increased hippocampal astrocyte density in both

depressed and healthy patients (Cobb et al., 2013; Saur et al., 2014). A study in rodents related the lower hippocampal volume in anxious and depressed animals to the high levels of glucocorticoids found, which may be responsible for the anatomical and physiological pathology of the hippocampus, in addition to the lower GFAP marking in astrocytes (Zhang et al., 2015).

The relationship between physical exercise and the morphological alterations of astrocytes in the hippocampus may be related to the increase in cellular fibroblast growth factor (FGF) and nerve growth factor (NGF) (Neeper et al., 1996), as reported in previous works, their presence promoted the proliferation of glial cells in animals subjected to exercise and the consequent increase in hippocampal volume (Gomez-Pinilla et al., 1995; Erickson et al., 2011). The increase in astroglial metabolism generated by physiological changes in exercise and the expression of proteins associated with cell hypertrophy also support the hypothesis of increased density of astrocytes.

After assessing the influence of flaxseed oil on the cellular plasticity of the hippocampus, we observed plastic maintenance, regardless of the submission to physical exercise, by the OLSE and OLEX groups. Omega 3 substances and their derivatives, such as DHA, act directly in the neuroprotection and plasticity of hippocampal cells. In addition, flaxseed oil is considered an antioxidant substance capable of preventing the harmful effects of oxidation by inhibiting lipoperoxidation, free radicals scavenging and/or metal ion chelation, reduction of reactive oxygen species (ROS), and its deleterious effects of oxidation (Moreira and Mancini-Filho, 2004).

ROS levels are consequential by-products of the physiological metabolism of aerobic exercise and in the brain. They are related to adaptation to exercise since this practice causes oxidative damage in the initial adaptive period and acts by preventing several diseases when performed regularly. Radak and Taylor (2005) propose the hormesis theory to explain this fact. They stated that the preventive effect of regular exercise is partially due to brief and intermittent increases in ROS formation, thus altering signaling pathways that induce adaptive neuroprotective responses.

Therefore, regular exercise provides protective benefits to cellular metabolism, making ROS levels regular and non-harmful, which can also be significantly reduced by supplementing antioxidant substances during the exercise period, such as flaxseed oil and its derivatives.

CONCLUSION

Overall, physical exercise associated with flaxseed oil supplementation was able to alter the plasticity of GFAP positive astrocytes, the expression of GFAP in astrocytes in the CA1, CA3, and dentate gyrus regions of the hippocampus. Such alterations are accompanied by morphological changes suggesting that these cells actively participate in hippocampal plasticity promoted by physical exercise and supplementation.

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