UNIVERSIDADE FEDERAL DO PAMPA

JOSSANA RODRIGUES RUFF

EFFECT OF DIET ENRICHED WITH FATTY ACIDS IN ELDERLY MICE: FOR BIOCHEMICAL AND COGNITIVE PARAMETERS.

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Trabalho de Conclusão de Curso apresentado ao Curso de Ciência e Tecnologia de Alimentos da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Bacharel em Ciência e Tecnologia de Alimentos.

Orientador: Cristiano Ricardo Jesse

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RESUMO

Com o aumento da idade, há um declínio progressivo das funções fisiológicas e comportamentais, essas mudanças podem levar a um alterações nas reações do metabolismo, associados com a perda de memória, ou a perda de várias funções de controle. Vários estudos apoiam a teoria de que existe um aumento dos danos oxidativos associados com a idade, em muitos sistemas celulares. Este estresse oxidativo pode ocorrer devido ao aumento da produção de espécies reativas de oxigénio, e de um decréscimo de reparar o dano e atividades antioxidantes. O cérebro é particularmente vulnerável a danos oxidativos, como resultado de seus altos níveis de ácidos graxos poliinsaturados, alto consumo de oxigênio, alto teor de metais de transição, e pobres defesas antioxidantes. A via de quinurenina (KP) que metaboliza o triptofano, vem gerando considerável interesse entre os neurocientistas, após a observação consistente que seus níveis e metabolitos estão envolvidos em várias condições neuropatológicas e doenças envolvendo ativação imune, estando relacionada com a doença de Huntington, doença de Alzheimer, isquemia, epilepsia, e relacionadas ao processo de envelhecimento. Existe uma procura de substâncias que reduzam ou anulem os danos causados pelo estresse oxidativo e o óleo de peixe tem se mostrado ser um bom candidato para satisfazer estas exigências. No entanto, poucos estudos têm investigado a correlação entre a Via da Quinurenina, o envelhecimento e mecanismos para atenuar os danos causados por essa via, de modo que este estudo teve por objetivo avaliar as alterações nos níveis das enzimas-chave e metabolitos da KP, e os níveis de degradação de algumas substâncias que indicam danos ao tecido cerebral, e a capacidade de óleo de peixe para atenuar esses danos durante o processo de envelhecimento. O óleo de peixe conseguiu reverter parcialmente diversos parâmetros como a atividade da enzima KMO, do triptofano e outros produtos da KP, marcadores de dano oxidativo em proteínas, lipídeos e DNA, e em alguns parâmetros houve uma redução completa, como na enzima IDO, da glutationa, e ácido quinurênico em pelo menos um dos tecidos analisados, demosntrando que a suplementação com óleo de peixe é eficaz para reduzir os danos causados pela idade.

Palavras-Chave: Via da quinurenina, estresse oxidativo, IDO, senescência.

ABSTRACT

With increasing age, there is a progressive decline in physiological and behavioral functions, these changes may lead to changes in metabolism reactions associated with memory loss or the loss of several control functions. Several studies support the theory that there is an increase in oxidative damage associated with age in most cellular systems. This oxidative stress can occur due to increased production of reactive oxygen species, and a decrease to repair the damage and antioxidant activities. The brain is particularly vulnerable to oxidative damage as a result of its high levels of polyunsaturated fatty acids, high oxygen consumption, high levels of transition metals, and poor antioxidant defenses. The route of kynurenine (KP) which metabolises tryptophan, has generated considerable interest among neuroscientists, after the consistent observation that their levels and metabolites are involved in various neuropathological and diseases involving immune activation conditions being associated with Huntington's disease, Alzheimer's disease, ischemia, epilepsy, and related to the aging process. There is a search for substances that reduce or nullify the damage caused by oxidative stress and the fish oil has been shown to be a good candidate to meet these requirements. However, few studies have investigated the correlation between via the kynurenine, aging and mechanisms to mitigate the damage caused in this way, so this study was to evaluate changes in the levels of key enzymes and metabolites of KP, and degradation levels of some substances to indicate damage to brain tissue, and fish oil ability to alleviate this damage during the aging process. The fish oil could partially reverse various parameters such as the activity of KMO enzyme, tryptophan and other products KP, markers of oxidative damage to proteins, lipids and DNA, and in some parameters there was a complete reduction as the enzyme IDO, the glutathione, and quinurênico acid in at least one of the tissues analyzed, demosntrando that supplementation with fish oil is effective in reducing damage caused by age.

Keywords: Kynurenine Pathway, oxidative stress, IDO, senescence.

LISTA DE ABREVIATURAS E SIGLAS

3-HK: 3-hydroxy-L-kynurenine

3-NT: 3-nitrotyrosine

4-HNE: 4-Hydroxynonenal

8oHdG : 8-hydroxy-2' -deoxyguanosine

DNA: Deoxyribonucleic acid

IDO: indoleamine 2,3-dioxygenase

GSH: glutathione

KAT: kynurenine aminotransferase

KMO: kynurenine monooxygenase

KP: kynurenine pathway

KYNA: kynurenic acid

KYN: kynurenine

QA: quinolinic acid

RNA: ribonucleic acid

ROS: reactive oxigen species

TRP: Tryptophan

Sumário

Effect of diet enriched with fatty acids in elderly mice: for biochemical and cognitive parameters

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1. Introduction

For the first time in history, most people can expect to live beyond 60 years and the health consequences will be profound (WHO, 2015). Since with increasing age, there is a progressive decline of physiological and behavioral skills functions, these changes can lead to an altered behavior such as memory loss, or loss of various control functions (Bernhardi, 2015). The aging should not be considered as a disease, because this is the natural course of life, although many diseases are associated with this process.

Several studies have been supporting the theory that there is an increase in oxidative damage associated with age in many cellular systems (Cunningham, 2015). Ie oxidative stress plays major role in brain aging (Chakrabarti, 2011). This oxidative stress causes the protein

oxidation levels (Stadtman, 2006), DNA / RNA and lipid peroxidation have their values increased due to increased production of reactive oxygen species (ROS) (Santos, 2013), and , a decrease of repairing the damage and antioxidant activities (Stadtman, 2006).

The brain is particularly vulnerable to oxidative damage as a result of its high levels of polyunsaturated fatty acids, high oxygen consumption, high content of transition metals, and poor antioxidant defenses (NUNOMURA, 2006).

The kynurenine pathway (KP) that metabolizes Tryptophan (TRP) is related to Huntington's disease, Alzheimer's disease, Ischaemia, Epilepsy, those that are often linked on the aging process.

There is a search for substances that reduce or nullify the damage caused by oxidative stress, fish oil has been shown to be a good candidate to meet these demands.

So this study was to evaluate the levels of some activity of enzymes of the KP and its products, and degradation levels of some substances that indicate damage to brain tissue, and the ability of fish oil to mitigate these damages during the aging process.

2. Materials and methods

2.1. Animals

Experiments were performed using male C57/BL6 mice (25-35g, 60 days old). Animals were maintained at 22-25ºC with free access to water and food, under a 12:12h light/dark cycle, with lights on at 7:00 a.m. All manipulations were carried out during light phase on the day. All experiments were performed in separate groups of animals and each animal was used only once in each test. The procedures of this study were conducted according to the guidelines of the Committee on Care and Use of Experimental Animals Resources.

2.2. Drugs

The biochemicals STZ and Minocycline were purchased from Sigma-Aldrich (USA) and were dissolved in saline solution before the administration.

2.3. Experimental design

Two experiments were conducted. In the first experiment, mice (n=6-8 animals per group) received an i.c.v. injection of STZ (ICV-STZ) to investigate the depressive-like behaviour and IDO activity in a time-course curve (times: 1, 6, 24h and 1 week), similarly to a previous study of our laboratory (Souza et al., 2013).

In the second experiment, mice were divided into four groups (n=6-8 animals per group): saline + saline (sham), saline + minocycline, STZ + saline and STZ + minocycline. Six hours after the ICV-STZ (peak effect), mice were subjected to behavioural tests. Afterwards, they were euthanized and the hippocampus was removed to neurochemical determinations (Fig. 1).

2.4. Intracerebroventricular injection of streptozotocin (ICV-STZ) and minocycline administration

STZ groups were administered an ICV-STZ (0.1mg/site, total volume of 4µl), whereas the sham groups received an i.c.v. injection of saline solution (total volume of 4 µl) as described earlier (Pinton et al., 2011; Souza et al., 2013). Mice were anaesthetized by an i.p. injection of sodium pentobarbital (0.067 mg/g). A single ICV-STZ or saline was injected into the left ventricle of the brain using a stereotaxic apparatus. The bregma coordinates used for injection were −1.0 mm lateral, −0.3 mm posterior, and −2.5 mm below.

Minocycline was administered at a dose of 50 mg/kg (i.p.) once daily for 2 consecutive days prior to and on the same day as STZ injection, according to O'connor et al. (2009).

2.5. Behavioural assessment

2.5.1. Open-field test (OFT)

The OFT was carried out to evaluate if the drugs produced effects on locomotor activity. The animals were submitted individually for a period of 5 min to an OFT apparatus (Insight model EP 154C). The total distance (unit: mm) was computed (Pru et al., 2003; Goes et al., 2014).

2.5.2. Forced swimming test (FST)

The test was conducted using the method described by Porsolt et al. (1977). Briefly, mice were individually forced to swim in open cylinders (25 cm height×10 cm diameter) containing 19 cm of water at 25±1 °C. The duration of immobility was scored during the 6 min test period as described previously (Rodrigues et al., 2002). Each mouse was recorded as immobile when floating motionless or making only those movements necessary to keep its head above water.

2.5.3. Splash test

Ten minutes after the open-field test the splash test was carried out. This test consists of squirting a 10% sucrose solution on the dorsal coat of a mouse placed individually in clear Plexiglas boxes (9 × 7 × 11 cm) (Rosa et al., 2014). Because of its viscosity, the sucrose solution dirties the mouse fur and animals initiate grooming behavior. After applying sucrose solution, the total amount of time spent grooming were manually recorded for a period of 5 min as an index of self-care and motivational behavior, considered to be parallel with some symptoms of depression such as apathetic behavior (Willner, 2005). The apparatus was cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.6. Biochemical assays

After behavioral tests, mice were euthanized, and blood and hippocampus were removed. Hippocampus was homogenized in 50mM Tris-Cl, pH 7.4. The homogenate was centrifuged at 2,400 × g for 15 min at 4 °C and a low-speed supernatant fraction (S1) was used for assays. Blood samples were collected directly from the ventricle of the heart in anesthetized animals, using heparin as the anticoagulant, and plasma was separated by centrifugation (2,400 × g) for 15 min.

2.6.1. Blood glucose determination

To confirm that ICV-STZ (0.1 mg/site) is a subdiabeticogenic dose, plasma glucose level was determined by enzymatic colorimetric methods using commercial kit (Labtest Diagnostica, MG, Brazil). Glucose level was expressed as mg/dl.

2.6.2. Pro-inflammatory cytokines levels

Levels of tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) in the hippocampus were determined using commercially available ELISA assays, following the instructions supplied by the manufacturer (DuoSet Kits, R&D Systems; Minneapolis). Results are shown as pg/mg tissue.

2.6.3. Tryptophan (TRP) and kynurenine (KYN) levels

The levels of TRP and its metabolite KYN in the hippocampus were performed in a Shimadzu LC-10A liquid chromatograph, according to Silva et al. (2002). The chromatographic separation was achieved using a 250- by 4.6-mm (inner diameter) C_{18} reverse-phase column (particle size, 4 µm; Aquapore RP-300 C-18). For TRP measurement, the column was eluted isocratically at flow rate of 1.0 ml/min with 0.015 M sodium acetate (pH 4.5) containing 15% methanol. For KYN determination, the column was eluted with acetonitrile at a 1:47 dilution in 0.1 M acetic acid–0.1 M ammonium acetate (pH 4.65). The absorbance of the column effluent was monitored at 280 and 365 nm for TRP and KYN respectively. The peaks of TRP or KYN were identified by comparison with the retention times of standard compounds (Sigma), and quantification was based on the ratios of the peak areas of compound to the internal standard. The tissue levels were expressed in pg/mg tissue.

2.6.4. Serotonin (5-HT) and 5-Hydroxyindoleacetic acid (5-HIAA) levels

The levels of 5-HT and its metabolite 5-HIAA in the hippocampus were analyzed by high performance liquid chromatography (HPLC) with electrochemical detection, as described by Ferraz et al. (2002). The mobile phase, used at a flow rate of 0.8 ml/min, consisted of 0.02 M phosphate/citrate buffer and 90/10 methanol (v/v), 0.12 mMNa2 EDTA, and 0.0556% heptane sulphonic acid as ion pair. The pH was adjusted to 2.64 with H_3PO_4 at 22 °C. A 5-µm (220 × 4.6) Spheri-5 RP-18 column from Brownlee Laboratory was used. Electrochemical detection was performed with a Shimadzu L-ECD-6A electrochemical detector with a potential of 0.75 V. The peak area of the internal standard (DHBA) was used to quantify the sample peaks. The tissue levels were expressed in pg/mg tissue.

2.6.5. Indoleamine-2,3-dyoxigenase (IDO) activity

IDO activity in the hippocampus was determined as previously described (Lestage et al., 2002). The supernatants (0.2 ml) were added to 0.8 ml of the reaction mixture containing 400 µM L-tryptophan, 20mM ascorbate, 10 µM methylene blue, and 100 µg catalase in 50mM potassium phosphate buffer pH 6.5. The reaction was carried out at 37ºC under agitation for 60 min. Then, it was blocked by adding 0.2 ml of 30% trichloroacetic acid and further incubated at 50ºC for 30 min to convert the N-formylkynurenine to L-kynurenine. Samples were centrifuged at 13,000g for 10 min at 4ºC. The supernatants were filtered through microspin ultrafiltrates with a cut-off of 10,000M_r before being taken for measurement of IDO.

The amount of L-kynurenine formed from tryptophan was determined by reversed phase high pressure liquid chromatography (HPLC). One hundred µl of the reaction product was injected onto a Merck LiChrospher column (150mm long, 4.6mm diameter, packed with 5 lm silica beads holding 18C long carbon chains). A cartridge guard column containing the same material as the analytical column was used. The mobile phase consisted of 0.1M ammonium acetate buffer (pH 4.65) with 5% acetonitrile. Flow rate was 1 ml/min. Kyn was detected using a spectrometer measuring absorbency at a wavelength of 365nm and was quantified using known amounts of L-kynurenine. The retention time of kyn was around 5.35 min. All determinations were performed in duplicate. One unit of the activity was defined as 1 pmol Kyn/h/mg protein at 37ºC.

2.6.6. Protein determination

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

2.7. Statistical analysis

Results are presented as means ± standard error medium (SEM). Comparisons between the experimental and the control groups were performed by one-way (experiment 1) or two-way (experiment 2) analysis of variance (ANOVA), followed by Newman-Keuls test when appropriate. A value of $P < 0.05$ was considered to be significant. All tests were carried out using the GraphPad software (San Diego, California, USA).

3. Results

3.1. The behavioural alterations in Sucrose Preference Test

Statistical analysis revealed no significant differences in sucrose preference test basal (FO X LPS, $F_{1,16} = 0.07$, $p < 0.78$; **Fig. 1**A).

Two-way ANOVA showed a significant effect for FO/LPS interaction ($F_{1,16} = 8.61$, $p < 0.01$; **Fig. 1**B) in SPT. The increase of sucrose intake indicates that supplementation with FO was able to protect account anhedonia-like effect.

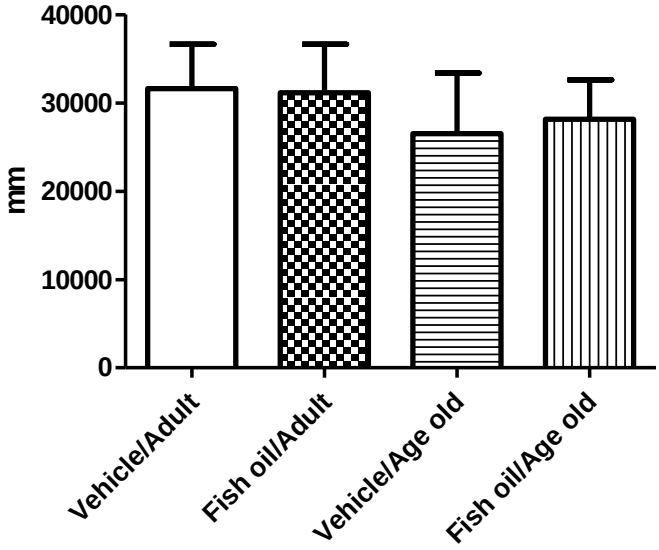
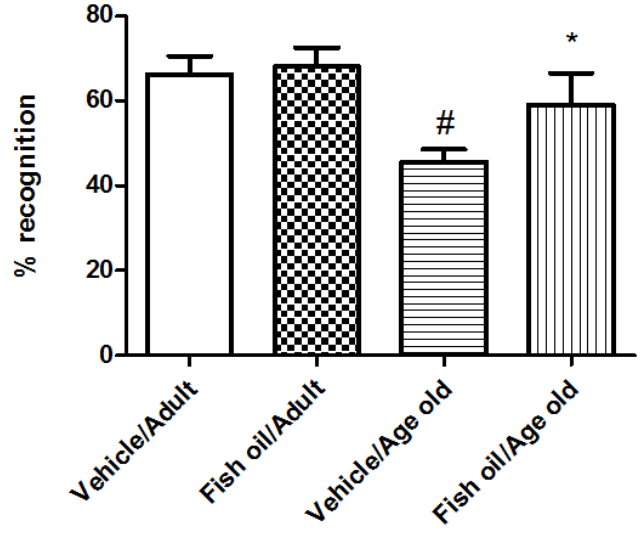


Figure.1. Effect of ICV-STZ (0.1 mg/site) on immobility time in the FST (A), total time of grooming in the splash test (B), total distance in the OFT (C) and plasma glucose levels (D) in groups tested 1, 6, 24h and 1 week after an STZ injection. Values are mean ± S.E.M. (n=6-8). *P<0.05, **P<0.01, ***P<0.001 compared with the sham group.



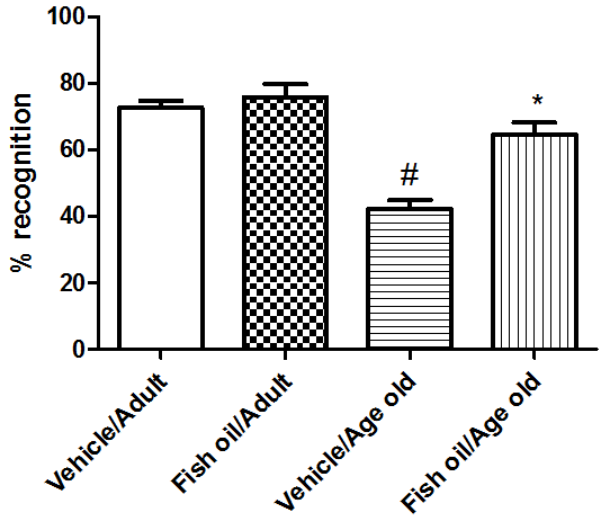


Figure.2. Effect of ICV-STZ (0.1 mg/site) on the levels of TNF-α (A) and IFN-γ (B) in hippocampus of mice in groups tested 1, 6, 24h and 1 week after an STZ injection. Values are mean ± S.E.M. (n=6-8). *P<0.05, **P<0.01, ***P<0.001 compared with the sham group.

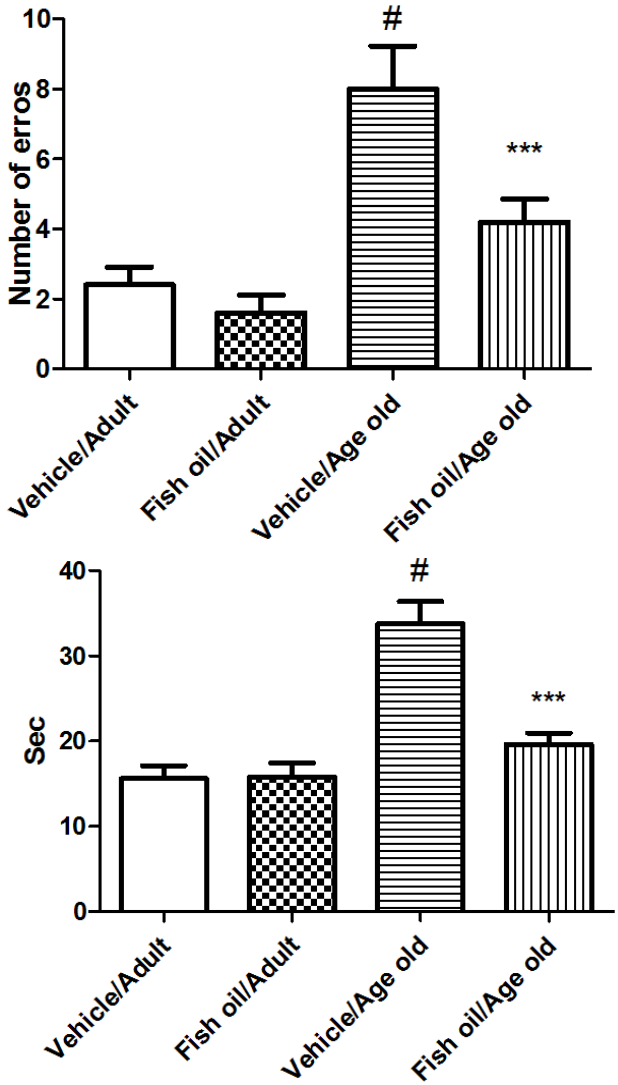
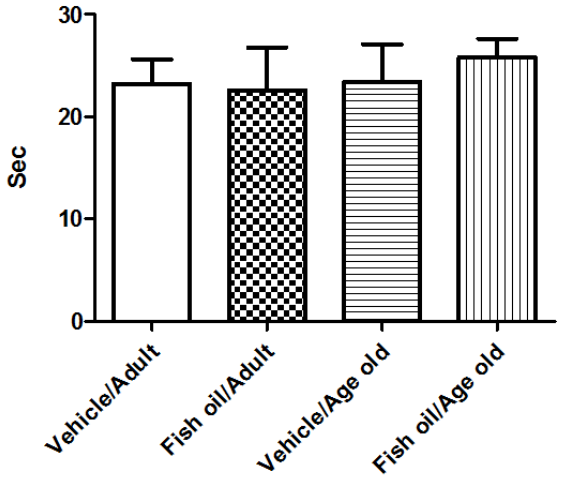


Figure.3. Effect of ICV-STZ (0.1 mg/site) on the IDO activity (A), TRP levels (B), KYN levels and KYN/TRP ratio (D) in hippocampus of mice in groups tested 1, 6, 24h and 1 week after an STZ injection. Values are mean ± S.E.M. (n=6-8). *P<0.05, **P<0.01, ***P<0.001 compared with the sham group.

3.2. Open Field Test

Statistical analysis revealed no significant differences in OFT of mice at all groups (FO X LPS, $F_{1,16} = 0.41$, $p < 0.53$; **Fig. 1**C).



3.3. Tail Suspension Test

Two-way ANOVA of immobility time in TST demonstrated a significant effect of FO/LPS interaction ($F_{1,16} = 11.1982$, $p < 0.001$; **Fig. 1**D). The decreased of immobility time is interpreted as a depressive-like effect caused by supplementation of FO.

3.4. Body Weight

Statistical analysis showed a significant effect against weight loss of FO/LPS interaction ($F_{1,16} = 42.23$, $p < 0.001$; **Fig. 1**E). The weight loss is a feature of the sickness behavior model, the supplementation with FO was able to protect against this characteristic pathology.

3.5. Corticosterone Levels

Two-way ANOVA demonstrated a significant main effect of LPS ($F_{1,12}$ = 66.41, p < 0.001; **Fig. 1**F), and FO supplementation ($F_{1,12}$ = 6.07, p < 0.02; **Fig. 1**F). Post hoc comparisons revealed that LPS significantly increasead corticosterone levels compared to control group (p < 0.001). The supplementation with FO was able to partially protect against this increase corticosterone levels caused by LPS (p < 0.02; **Fig. 1**F).

3.6. Neuroinflammatory Markers

Table 1. Effect of fish oil in oxidative parameters in the brain structures of age old mice.

(pg/mg protein)	Adult		Age old	
	Vehicle	Fish oil	Vehicle	Fish oil
Prefrontal Cortex				
ROS	170.55±5.66	131.7±5.66[a]	359.6±3.55[a]	268.5±6.33[bc]
GSH	3.05±0.11	3.22±0.19	1.66±0.21[a]	2.45±0.24[bc]
4-HNE	24.68±1.69	26.71±2.31	48.33±4.79[a]	37.03±3.44[bc]
3-NT	167.8±8.65	157.9±2.33	265.3±10.31[a]	219.2±4.66[bc]
8oHdG	34.67±2.87	37.01±3.22	59.86±3.94[a]	46.7±2.67[bc]
Hippocampus				
ROS	356.8±8.68	291.5±7.55[a]	568.6±13.22[a]	426.8±11.61[bc]
GSH	2.11±0.21	2.17±0.22	1.24±0.11[a]	2.01±0.23[c]
4-HNE	35.6±2.11	31.4±2.33	52.35±4.61	43.55±3.55[bc]
3-NT	279.6±12.73	301.5±9.65	484.9±9.83[a]	375.1±7.55[bc]
8oHdG	28.64±2.18	26.24±2.77	54.27±2.37[a]	40.50±3.55[bc]
Striatum				
ROS	286.9±12.3	244.4±5.66[a]	567.5±7.08[b]	396.4±11.21[bc]
GSH	2.87±0.15	3.01±0.22	1.77±0.22	2.45±0.22[bc]
4-HNE	36.54±2.12	34.44±3.37	70.14±3.24[a]	49.21±3.01[bc]
3-NT	245.6±7.32	258.7±6.31	404.7±12.3[a]	277.2±6.31[c]
8oHdG	42.31±3.40	40.77±3.21	68.98±6.04[a]	51.22±3.29[bc]

The values were analyzed by two-way ANOVA and Bonferroni multiple comparison test.

Each value is expressed as the mean ± S.E.M. (n =6).

a P < 0,05 when compared with Vehicle/Adult.

b P < 0.05 when compared with Fish oil/Adult.

c P < 0.05 when compared with Vehicle/Age old.

Statistical analysis of IL-1β levels in hippocampus demonstrated a significant FO/LPS interaction ($F_{1,12}$ = 13.03, $p < 0.001$). Post hoc comparisons revealed that FO supplementation attenuated the increase of IL-1β levels caused by LPS (**Table. 1**).

Similarly, two-way ANOVA of IL-1β levels in the striatum yielded a significant FO/LPS interaction ($F_{1,12}$ = 14.15, $p < 0.001$). Post hoc comparisons showed that the increased IL-1β levels observed in the striatum of LPS -treated mice was mitigated by FO supplementation (**Table. 1**).

Significant results were revealed by statistical analysis of IL-1β levels in cortex for FO/LPS interaction ($F_{1,12}$ = 22.89, $p < 0.001$). Bonferroni post-hoc testing revealed that FO supplementation attenuated the increase of IL-1β levels caused by LPS (**Table. 1**).

Statistical analysis of IL-6 in hippocampus demonstrated a significant main effect of LPS ($F_{1,12}$ = 93.08, $p < 0.001$) and FO supplementation ($F_{1,12}$ = 11.13, $p < 0.001$). FO supplementation partially protected against the decrease of IL-6 induced by LPS (**Table. 1**).

Significant results were revealed by statistical analysis of IL-6 levels revealed that LPS increase levels in striatum of mice compared to control group ($F_{1,12}$ = 104.32, $p < 0.001$) and FO supplementation was not capable of attenuating the increase of IL-6 levels caused by LPS in striatum (**Table. 1**).

Similarly, two-way ANOVA revealed that LPS significantly increase IL-6 levels in cortex of mice compared to control group ($F_{1,18}$ = 69.77, $p < 0.001$). Statistical analysis showed a FO supplementation was not capable of attenuating the increase of IL-6 levels caused by LPS (**Table. 1**).

Statistical analysis of TNF-α in hippocampus demonstrated a significant FO/LPS interaction ($F_{1,12}$ = 15.09, $p < 0.001$). Post hoc comparisons revealed that FO supplementation attenuated the increase of TNF-α levels caused by LPS (**Table. 2**).

Similar results were revealed by two-way ANOVA of TNF-α levels in striatum for FO/LPS interaction ($F_{1,12}$ = 26.80, $p < 0.001$). Bonferroni post-hoc testing revealed that FO supplementation protect against the increase of TNF-α levels caused by LPS in striatum (**Table. 2**).

Significant results were revealed by statistical analysis of TNF-α levels in the cortex of FO/LPS interaction ($F_{1,12}$ = 32.06, $p < 0.001$). Bonferroni post-hoc testing revealed that FO supplementation attenuated the increase of TNF-α levels caused by LPS (**Table. 2**).

Two-way ANOVA revealed that LPS significantly increase IFN-γ levels in hippocampus of mice compared to control group ($F_{1,12}$ = 66.35, p < 0.001). Statistical analysis showed a FO supplementation was not capable of attenuating the increase of IFN-γ levels caused by LPS (**Table. 2**).

Statistical analysis of IFN-γ levels in striatum demonstrated a significant FO/LPS interaction ($F_{1,12}$ = 10.40, p < 0.001). Post hoc comparisons revealed that FO supplementation attenuated the increase of IFN-γ levels caused by LPS in striatum (**Table. 2**).

Two-way ANOVA revealed that LPS significantly increase IFN-γ levels in cortex of mice compared to control group ($F_{1,12}$ = 38.78, p < 0.001). Bonferroni post-hoc testing showed a FO supplementation was not capable of attenuating the increase of IFN-γ levels caused by LPS in cortex (**Table. 2**).

3.7. Serotonergic 5-HT receptors and 5-HIAA metabolite

Statistical analysis of 5-HT levels revealed that LPS significantly increase levels in hippocampus of mice compared to control group ($F_{1,12}$ = 5.23, p < 0.04; **Fig. 2**A). Post hoc comparisons of 5-HT levels showed a FO supplementation was not capable of attenuating the increase of 5-HT levels caused by LPS in hippocampus (p < 0.41; **Fig. 2**A).

Two-way ANOVA of 5-HT levels revealed no significant differences in striatum (FO X LPS, $F_{1,12}$ = 4.16, p < 0.06; **Fig. 2**B).

Significant results were revealed by statistical analysis of 5-HT levels in the cortex of FO/LPS interaction ($F_{1,12}$ = 7.23, p < 0.01). Bonferroni post-hoc testing revealed that FO supplementation attenuated the increase of 5-HT levels caused by LPS (**Fig. 2**C).

Two-way ANOVA of 5-HIIA levels in hippocampus demonstrated a significant FO/LPS interaction ($F_{1,12}$ = 8.09, p < 0.01). Post hoc comparisons revealed that FO supplementation attenuated the increase of 5-HIIA levels caused by LPS (**Fig. 2**E).

Statistical analysis demonstrated by statistical analysis of 5-HIIA levels in the striatum of FO/LPS interaction ($F_{1,12}$ = 6.68, p < 0.02). Bonferroni post-hoc testing revealed that FO supplementation attenuated the increase of 5-HIIA levels caused by LPS (**Fig. 2**F).

Two way ANOVA reveled that LPS significantly increase 5-HIIA levels in cortex of mice compared to control group ($F_{1,12}$ = 58.30, p < 0.001). Post hoc comparisons revealed a FO supplementation was not capable of attenuating the increase of 5-HIIA levels caused by LPS (p < 0.18; **Fig. 2**G).

3.8. Tryptophan and Kynurenine levels

Table 2. Effect of fish oil on kynurenine pathway metabolites in the brain structures of age old mice.

(pg/mg protein)	Adult		Age old	
	Vehicle	Fish oil	Vehicle	Fish oil
Prefrontal Cortex				
TRP	312.5±12.31	313.8±20.51	247.5±10.2[a]	301.1±11.5[c]
KYN	81.5±6.22	89.5±5.22	194.8±10.75[a]	149.8±6.96[bc]
KYNA	3.56±0.21	3.66±0.19	2.55±0.24[a]	3.21±0.19[bc]
3-HK	79.5±6.21	82.7±5.12	201.2±8.72[a]	122.5±7.08[bc]
QA	7.25±0.57	8.35±1.14	19.55±1.34[a]	11.23±1.25[bc]
Hippocampus				
TRP	325.5±10.2	336.8±14.2	241.1±11.9[a]	287.3±12.1[bc]
KYN	128.5±5.68	157.4±5.22	268.8±7.33[a]	205.5±8.4[bc]
KYNA	4.55±0.44	4.17±0.41	2.85±0.25[a]	4.11±0.53[c]
3-HK	92.5±6.55	99.5±6.54	165.3±7.44[a]	119.2±5.87[bc]
QA	6.01±0.60	6.55±0.55	16.87±1.26[a]	12.91±0.88[bc]
Striatum				
TRP	306.5±10.76	292.2±9.23	215.5±7.49[a]	250.5±8.29[bc]
KYN	98.7±6.85	108.6±8.33	175.5±9.19[a]	148.5±8.22[bc]
KYNA	4.77±0.44	4.22±0.43	2.47±0.24[a]	3.88±0.31[c]
3-HK	85.6±8.61	101.2±6.55	158.8±4.12[a]	132.5±6.88[bc]
QA	7.65±0.57	8.01±0.72	17.6±1.11[a]	11.41±0.88[bc]

The values were analyzed by two-way ANOVA and Bonferroni multiple comparison test.

Each value is expressed as the mean ± S.E.M. (n =6).

[a] $P < 0,05$ when compared with Vehicle/Adult.

[b] $P < 0.05$ when compared with Fish oil/Adult.

[c] $P < 0.05$ when compared with Vehicle/Age old.

Statistical analysis of TRP levels in hippocampus demonstrated a significant FO/LPS interaction ($F_{1,12} = 4.79$, $p < 0.04$). Post hoc comparisons revealed that FO supplementation attenuated the increase of TRP levels caused by LPS in hippocampus (**Fig. 3**A).

Similary, two-way ANOVA of TRP levels in striatum demonstrated a significant FO/LPS interaction ($F_{1,12}$ = 14.26, p < 0.001). Statistical analysis revealed that FO supplementation attenuated the increase of TRP levels caused by LPS (**Fig. 3**B).

Similar results were revealed by statistical analysis of TRP levels in cortex for FO/LPS interaction ($F_{1,12}$ = 6.57, p < 0.02). Bonferroni post-hoc testing revealed that FO supplementation attenuated the increase of TRP levels caused by LPS in cortex (**Fig. 3**C).

Two-way ANOVA of KYN levels in hippocampus demonstrated a significant FO/LPS interaction ($F_{1,12}$ = 8.23, p < 0.01). Statistical analysis revealed that FO supplementation attenuated the increase of KYN levels caused by LPS (**Fig. 3**D).

Similarly, statistical analysis of KYN levels in the striatum yielded a significant FO/LPS interaction ($F_{1,12}$ = 25.31, p < 0.001). Bonferroni post-hoc testing revealed that FO supplementation attenuated the increase of KYN levels caused by LPS in striatum (**Fig. 3**E).

Similar results were revealed by two-way ANOVA of KYN levels in cortex for FO/LPS interaction ($F_{1,12}$ = 20.99, p < 0.001). Statistical analysis revealed that FO supplementation attenuated the increase of KYN levels caused by LPS (**Fig. 3**F).

Statistical analysis revealed that FO/LPS interaction significantly at KYN/TRP ratio in hippocampus ($F_{1,12}$ = 5.83, p < 0.03). Bonferroni post-hoc testing showed a FO supplementation attenuating the KYN/TRP ratio caused by LPS in hippocampus (**Fig. 3**G).

Two-way ANOVA revealed that LPS significantly KYN/TRP ratio in striatum of mice compared to control group ($F_{1,12}$ = 102.64, p < 0.001). Statistical analysis showed a FO supplementation was not capable of attenuating the KYN/TRP ratio caused by LPS in cortex (p < 0.23; **Fig. 3**I).

Significant results by statistical analysis revealed that FO/LPS interaction at KYN/TRP ratio in cortex ($F_{1,12}$ = 10.,79 p < 0.001). Bonferroni post-hoc testing showed a FO supplementation attenuating the KYN/TRP ratio caused by LPS in cortex (**Fig. 3**G).

3.9. Kynurenic Acid levels and Kynurenic Acid/Kynurenine ratio

Statistical analysis demonstred no significant differences that KYNA levels in hippocampus of mice (FO X LPS, $F_{1,12}$ = 0.05, p < 0.82; **Fig. 4**A). Post hoc comparisons revealed that FO supplementation increased KYNA levels when compared to control group ($F_{1,12}$ = 23.72, p < 0.001).

In similar manner, two-way ANOVA revealed no significant differences that KYNA levels in striatum (FO X LPS, $F_{1,12}$ = 1.48, p < 0.24; **Fig. 4**B).

The statistical analysis revealed no significant differences that KYNA levels in cortex of mice (FO X LPS, $F_{1,12} = 0.57$, $p < 0.46$; **Fig. 4**C). Bonferroni post-hoc testing showed a FO supplementation increased KYNA levels when compared to control group ($F_{1,12} = 5.27$, $p < 0.04$).

Two-way ANOVA revealed that LPS significantly KYNA/KYN ratio in hippocampus of mice compared to control group ($F_{1,12} = 65.08$, $p < 0.001$). Statistical analysis showed a FO supplementation attenuating the KYNA/KYN ratio in hippocampus ($F_{1,12} = 7.52$, $p < 0.01$;**Fig. 4**D).

Similary, statistical analysis revealed that LPS significantly KYNA/KYN ratio in striatum of mice compared to control group ($F_{1,12} = 32.05$, $p < 0.001$). Bonferroni post-hoc testing showed that FO supplementation attenuating the KYNA/KYN ratio in striatum ($F_{1,12} = 7.21$, $p < 0.01$; **Fig. 4**E).

Similar results were revealed by two-way ANOVA that LPS significantly KYNA/KYN ratio in cortex of mice compared to control group ($F_{1,12} = 26.06$, $p < 0.001$). Statistical analysis revealed that a FO supplementation attenuating the KYNA/KYN ratio in cortex of mice ($F_{1,12} = 8.81$, $p < 0.01$; **Fig. 4**F).

3.10. 3-Hydroxykynurenine, Quinolinic Acid levels and 3-Hydroxykynurenine/kynurenine ratio

Two-way ANOVA of 3-HK levels in hippocampus demonstrated a significant FO/LPS interaction ($F_{1,12} = 51.84$, $p < 0.001$). The statistical analysis revealed that FO supplementation attenuated the increase of 3-HK levels caused by LPS (**Fig. 5**A).

Similarly, statistical analysis of 3-HK levels in the striatum yielded a significant FO/LPS interaction ($F_{1,12} = 16.48$, $p < 0.001$). Bonferroni post-hoc testing revealed that FO supplementation attenuated the increase of 3-HK levels caused by LPS in striatum (**Fig. 5**B).

Two-way ANOVA revealed that LPS significantly 3-HK levels in cortex of mice compared to control group ($F_{1,12} = 62.30$, $p < 0.001$; **Fig. 5**C). Statistical analysis revealed that a FO supplementation was not capable of attenuating the 3-HK levels caused by LPS in cortex of mice (**Fig. 5**C).

The statistical analysis revealed no significant differences that 3-HK/KYN ratio in hippocampus of mice ($F_{1,12} = 0.08$, $p < 0.77$; **Fig. 5**D).

Two-way ANOVA revealed that FO supplementattion significantly 3-HK/KYN ratio in striatum of mice compared to control group ($F_{1,12} = 8.01$, $p < 0.01$; **Fig. 5**E).

Statistical analysis showed no significant differences that 3-HK/KYN ratio in Cortex of mice ($F_{1,12} = 0.20$, $p < 0.66$; **Fig. 5**F).

Two-way ANOVA of QUIN levels in hippocampus demonstrated a significant FO/LPS interaction ($F_{1,12} = 11.38$, $p < 0.001$). Bonferroni post-hoc testing showed that FO supplementation attenuated the increase of QUIN levels caused by LPS in hippocampus (**Fig. 5**G).

Similary, statistical analysis of QUIN levels in striatum showed a significant FO/LPS interaction ($F_{1,12} = 40.43$, $p < 0.001$). Two-way ANOVA revealed that FO supplementation attenuated the increase of QUIN levels caused by LPS (**Fig. 5**H).

The results were revealed significant by statistical analysis of QUIN levels in cortex for FO/LPS interaction ($F_{1,12} = 36.99$, $p < 0.001$). Bonferroni post-hoc testing revealed that FO supplementation attenuated the increase of QUIN levels caused by LPS in cortex (**Fig. 5**I).

3.11. Indoleamine-2,3-dioxygenase, Kynurenine 3-monoxygenase and Kynurenine aminotransferase activities

Table 3 Effect of fish oil on kynurenine pathway enzymes in the brain structures of age old mice.

(pg/mg protein)	Adult		Age old	
	Vehicle	Fish oil	Vehicle	Fish oil
Prefrontal Cortex				
IDO	101.2±4.66	115.5±3.24	164.5±6.03[a]	125.6±4.03[c]
KMO	103.8±7.12	113.4±5.56	181.2±7.22[a]	127.2±3.88[bc]
KAT	107.6±9.07	114.1±2.39	71.77±3.87[a]	85.93±6.24[bc]
Hippocampus				
IDO	104.5±5.68	108.6±6.23	153.6±7.56[a]	114.5±7.22[c]
KMO	101.3±3.66	112.8±8.65	159.8±4.12	118.5±10.2[c]
KAT	102.2±6.33	95.6±7.12	71.23±5.75[a]	89.9±5.01[c]
Striatum				
IDO	97.5±3.11	102.5±6.22	167.2±6.33[a]	108.6±8.66[c]
KMO	101.3±4.55	108.5±6.78	177.6±8.01[a]	137.6±3.78[bc]
KAT	110.2±4.56	122.5±8.79	62.57±4.11[a]	101.5±4.68[c]

The values were analyzed by two-way ANOVA and Bonferroni multiple comparison test.

Each value is expressed as the mean ± S.E.M. (n =6).

a P < 0,05 when compared with Vehicle/Adult.

b P < 0.05 when compared with Fish oil/Adult.

c P < 0.05 when compared with Vehicle/Age old.

Statistical analysis of IDO activity in hippocampus demonstrated a significant FO/LPS interaction ($F_{1,12}$ = 12.81, p < 0.001). Bonferroni post-hoc testing showed that FO supplementation attenuated the increase of IDO activity caused by LPS in hippocampus (**Fig. 6**A).

Similary, two-way ANOVA of IDO activity in striatum demonstrated a significant FO/LPS interaction ($F_{1,12}$ = 16.91, p < 0.001). Statistical analysis revealed that FO supplementation attenuated the increase of IDO activity caused by LPS (**Fig. 6**B).

The statistical analysis of IDO activity in the cortex yielded a significant main effect of LPS ($F_{1,12}$ = 46.12, p < 0.001) and FO supplementation ($F_{1,12}$ = 14.86, p < 0.001). FO supplementation partially protected against the decrease IDO activity induced by LPS in cortex (p < 0.001; **Fig. 6**C).

Two way of KMO activity in hippocampus demonstrated a a significant main effect of LPS ($F_{1,12}$ = 30.16, p < 0.001) and FO supplementation ($F_{1,12}$ = 17.30, p < 0.001). Bonferroni post-hoc testing revealed that FO supplementation partially protected against the decrease KMO activity induced by LPS in cortex (p < 0.001; **Fig. 6**D).

Statistical analysis of KMO activity in the striatum yielded a significant main effect of LPS ($F_{1,12}$ = 121.96, p < 0.001) and FO supplementation ($F_{1,12}$ = 25.69, p < 0.001). FO supplementation partially protected against the decrease KMO activity induced by LPS (**Fig. 6**E).

In similar manner, two-way ANOVA of KMO activity in cortex demonstrated a significant main effect of LPS ($F_{1,12}$ = 136.33, p < 0.001) and FO supplementation ($F_{1,12}$ = 9.89, p < 0.001). FO supplementation partially protected against the decrease KMO activity induced by LPS in cortex (p < 0.001; **Fig. 6**F).

Two-way ANOVA of KAT activity in hippocampus showed a FO supplementation caused increase enzyme activity when compared a control group ($F_{1,12}$ = 55.40, p < 0.001; **Fig. 6**G).

Statistical analysis of KAT activity in striatum demonstrated a significant FO/LPS interaction ($F_{1,12}$ = 12.01, p < 0.001). Bonferroni post-hoc testing showed that FO supplementation attenuated the increase of KAT activity caused by LPS (**Fig. 6**H).

Post hoc comparisons of KAT activity in cortex showed a FO supplementation caused increase enzyme activity when compared a control group ($F_{1,12}$ = 88.79, p < 0.001; **Fig. 6**I).

4. Discussion

The aging is a complex process cumulative changes which can affect many tissues and processes leading to highly complex functional alterations in recent years, the KP has generated considerable interest among neuroscientists, following the consistent observation of elevated levels of KYN and other metabolites of the KP of various neuropathological conditions and other diseases involving immune activation (Wu, 2013; BERTAZZOA, 2001;). However, few studies have investigated changes in TRP metabolism with aging, so this study aimed to evaluate changes in the levels of key enzymes, IDO, KMO and KAT involved in the metabolism of the amino acid tryptophan via the kynurenine (KP), that under conditions immune activation is mentioned in the aging process, as their products and TRP products, KYN, KYNA, 3-HK and QA, and compounds indicators of cell damage by oxidative stress in brain tissue prefrontal cortex, hippocampus and striatum, and the possible protective effect of fish oil on these parameters.

In the open field memory test there was no difference in the test object recognition in time of 4:24 hours, the group of elderly mice treated with fish oil spent more time recognizing the newly inserted object, such as the youth group treated mice with fish oil, causing the animals to explore longer the new object, as is its exploratory nature, than the old one that had already been explored. In the Barnes maze test the old mice treated with fish oil had fewer errors and found the right place more easily than the elderly control group. These tests demonstrate that the fish oil assists in preserving both the short term memory and long in mice.

In Table 1 are shown the levels of certain enzymes KP, they are IDO, KMO and KAT, brain tissue prefrontal cortex, hippocampus and striatum.

IDO enzyme had its increased levels (Table 1) in the elderly group in all tissues compared to the new control group, contrary to that report (Badawy, 2015; COMAI, 2005) indicating, under normal conditions, that with aging to this enzyme levels will decaying, but their increase in the aging process has been described (OXENKRUG, 2011; THEOFYLAKTOPOULOU, 2013) by increasing inflammation activation of IFN-γ which acts by increasing the activity of IDO, as well as proinflammatory cytokines IL 1b and tumor necrosis factor TNF-α which exert no effect on IDO, but potentiate its induction by IFN-γ on

the other hand, the anti-inflammatory cytokine IL 4 and IL-10 and growth factor TGF inhibit induction of IDO by IFN-γ (Badawy, 2013). The increase in enzyme levels is also related to immune activation in viral, parasitic infections, autoimmune disease and certain neurological diseases (Mackenzie, 1999). The supplementation with fish oil could protect all tissues increasing the IDO enzyme activity, possibly due to its antioxidant capacity that can modulate inflammation levels.

The KMO is a dependent FAD monooxygenase and is located on the outer mitochondrial membrane where it converts L-kynurenine to 3-hydroxykynurenine (Smith, 2016), had their levels increased in the group without supplementation elderly, the prefrontal cortex tissues and striatum, the hippocampus there was no significant difference when compared with the control and fish oil had partial effect on their levels probably its increase is related to levels of IDO in KP was increased. Being a KMO branch point in KP studies (BREDA, 2016; AMARAL, 2013) is seeking to develop inhibitors for this enzyme because it is an attractive point for the treatment of neurodegenerative diseases one and neuroinflammatory conditions.

The kynurenine aminotransferase (KAT), an enzyme that catalyzes the synthesis of kynurenic acid (KYNA) by KP (HAN, 2010), had a decrease in their levels in the elderly group of mice in all tissues analyzed.

Comai, 2015 found that there was a decrease of this enzyme in hepatic tissues without aged rats 18 months, although the testing has been realisadas in different tissues, our results support that these levels may be related to cell age and tissue damage probably caused by reactive oxygen species or alteration of enzyme activity with increasing age. Supplementation with fish oil managed to reverse the values to control levels, except in the prefrontal cortex, where he obtained partial control. Vamos, 2009 describes that studies on KP for possible inhibition of KMO and overactivation of KAT, as the reason for its metabolites are essences for the balance and performance of the cell, to offer new therapeutic opportunities, such as development of new and powerful compounds as a promising outlook for brain neuroprotection.

The levels of TRP (Table 2) were aged at low levels in the vehicle group, studies corroborate our data BRAYDI 2011, they found a significant decrease in age-related TRP content in brain, liver and kidney. In humans it was observed the entire contents of the TRP at least 12% plasma about the elderly. This decrease in TRP can contribute to the development of immunodeficiency observed in elderly subjects (FRICK, 2004). TRP is a parameter that

can be influenced by many factors, including nutritional, hormonal, psychological and pharmacological agents (Badawy, 2015). The high levels of the enzyme IDO found may be related to the reduction of TRP in the brain tissue, since this is the key enzyme for its metabolism by KP was at high levels in the elderly control group. Therefore, the altered tryptophan metabolism in aged subjects was related to vitamin B6 and nicotinamide deficiency. The values found in the elderly group treated with fish oil showed a complete protection only in the prefrontal cortex, in other tissues there was only partial protection. TRP levels reduced either by increasing its catabolism or reduced absorption can decrease the anabolism of serotonin and thus may be associated with increased vulnerability to depression and neuropsychiatric conditions (braidy, 2009).

The Particularly KP is modulated by the regulatory mechanisms of the immune response and by the redox status. The KYN presented was high in all tissues analyzed in the elderly control group, the main hypothesis is the action of IDO enzyme which metabolizes about 95-99% of the TRP (Myint and Kim, 2014; ESQUIVEL, 2016) encontrou- is high in the elderly group vehicle through degradation by KP a series of reactions will be triggered, forming different products with different functions. Supplementation with fish oil exerted partial protection in all tissues.

4.1. KYNA

It is synthesized from L-KYN in enzymatic reactions mediated by kynurenine aminotransferase (KAT). KYNA levels presented decreased in the old control group, as in the old fish oil group had total protection in the hippocampus and striatum, this is due to KAT levels that were also full protection in these tissues, as in the prefrontal cortex where the enzyme was obtained partial protection, it occurred to its metabolite to KYNA where there was partial protection. It is known that KYNA level changes in several neurodegenerative disorders: it decreases in epilepsy, infantile spasms and Huntington's disease (HD), and increases Alzheimer's disease (AD) and viral infections, among others (WE 2009) .

4.2. 3-HK

In the elderly control group had increased levels with supplementation there was a partial protection of tissues, but contrary to what happened with KAT and its KYNA product in the tissue where there was a total protection of KMO in the hippocampus, this protection

was not constant product showed partial protection. The generation of 3-HK is potentially toxic and is linked to ROS production.

4.3. QA

In the elderly control group in all tissues increased by QA and supplementation with fish oil could partially reverse these levels. QA can increase release of glutamate by neurons, promote lipid peroxidation, resulting in the formation of reactive oxygen species which mediate lipid peroxidation, and can potentiate their own toxicity and other excitotoxins.

In Table 3 there quantification of substances involved in oxidative stress, which indicate the levels of reactive oxygen species, lipid peroxidation, protein oxidation, DNA and antioxidant enzyme in the brain. oxidation levels measured by these different compounds were elevated in the elderly control group, and reduced antioxidant enzyme supplementation with most showed partial protection, except for the full protection of GSH in the hippocampus and the striatum 3NT. Although the cause of these effects is unknown, possibly it is related to cellular damage and age-dependent tissues, probably caused by reactive oxygen species and a change in enzyme activity with increasing age.

With the possibility of influencing the path kynurenine, which is the primary pathway for the metabolism of essential amino acid TRP in mammalian tissues including the brain, to reduce the 3-HK and increase the level of KYNA in the brain there is a new target for acting drugs that alter this balance, reducing excitotoxins and increasing neuroprotective. Through the analysis performed you can check that aging causes an inflammatory process that alters the functioning of various systems such as the KP that play a key role in immune tolerance and its activation has been implicated in the pathogenesis of neuroinflammatory and neurodegenerative disorders. The fish oil proved to be valid as a supplement lifelong seen that in all analyzed parameters could protect tissues of older mice partially and sometimes complete.

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