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ORIGINAL ARTICLE

Behavioral, Oxidative, and Biochemical Effects of Omega-3 on an Ovariectomized Rat Model of Menopause

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Objectives: Menopause induces changes in neuronal transmission, leading to anxiety and depression. Changes in the brain's glutamate levels cause psychological behavior in postmenopausal women. Omega-3 has been studied to improve some of these behaviors.
Methods: Twenty-four female Wistar rats were divided into four groups: sham-operated treated with water (SO-W), sham-operated treated with omega-3 (SO-O), ovariectomized (OVX) treated with water (OVX-W), and bilateral OVX treated with omega-3 (OVX-O). These treatments were performed for 20 days via gavage, before and after surgery, totaling 40 days.
Results: In the forced swimming, elevated plus-maze, and open field tests to assess behaviors, such as depression and anxiety, omega-3 improved these behaviors in both treated groups. The levels of thiobarbituric acid reactive substances (TBARS) in the brain were not different between the groups; however, there was a significant decrease in the catalase activity in the SO-O group compared with the SO-W group ($P < 0.05$). The glutamate level in the cerebrospinal fluid (CSF) was elevated in the SO-O group ($P < 0.001$) but not in the OVX-W or OVX-O groups.
Conclusions: These results bring novel data when related to the glutamatergic system in the SO-O group. This has suggested that the action mechanism of omega-3 was not dependent on glutamate levels in the CSF of the OVX group, but it played a regulatory role in the sham-operated animals. To confirm this, more studies are needed to explore this field when relating to the estrogen and glutamate receptor changes in specific brain regions.

Key Words: Anxiety, Brain, Depression, Glutamate

INTRODUCTION

In the postmenopausal period, there is a decrease in ovarian hormone production, and physiological and psychological effects occur in women, such as depression, anxiety, irritability, and nervousness [1]. Bilateral ovariectomy (OVX), a surgical model of menopause, has been proven to induce anxiety [2] and depressive-like behavior [3] in rodents. This fact could be related to a significant decrease in the dopamine and serotonin release in the brains of the OVX group of rats [4]. The role of the glutamate neurotransmitter was currently evidenced by the study of Lipsitz et al. [5], where four intravenous injections of ketamine (N-methyl D-aspartate receptor antagonist) were able to cause improvements in social functions and suicidal ideation in postmenopausal women. Many women have specific contraindications to the use of hormone replacement therapy during the menopause, which is based on the use of synthetic forms of estrogen [6]. The search for alternative resources that treat, or ameliorate, the symptoms of the menopause, without side effects or contra-

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indications, is increasing.

Linolenic acid belongs to the group of polyunsaturated omega-3 fatty acids, as does eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [7]. Larrieu and Layé [8] reviewed that omega-3 could act on the brain through the effect of members of the "rhodopsin-like" GPCR family, the endocannabinoid system, and the hypothalamic-pituitary-adrenal axis systems, all of which modulate mood-related behaviors. The antidepressant effect caused by omega-3 could be attributed to its immune-modulating actions and inflammatory progression, as seen in other depressive-like-induced behavior, by either chronic stress, lipopolysaccharide, or OVX [9]. The anxiolytic effect of omega-3 in the OVX rats was associated with higher hippocampal concentrations of L-Dopa and 5-HIAA, and with an elevated serotonin turnover [10].

In this sense, omega-3 has shown similar vascular beneficial effects in both postmenopausal women and an animal model of OVX, which could be related to the antioxidant and/or anti-inflammatory effects [11]. Manlapaz-Mann et al. [12] supported that daily oral supplementation with antioxidants, like omega-3, coenzyme Q10, and glutathione nanoparticles, from the first post-natal day, until the 14th day, in a neonatal intermittent hypoxia model, had therapeutic benefits during the treatment, by decreasing the oxidative damage and the inflammatory prostanoids in the brain. The anxiety and depression-like behaviors in the OVX rats were associated with an increase in neural apoptosis, microglial activation in the hippocampus, enhancement of the proinflammatory cytokine expression, and the suppression of the expression of the anti-inflammatory cytokine, interleukin-10 [9]. The same study also showed that supplementation with omega-3 exerted antidepressant and neuroprotective activities, which were accompanied by neuroimmune-modulating actions, like maintaining the normal homeostatic balance between the M1 and the M2 microglial phenotypes. These activities are linked to the production of proinflammatory or anti-inflammatory cytokines, respectively. According to Diaz Brinton [13], it remains unclear if the changes that are caused by the surgical procedures that are conducted in preclinical translational animal models mirror those that women experience at a comparable age (6–10 months of age in the rodents). OVX in reproductively capable rodents provides a remarkably predictive model of ovariectomy in premenopausal women [14]. Preclinical models of surgical interventions have the potential to advance the understanding of medium-term OVX in rats [15]. It is known that there is an increase in anxiety-like and depression-like behavior, 3 weeks post-OVX in the rats, which might be related to a reduction of the steroid hormones at 2 weeks post-OVX, as this is a status feature of postoperative menopause [15].

As a consequence, the present study aimed to investigate the behavioral cognitive effects of omega-3 treatments in rats, such as anxiety and depression, as well as the involvement of the glutamate levels that were subsequently measured in the cerebrospinal fluid (CSF).

MATERIALS AND METHODS

Animals

The animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at the Lutheran University of Brazil (ULBRA) (protocol No. 2015.31) and they were carried out in accordance with the National Institutes of Health and University Guidelines for the Care and Use of Laboratory Animals. Twenty-four healthy female adult Wistar rats, weighing about 200 g, at 8 weeks of age, were maintained under standard environmental conditions, with a 12 h day-night cycle, and with a temperature of 22°C ± 2°C. The animals were provided with a standard pellet diet, and with water ad libitum.

Surgical procedure and supplementation

The animals were anesthetized by using ketamine and xylazine, at 50 mg and 5 mg/kg (intraperitoneal), respectively. Bilateral OVX was carried according to Parhizkar et al. [16]. The rats were randomly assigned into four groups, as follows: sham-operated treated with water (SO-W, n = 5); sham-operated treated with omega-3 (SO-O, n = 5); OVX treated with water (OVX-W, n = 6); and OVX treated with omega-3 (OVX-O, n = 6). The supplementation was performed 20 days before and 20 days after the surgical procedure, at a daily dose of 500 mg/kg/day of omega-3 (1,000 mg capsules, containing 180 mg of EPA and 120 mg of DHA; Multiphytus, Porto Alegre, Brazil), according to the study by Lakhwani et al. [17].

The animals had a postoperative stabilization period to minimize the possible complications due to the surgery. After surgery, the rats received meloxicam (0.2 mg/kg, subcutaneous) for 3 days. During the recovery from anesthesia, they remained under a heated mat-

tress. In the postoperative period, the surgical wounds were inspected daily and treated topically with rifampicin for seven days. The supplementation with omega-3 was maintained during this period.

The weight assessment was carried out on days 0, 20, and 40. On the 40th day, the behavioral study was evaluated. Upon completion of the behavioral study, the animals were sacrificed by an overdose inhalation of isoflurane, and their CSF was analyzed.

Open field test

Each animal was placed in the center of a white open-field arena (60 cm × 40 cm × 50 cm), with the background equally divided into 16 squares by black lines. The horizontal locomotor (crossings) and vertical exploratory (rearings) activities were analyzed [18].

Forced swimming test

This test was performed according to Slattery and Cryan [19], where immobility was considered as an indicator of the depressive state. The rats were individually placed in a polyvinyl chloride cylinder (height, 45 cm; diameter, 20 cm) that was filled with water to a depth of 30 cm, at 25°C ± 1°C, for 5 minutes.

Elevated plus–maze test

The maze consisted of two open arms (51 cm × 10 cm) and two enclosed arms (51 cm × 10 cm × 41 cm), extending from a common central platform (10 cm × 10 cm) that was elevated 55 cm above the floor. The animals were placed in the center of the maze, facing an open arm to begin the test. The behavior of the animals was observed for 5 minutes. The frequency of the open and closed arm entries was counted [20].

Thiobarbituric acid reactive substances (TBARS) and catalase (CAT) in the brain tissue

The brains were dissected and homogenized in a specific buffer and centrifuged. The concentration of the proteins in the brain homogenates was determined by the method of Lowry et al. [21] in a spectrophotometer (Shimadzu, Kyoto, Japan) at 625 nm. The supernatants were mixed with 10% trichloroacetic acid and 0.67% thiobarbituric acid. This mixture was incubated for 15 minutes in a dry block at 100°C and then cooled for 5 minutes. After the cooling of the samples, 1.5 mL of N-butyl alcohol was added to extract the formed pigment. They were placed on a shaker for 45 seconds and centrifuged for 10 minutes at 3,000 rpm. Finally, the samples were read on a spectrophotometer at 535 nm. The levels of the TBARS were reported as nmol TBARS per mg of protein [22].

To evaluate the CAT activity, the method was based on the ability of this enzyme to decompose hydrogen peroxide (H_2O_2). The reaction was started with the addition of H_2O_2 into the medium. The test consisted of measuring the decrease in absorption at 240 nm in the spectrophotometer. The CAT activity was expressed as pmol/g of tissue [23].

Glutamate in the CSF

The CSF was removed by a puncture in the cisterna magna. An analytical UV spectrophotometric method was performed, according to Ferreira et al. [24]. The measurements on the UV–Vis spectrophotometer were performed at the maximum absorption wavelength of 265 nm, after the derivatization reaction of the neurotransmitter.

Statistical analysis

The normality of data was carried out by the Kolmogorov–Smirnov test. The data analysis was conducted by one-way ANOVA, followed by the Student–Newman–Keuls post hoc test for all of the analyses, except for the weight assessment, which used two-way ANOVA, followed by Bonferroni's post hoc test. The results were expressed as mean ± standard error, and a P value < 0.05 was considered significant.

RESULTS

Weight assessment

For 20 days, the weight of the animals did not show any significant difference between the groups. The OVX-W group (302.0 ± 12.81 g, $P < 0.05$) showed a significant increase in body weight on the 40th day when compared with the SO-W (270.5 ± 3.15 g) and SO-O (268.0 ± 6.59 g) groups.

Open field test

The OVX-W group (29.67 ± 2.91) suggested more anxious behavior because it moved significantly less when compared with the OVX-O (55.33 ± 2.81, $P < 0.001$), SO-W (41.83 ± 2.90, $P < 0.05$), and SO-O (39.60 ± 1.99, $P < 0.05$) groups. There was also a significant difference between the OVX-O, SO-W, and SO-O ($P < 0.05$) groups (Fig. 1A). Concerning the exploratory behavior, there were no significant differences (Fig. 1B).

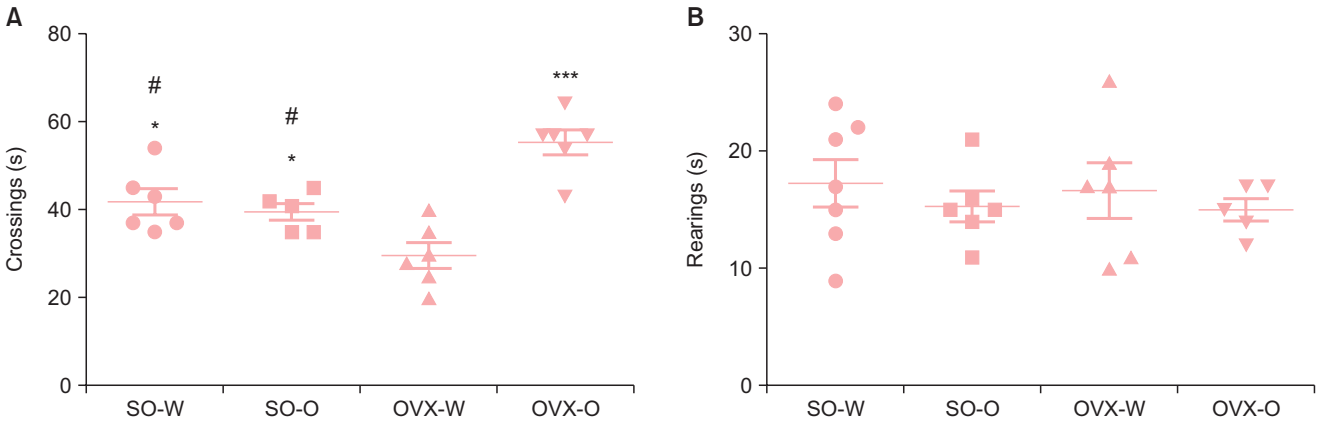

Fig. 1. Effects of omega-3 (per os [oral administration]) for 40 days on the (A) locomotor, and (B) exploratory activities of the sham-operated and ovariectomized rats in the open field test. Each bar represents the mean ± standard error of 5–6 animals. $^{*}P < 0.05$ and $^{***}P < 0.001$ when compared with the OVX-W group. $^{\#}P < 0.05$ when compared with the OVX-O group. One-way ANOVA, followed by the Student–Newman–Keuls test. SO-W: the sham-operated rats treated with water, SO-O: the sham-operated rats treated with omega-3, OVX-W: the ovariectomized rats treated with water, OVX-O: the ovariectomized rats treated with omega-3.

Forced swimming test

In the forced swimming test, the OVX-W group (22.50 ± 1.56 seconds) had a significantly longer immobility time when compared with the SO-W (11.83 ± 0.63 seconds, $P < 0.001$), SO-O (13.20 ± 1.11, $P < 0.001$), and OVX-O (16.20 ± 0.86, $P < 0.01$) groups.

Moreover, the group OVX-O also showed a significant increase in the immobility time when compared to the SO-W group (Fig. 2).

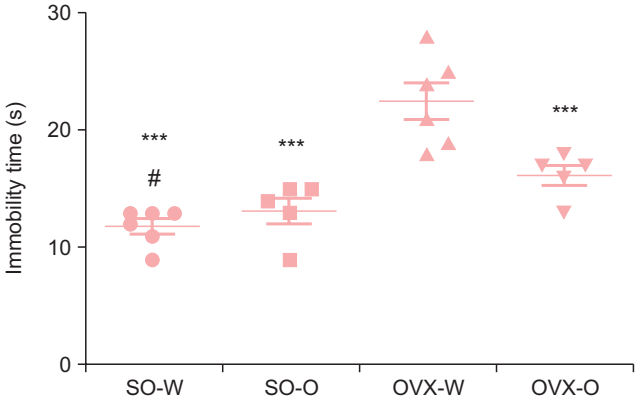

Fig. 2. Effects of omega-3 (per os [oral administration]) for 40 days on the forced swimming test for the sham-operated and ovariectomized rats. Each bar represents the mean ± standard error of 5–6 animals. $^{***}P < 0.001$ when compared with the OVX-W group. $^{\#}P < 0.05$ when compared with the OVX-O group. One-way ANOVA, followed by the Student–Newman–Keuls test. SO-W: the sham-operated rats treated with water, SO-O: the sham-operated rats treated with omega-3, OVX-W: the ovariectomized rats treated with water, OVX-O: the ovariectomized rats treated with omega-3.

Elevated plus-maze test

In the elevated plus-maze test, the OVX-W group (35.01 ± 7.96 seconds) remained a significantly shorter time in the open arms when compared with the SO-W group (79.33 ± 7.36 seconds, $P < 0.05$) (Fig. 3A). The number of entries into the open arms of the OVX-W group was lower (4.83 ± 1.25) but it was similar to the SO-W group (4.33 ± 1.08). The groups that were treated with omega-3 also showed similar behavior (SO-O, 9.33 ± 1.33; OVX-O, 9.71 ± 0.68) (Fig. 3B).

The OVX-W group remained longer in the closed arms (263.70 ± 8.26 seconds) (Fig. 3C). The animals that were treated with omega-3 (SO-O: 238.21 ± 4.21 seconds; OVX-O: 249.70 ± 11.45 seconds) did not show any significantly different behavior. The same happened with the number of entries into the closed arm (Fig. 3D).

Analysis of the TBARS and CAT in the brain and the liver

The levels of the TBARS in the brain of the animals were not different between the groups (Fig. 4A). Having said that, the levels of CAT in the same tissue as the SO-W group (6.13 ± 0.21 pmoles/mg of protein) were significantly different to the SO-O group (3.93 ± 0.57 pmoles/mg of protein, $P < 0.05$) (Fig. 4B).

Analysis of glutamate in the CSF

The SO-O group had an increase (0.55 ± 0.01 μmol/mL, $P < 0.001$) in the levels of glutamate in the CSF when compared with the OVX animals and the SO-W

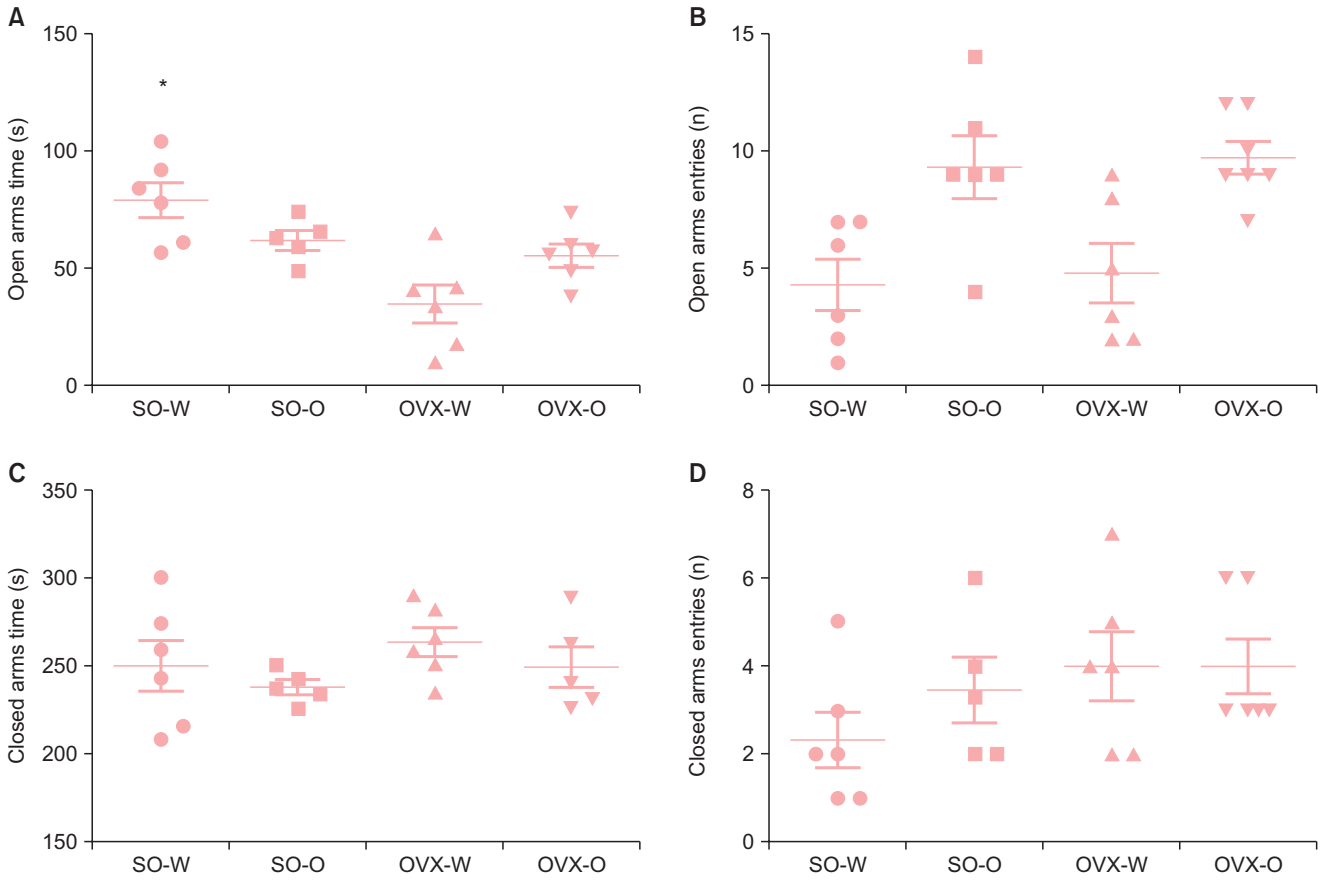

Fig. 3. Effects of omega-3 (per os [oral administration]) for 40 days on the elevated plus-maze test for the sham-operated and ovariectomized rats. Each bar represents the mean ± standard error of 5–6 animals. *$P < 0.05$ when compared with the OVX-W group. One-way ANOVA, followed by the Student–Newman–Keuls test. SO-W: the sham-operated rats treated with water, SO-O: the sham-operated rats treated with omega-3, OVX-W: the ovariectomized rats treated with water, and OVX-O: the ovariectomized rats treated with omega-3.

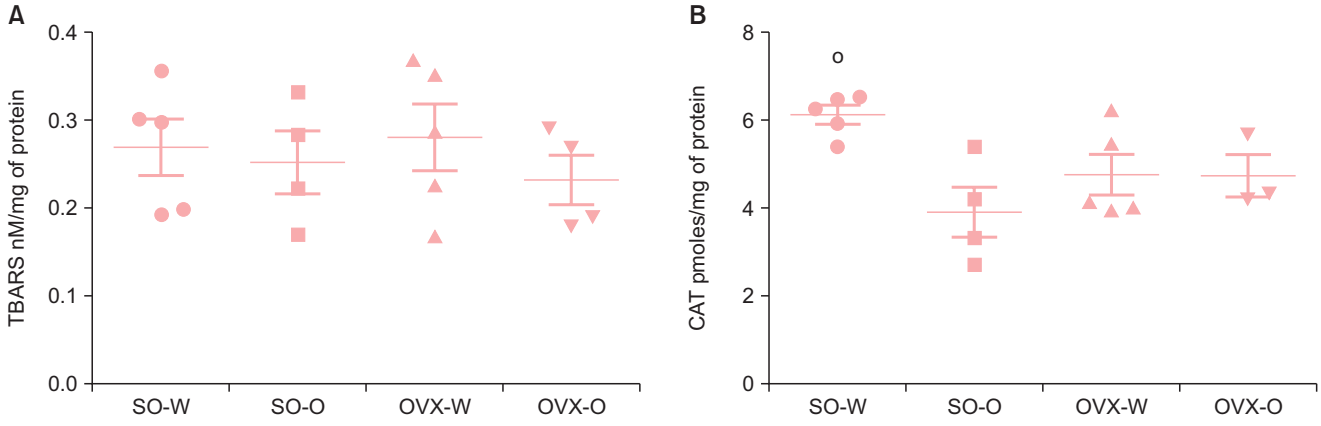

Fig. 4. Effects of omega-3 (per os [oral administration]) for 40 days on the levels of the thiobarbituric acid reactive substances (TBARS) (A), and catalase (CAT) (B), in the brain of the sham-operated and ovariectomized rats. Each bar represents the mean ± standard error of 5–6 animals. º$P < 0.05$ when compared with the group SO-O. One-way ANOVA, followed by the Student–Newman–Keuls test. SO-W: the sham-operated rats treated with water, SO-O: the sham-operated rats that were treated with omega-3, OVX-W: the ovariectomized rats treated with water, OVX-O: the ovariectomized rats treated with omega-3.

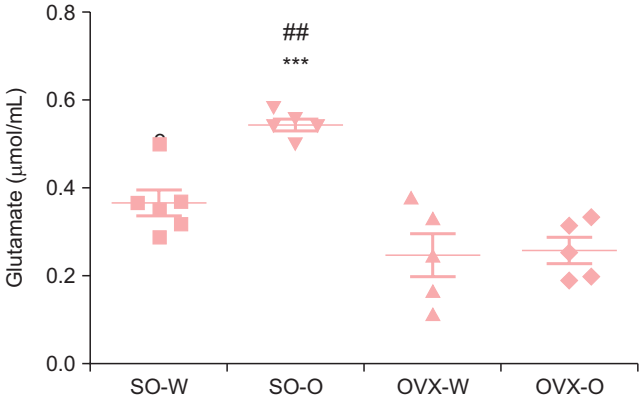

Fig. 5. Effects of omega-3 on the levels of glutamate in the cerebrospinal fluid (CSF) of the sham-operated and ovariectomized rats. Each bar represents the mean ± standard error of n = 5–6 animals. ***$P < 0.001$ when compared with the OVX-W group. ##$P < 0.01$ when compared with the OVX-O group. º$P < 0.05$ when compared with the SO-O group. One-way ANOVA, followed by the Student–Newman–Keuls test. SO-W: the sham-operated rats treated with water, SO-O: the sham-operated rats treated with omega-3, OVX-W: the ovariectomized rats treated with water, OVX-O: the ovariectomized rats treated with omega-3.

sham-operated water-treated animals (0.36 ± 0.30 μmol/mL, $P < 0.05$) (Fig. 5). The OVX-O (0.26 ± 0.03 μmol/mL) group showed similar values to the OVX-W group (0.25 ± 0.05 μmol/mL) but they were significantly lower than the SO-W group.

DISCUSSION

According to Yousefzadeh et al. [25], rats reach sexual and skeletal maturity at around 2.5 months and 10 months of age. Because of this, 6-month-old rats are preferred to 9-month-old rats, due to the lower age-related changes. This present study used 8-week-old rats, as the study did not intend to evaluate the osteoporosis parameters. To access the anxiety and depression-like behavior, many authors have used older animals for the OVX model [3,9,26,27]. The treatment period was based on the results of Puga-Olguín et al. [15], who demonstrated an increase in mood-change behavior, 3 weeks post-OVX. The current work hypothesized that a pre-treatment with omega-3 before ovariectomy could occur in clinical practice with humans. It could possibly minimize the effects of surgical menopause, although this has never been studied and described.

In the present work, the OVX animals substantially increased their body weight on the 40th day. The supplementation with omega-3 did not interfere with this parameter. The supplementation of omega-3 induced an increase in the locomotory activity, as seen in the open field test, at the same levels as the OVX animals. The depression-like behavior was improved in the forced swimming test, where the supplemented ovariectomized animals showed a resemblance to the behavior of the sham-operated animals. The sham-operated rats, or the ovariectomized supplemented rats with omega-3, did not show anxious behavior when evaluated in the elevated plus-maze.

The appropriate balance of excitation and inhibition is critical for normal brain function. This is achieved by the opposing actions of excitatory glutamate and the inhibitory gamma-aminobutyric acid (GABA) [28]. It is known that OVX animals develop depressive- and anxious-like behaviors [29], and this can be related to an imbalance between the excitatory and inhibitory neurotransmission [30]. Sandini et al. [31] suggested that there was a link between the estrogen activation and the neurotransmitters because it was observed that there was an increase in both the glutamate and GABAergic levels in the limbic regions of the intact female middle-aged rats (12 months).

Qu et al. [32] found that 1 week after the OVX, the rats showed a loss of neurons and synapses in the hippocampus, as well as a decrease of ERα but not with the ERβ expression. Both Jin and Park [33] and Choi and Park [34] stated that the EPA and DHA supplementation, plus or not the 17β-estradiol-3-benzoate (E2) injection, increased the ERα in the hippocampus but ERβ was increased only by the E2 injection. Overall, Gross and Mermelstein [35] revealed that there was a mechanism that has emerged in the coupling of ERα and ERβ with the mGluRs, to initiate the G protein signaling cascades, which ultimately influence neuronal physiology, structure, and behavior. According to the same authors, the heterogeneity of possible receptor pairings led to diverse molecular results, and this can conduce the biological processes.

Slowik et al. [36] revealed that psychiatric disorders, such as depression, could be modulated by estrogen. Estradiol modulates the glutamate transmission via a complex relationship that involves direct interactions between the subtypes of the estrogen receptor (ER) and the subtypes of the metabotropic glutamate receptor (mGlu). This can protect against glutamatergic excitotoxicity and it attenuates the amount of calcium entering the cell, following the glutamate release [37,38]. Recently, it was investigated that an activation of

$mGlu_5$ was necessary for the estradiol mitigation of the anxiety-related behaviors that are induced by an acute stressor [39].

In the present study, the results have indicated that there was a decrease in the level of glutamate in the CSF of the menopaused rats when compared with the sham-operated animals. The present data differs from the findings of Sandini et al. [31]. Since ovariectomy is a surgical model of menopause, this causes an abrupt cessation of estrogen that leads to complex changes in the homeostasis [40]. The data also differs from the results of Zhou et al. [41] who behaviorally demonstrated that the antidepressant and anxiolytic effects in the OVX mice that were submitted to chronic unpredictable mild stress, were possibly mediated via the restoration of the brain neurotransmitters, such as dopamine, serotonin, GABA, and glutamate, and their related biomarkers in the different brain regions. The authors verified that the OVX mice displayed higher levels in the hippocampal and the cortical glutamate than did the sham-operated mice. The current findings have suggested that the depressive and anxious behaviors in the OVX rats were related to the decrease in the ERα [32] and glutamate levels in the CSF.

The behavioral improvements in the OVX animals that were treated with omega-3 cannot be related to the glutamatergic changes but with its antioxidant and anti-inflammatory effects [11], although the current work did not verify the antioxidant effects. Some examples are that Behling et al. [42] showed that the omega-3 treatment in the OVX rats had a prooxidant effect on the brain. Avramovic et al. [43] showed that lipid peroxidation was significantly decreased in the supplemented animals with omega-3 and that the CAT activity was also decreased, but not significantly. Monteiro et al. [44] revealed that thirty days after ovariectomy, the animals presented a significant increase in the CAT activities but that they did not change the oxidative stress parameters (radical-trapping the antioxidant potential and the TBARS) when compared with the sham or the other rats. As a consequence, the present results have corroborated the idea that omega-3 does not improve membrane lipid peroxidation in the brain tissue.

Curiously, the present results have shown that there was a significant increase in the glutamate levels in the CSF of the sham-operated animals that were supplemented with omega-3. This might suggest that adequate levels of estrogen and ERα could protect against the excitotoxic effects of the glutamate, while not leading to anxious behavior. Aryal et al. [45] showed that the hippocampal synaptosomes of the omega-3 fatty acid-deficient mice had reduced concentrations of the glutamate receptor subunits. Under this scenario, the expressions of the ER and glutamate receptors in the omega-3 supplemented rats would need to be investigated, to correlate with the present findings of this study.

Estrogen potentiated the release of glutamate and acted on the postsynaptic membranes that are related to synaptic plasticity [46], and also increased the expression of the NMDA receptor and its sensitivity to glutamate [47]. Brinton [48] showed that this mechanism led to an increase in neuronal excitability, generating the morphological plasticity changes, such as an increase in spine density in the hippocampus, amygdala, and prefrontal cortex. The present observations are potentially interesting but the determination of the brain regions that showed changes in the glutamatergic activity, or how the ovarian hormones and omega-3 affected the neurotransmitter, would help in the understanding of the physiological significance of the data.

In summary, the results of the present work have reinforced the improvement of the anxious and depressive-like behavior that was caused by the supplementation of omega-3 in the post-menopause period. To the best of the authors' knowledge, this is the first report that has shown that the OVX model of menopause caused a decrease in the glutamate levels in the CSF. The glutamate levels were not improved by the omega-3 supplementation in the OVX rats, although in the sham-operated animals, the levels increased significantly. The exact mechanism by which the polyunsaturated fatty acids exerted an increase in the glutamate level in the CSF of the sham-operated rats is still not clear. Further studies need to be addressed to investigate the role of omega-3 in the brain regions, which are related to the anxious and depressive-like behavior, mainly regarding the glutamatergic projection. Therefore, the present results bring novel data related to the glutamatergic system and confirm that more studies are needed to explore this field.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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