Neuroprotective Effects of a Combined Therapy With Memantine, Donepezil and Vitamin D in Ovariectomized Female Mice Subjected to Dementia Model

Ana Daniela Coutinho Vieira
University of Southern Santa Catarina (UNESC)
Eduarda Behenck Medeiros
University of Southern Santa Catarina (UNESC)
Gabriel Casagrande Zabot
University of Southern Santa Catarina (UNESC)
Nathalia de Souza Pereira
University of Southern Santa Catarina (UNESC)
Natália Baltazar do Nascimento
University of Southern Santa Catarina (UNESC)
Adrielly Vargas Lidio
University of Southern Santa Catarina (UNESC)
Ândrea Kohlrausch Scheffer
University of Southern Santa Catarina (UNESC)
Lisienny Campoli Tono Rempel
University of Southern Santa Catarina (UNESC)
Bárbara Machado Naspolini Macarini
University of Southern Santa Catarina (UNESC)
Ewa Kucharska
Jesuit University Ignatianum
Matheus Scarpatto Rodrigues
Federal University of Rio Grande do Sul
José Cláudio Fonseca Moreira
Federal University of Rio Grande do Sul
Jade de Oliveira
Federal University of Rio Grande do Sul
Josiane Budni (josiane.budni@unesc.net)
University of Southern Santa Catarina (UNESC)

Research Article

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Abstract

The postmenopausal period is characterized by a decrease in the hormonal supply which is associated with Alzheimer's Disease (AD). Vitamin D is neuroprotective and can be used in combination with pre-existing medications to improve its effects. The objective was to evaluate the effect of vitamin D associated with memantine and donepezil in female mice submitted to ovariectomy (OVX) for 5 months and subjected to an AD-induced dementia model. Animals were divided into 5 groups who received 17 days of treatment and were subjected to behavioral tests. The animals underwent euthanasia at 18th day. OVX groups exhibit reduced levels of E2 and triple treatment group had high levels of vitamin D. The induction of dementia with OVX induced short- and long-term spatial and habituation memories damage. Also, induced reduction of BDNF and IL-4 levels in hippocampus, and increasing levels of TNFα in hippocampus and of IL-1β in hippocampus and frontal cortex of animals, as well as a significant increase on GFAP immunoreactivity. Triple-association treatment reversed the effects of long-term spatial and habituation memories damage, as well as reversed changes in TNFα, IL-1β, IL-4 and GFAP immunoreactivity levels in hippocampus of treated animals. Therapeutic association has beneficial effects on memory and inflammation parameters in female mice subjected to OVX and the AD animal model of dementia.

1 Introduction

Population aging tends to cause a demographic change in coming years, increasing the occurrence of senility-related pathologies, such as dementia (Annweiler et al, 2011; Shen e Ji, 2015; Alzheimer’s Association, 2017). Alzheimer's Disease (AD) is the most frequent cause of dementia (Vest and Pike, 2013; Reitz and Mayeux, 2014; Lobo et al, 2014; Shen e Ji, 2015; Hernando-Requejo, 2016; Alzheimer’s Association, 2017), and is recognized as a progressive and irreversible neurodegenerative disease (Cai, Hussain and Yan, 2013). Its pathophysiology is characterized by extracellular accumulation of senile plaques in brain tissue, along with the occurrence of intracellular neurofibrillary tangles derived from abnormal hyperphosphorylation of tau protein (Vest and Pike, 2013; Balin e Hudson, 2014; Durk et al, 2014; Lobo et al, 2014; Reitz e Mayeux, 2014; Bermejo-Pareja et al, 2016; Hamdan e Ramos, 2016). These alterations mainly affect the hippocampus and cerebral cortex regions (Vest and Pike, 2013) and, in addition to these findings, other pathogenic mechanisms may be related to the development of the disease, especially neuroinflammation, i.e., the process by which the brain responds to injuries through glial cells (e.g., astrocytes) activation (Cai, Hussain and Yan, 2013; Heppner et al, 2015). Both increase of proinflammatory cytokines such as TNFα, IL-1β, and IL-6, and imbalance between pro-and anti-inflammatory cytokines, such as IL-4, IL-10, and IL-13, are detrimental in this scenario (Cai, Hussain and Yan, 2013).

Women older than 60 years have a higher prevalence of dementia, aging-related cognitive decline and AD than the rest of the population (Reitz et al, 2011; Alzheimer’s Association, 2017; Davey, 2017). Some theories pointed to this higher risk with hormonal senescence in menopause (Bushra et al, 2014), characterized by the decline of estrogen levels, leading to a permanent cessation of ovulation and

menstruation (Takahashi et al, 2015; Davey, 2017; Siebert et al, 2017). Depletion of female sex steroid hormones, especially estradiol (E2) (Hansberg-Pastor et al, 2015), seems to increase vulnerability to diseases in tissues sensitive to these hormones, such as bones, cardiovascular system, and brain (Lobo et al, 2014). Therefore, it is necessary to search for effective alternatives to treat deleterious cognitive effects of post-menopausal E2 deprivation, which can lead to the onset of AD (Annweiler, 2011).

Currently, only four drugs are approved for the treatment of AD - memantine, donepezil, galantamine, and rivastigmine (Graham, Bonito-Oliva e Sakmar, 2017; Kim et al, 2017); however, the effectiveness of these drugs is limited. In addition, these drugs do not present clear evidence regarding both long-term safety and efficacy of treatment, since none of the drugs modify primary pathological processes of disease or prevent its progression, despite providing a symptomatic relief (Annweiler et al, 2012a; Matsuzono et al, 2015; Crous-Bou et al, 2017; Graham, Bonito-Oliva and Sakmar, 2017; Hung and Fu, 2017).

To minimize symptoms of AD, the development of new therapeutic strategies is essential (Annweiler et al, 2011), and one current alternative is to search for substances with neuroprotective properties, preferably, already approved by FDA (Food and Drug Administration) and with known toxicity, to facilitate access to AD patients (Landel et al, 2016). Currently, links between vitamin D and AD have been highlighted. In fact, deficiency of this vitamin is prevalent both in AD patients and individuals who have dementia in general (Afzal et al, 2014; Morley, 2014; Shen and Ji, 2015; Landel et al, 2016). 1.25$(OH)_2$D3 (active vitamin D) acts on the central nervous system (CNS) through vitamin D-specific nuclear receptors (VDR) (Landel et al, 2016), which are present in neurons and glial cells (Annweiler, 2016), especially in cognition-related regions such as the hippocampus, hypothalamus, cortex, and subcortum (Annweiler et al, 2014). Thus, modulation in these receptors by vitamin D exposure could regulate the expression of structural proteins, neurotrophic factors (Annweiler, 2016), and neurotransmitters deficient in neurodegenerative diseases (Mpandzou et al., 2016), attenuates inflammation related to natural senescence and Aβ peptide toxicity (Annweiler, 2016).

Based on this, new therapeutic approaches have been studied aiming at the use of this vitamin alone (Briones e Darwish, 2012) or in association with other substances (Annweiler et al, 2011), contributing to multiple drug treatment and a possible enhancement of favorable effects in the management of AD. In this study, we submitted female mice ovariectomized (OVX) and injected with Aβ peptide to memantine, donepezil, and vitamin D combination treatment.

2 Materials And Methods

2.1 ANIMALS

70 female Balb/c mice (8 months old, weighing 25–30g) were used in this study after 5 months of OVX. The animals were kept under standard conditions of a 12-h light/dark cycle, with food and water available *ad libitum*.

All animal experiments were carried out in compliance with ARRIVE guidelines and were performed in accordance with the National Council for the Control of Animal Experimentation (Conselho Nacional de Controle de Experimentação Animal – CONCEA) were approved (protocol 063/2018-2 - version 02) by the Ethics Committee on Animal Use (Comissão de Ética no Uso de Animais - CEUA) of the University of Southern Santa Catarina (Universidade do Extremo Sul Catarinense - UNESC).

2.2 OVX SURGERY FOR HORMONE DEFICIENCY INDUCTION

Female mice 3-month-old were anesthetized with ketamine/xylazine (ketamine: 60 mg/kg and xylazine: 4 mg/kg intramuscular, i.m). After disinfection with chlorhexidine, a cutaneous incision in the dorsal midline was made caudal to the back of the ribs. Using blunt dissection to the subcutaneous tunnel, lateral to skin incision, the posterior abdominal wall muscles were separated to expose the abdominal cavity. The ovary was located on a fat pad 1 to 2 cm below muscles. The periovarial fat was clamped with forceps to lift and externalize the ovary. The fallopian tube was crushed, and the ovary was removed by cauterization. The skin incision was closed using suture number 6. The control (Sham) animals only underwent surgery without cauterization of ovaries. After surgery, to ensure the prolonged absence of ovarian hormones, animals remained in the vivarium for 5 months (Rigalli and Loreto, 2009; Fatemi et al, 2018).

2.3 INDUCTION OF ANIMAL MODEL BY ADMINISTRATION OF AB1-42

Aβ1-42 peptide (Tocris Bioscience, Bristol, UK) was dissolved in a phosphate buffer solution (137 mM NaCl, 10 mM Na_2 HPO_4, 1.8 mM KH_2 PO_4, 2.7 mM KCl_2, pH 7.5) in a concentration of 1 µg/µL. The solution was incubated for 7 days at 37°C and 5% CO_2 to allow aggregation of Aβ1-42 peptides in oligomers (Ueda, Fukui and Kageyama et al., 1994; Resende et al., 2008; Ruiz-Muñoz et al., 2011; Garcez et al., 2017), and after aggregation, was stored at -20ºC until use. Before administration, solution was diluted to final dose of 100 pmol/µL in ACSF (24 mM NaCl, 2.5 mM KCl, 2.0 mM $MgSO_4$, 1.25 mM KH_2 PO_4, 26 mM $NaHCO_3$, 10 mM glucose and 4 mM sucrose). Animals were anesthetized with isoflurane (1.5%) using an inhalation anesthesia apparatus. Aβ oligomers were injected through a 3 mm long 28-gauge stainless steel needle coupled to a Hamilton syringe. The needle was inserted unilaterally through the skin and skull directly into the left lateral ventricle via i.c.v. Aβ oligomers were injected in a volume of 4µl (400 pmol/site) for 10 seconds, followed by a 10-second delay to allow full diffusion of the solution. After administration, animals were placed in a community cage with *ad libitum* access to food and water and then recovered from anesthesia.

2.4 TREATMENT

Animals were treated with vitamin D3 (420UI/kg) (Adera®) associated with memantine (5 mg/kg) (Eurofarma®) and donepezil (1 mg/kg) (Eurofarma®) or water, administered orally (by gavage) for 17 days.

2.5 BEHAVIORAL TESTS

On the 14th day of treatment, animals were submitted to the behavioral test of the radial maze, which persisted for 5 days to evaluate spatial memory. Another independent group of animals, on the 11th and 12th days of treatment, were submitted to open field test for habituation evaluation, and on the 13th day, short-term spatial memory was evaluated with the Y maze test. After behavioral tests, the animals were induced to euthanasia.

2.6 RADIAL MAZE TEST

The radial maze test was performed to evaluate the spatial memory. On the 14th day of treatment, the task began. The radial maze apparatus had 8-arms, which were numbered from 1 to 8 (48 × 12 cm) and extended radially from a central area (32 cm diameter). The apparatus was placed 50 cm above the floor, and geometric shapes were positioned in the straight arms where the food was placed (visual cues). On the first day, each animal was placed in the apparatus for a total of 10 min, allowed only to explore, and then returned to its cage. On the 2nd day (or the true first day of test), the animals were placed in the apparatus, where food (cereal) had already been deposited in four of the eight arms. The food bearing arms had visual cues at the end of each arm. Over a period of 10 min, the entry into each arm (total errors to find food) and the time each animal took to find the four pieces of cereal were recorded (latency to find food). The same test was held over four consecutive days, with one trial occuring per day. At the end of the test, the animals were euthanized (Foyet et al., 2011; Hritcu et al., 2012).

2.7 Y-MAZE TEST

The Y-maze test was performed to evaluate short-term spatial memory. It was performed at the 13th day of treatment, the apparatus consists in a 3 armed labyrinth (50 x 10 x 20) at a 120° angle, being similar to the shape of the letter “Y”. The first arm has a visual clue to ease the spatial location of the animals. This behavioral test is made at a dark room, only lighted by a red bulb, and it occurs in two sessions, divided by a 2-hour break. At the first session, the animal starts at the first arm (start) and has free access to explore the second arm (other) during five minutes, while the third arm (novel) is closed (Dellu et al., 1992). The animal returns to his cage at the end. 2 hours later, the animal returns to the apparatus with all 3 arms open to explore freely during five minutes. His permanence at all arms is timed separately, and the short-term special memory is evaluated through his permanence at the “novel” arm (Dellu et al., 1992).

2.8 HABITUATION TO OPEN FIELD TEST

The habituation to open field test was performed to evaluate the habituation memory, at the 11th and 12th days of treatment. The apparatus is made with 4 walls (60 x 40 x 50 cm), one of them is made of glass, its floor is divided in 9 equal squares divided by lines drawn. It’s done in 2 separate sessions divided by a 24-hour break. At the first day, the animal is put inside the posterior left square of the open field apparatus, once inside, the times the animal crosses lines (crossings) and stands on his hind legs (rearings) are counted separately during 5 minutes. (Vianna, 2000). After this period, the animal is removed from the apparatus and returns to it’s cage. 24 hours later, the protocol is repeated. His

locomotor activity is evaluated through the number of crossings and his habituation memory is evaluated through the number of rearings (Vianna, 2000).

2.9 BIOCHEMICAL ANALYZES

Animals were subjected to euthanasia on the 18th day of the experiment by cervical dislocation. The frontal cortex and hippocampus were dissected and homogenized in phosphate buffer to analyze cytokine (TNFα, IL-1β, and IL-4) levels by enzyme immunoassay kits (ELISA). Hippocampus was also used for the analysis of BDNF levels by ELISA. Blood was collected to analyze 25-hydroxyvitamin D and estrogen (E2) levels by chemiluminescent immunoassay in serum. The samples were stored under refrigeration at -80°C until use.

2.10 IMMUNOFLUORESCENCE MICROSCOPY

After the end of the experimental protocol, mice were anesthetized (75 and 10 mg/kg of xylazine mixture, respectively, intraperitoneally), perfused through the left cardiac ventricle with 0.9% saline solution, and by 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS), pH 7.4. The brains were collected and post-fixed in the same fixative solution for 24 h at room temperature (RT), and cryoprotected by immersion in a 30% sucrose solution in PBS at 4ºC. Serial coronal sections (40 µm) of hippocampi were obtained with a Vibratome. The free-floating sections were first blocked using 5% horse serum (HS) diluted in PBS containing 2% Triton X-100 (PBS-Tx) for 2 h at RT. Then, the sections were incubated 24 hours at 4ºC with mouse anti-GFAP (glial fibrillary acidic protein) (Sigma, 1:400) in 1% HS diluted in 0.5% PBS-Tx. After three washes in PBS, tissue sections were incubated with anti-mouse Alexa 488 (Invitrogen, 1:400) in 1% HS diluted in 0.5% PBS-Tx for 2 h at RT. After incubation in secondary antibody, the sections were washed three times in PBS. Thereafter, the sections were washed several times in PBS, mounted on slides with CC/Mount (Sigma), and covered with coverslips. Finally, images from mouse hippocampi were obtained with a Microscopy EVOS® FL Auto Imaging System (AMAFD1000 - Thermo Fisher Scientific; MA, USA) (de Oliveira et al., 2014).

2.11 STATISTICAL ANALYSIS

The results were analyzed using STATISTICA version 8.0 (StatSoft, Inc., USA). Shapiro-Wilk normality test was performed to confirm that the data had a normal distribution. The results of the radial maze test were confirmed by analysis of variance of one-way repeated measures analysis of variance (ANOVA), followed by Newman-Keuls post-hoc test when appropriate. Open field, and Y-maze data were analyzed by Student's T-test. Data on levels of estrogen, vitamin D, BDNF, and cytokines were analyzed by one-way ANOVA, followed by the Newman-Keuls post-hoc test when appropriate. Data were expressed as mean ± standard error of the mean (SEM). The values of p are considered significant when <0.05.

3 Results

Our results demonstrated that OVX surgery effectively reduced E2 levels since the hormonal dosage of Sham or OVX animals showed significant differences [$F(4.25)=521.45$, $p<0.01$], indicating that OVX

animals had E2 levels ≤9.9 pg/mL (Figure 1). Figure 2 showed that animals treated with vitamin D3 (420 IU/kg) had an increase of the serum 25-hydroxyvitamin D levels [F(4.22)=57.10, p<0.01].

The radial labyrinth data show a significant effect [F(12.126)=2.09, p<0,05] on latency to find the rewards. The control group (Sham + ACSF + water) learned the task on the third and fourth test days. The Sham + Aβ1-42 + water group learned only on the last day, indicating long-term spatial memory damage. Animals from OVX + ACSF + water group learned the task on the third and fourth days, and the control group, indicating that OVX did not damage long-lived spatial memory. However, the OVX + Aβ1-42 + water group showed spatial memory damage on all test days, and this damage was even more significant when comparing Sham + Aβ1-42 + water group to OVX + Aβ1-42 + water group. This result shows that OVX associated with peptide causes more severe impairment in memory. It was possible to observe that the treatment with the triple association of vitamin D + memantine + donepezil effectively reversed spatial memory damage caused by peptide associated with OVX since the animals in this group learned on the third day of training as control. However, data on the errors that animals committed until they found the reward did not reveal any significant difference [F(12.126)=0.53, p=0.05] (Figure 3).

The Y-maze data showed a significant difference only in the Sham + ACSF + water group [t(9): -2.78, p<0.05], indicating that only the control learned the task, showing no short-term spatial memory impairment. While other groups, including the treatment group, did not show significant differences between the other and the new arm, indicating that animals did not learn the task, i.e., short-term spatial memory damage and treatment could not reverse it (Figure 4).

The results of open-field habituation test showed significant differences in number of crosses for Sham + ACSF + water group [t(10): 2.95, p<0.05], OVX + ACSF + water [t(9): 2.47, p<0.05] and OVX + Aβ1-42 + vitamin D + memantine + donepezil [t(7): 3.02, p<0.05]. There was a significant difference in the number of withdrawals only for the group supplemented with the triple treatment [t(7): 2.94, p<0.05]. These results demonstrate that animals of Sham + ACSF + water and OVX + ACSF + water groups learned in the number of crosses but not in the number of surveys. However, groups Sham + Aβ1-42 + water and OVX + Aβ1-42 + water did not learn in the number of crosses or the number of surveys, showing damage to habituation memory in the open field test. However, treatment with vitamin D + memantine + donepezil combination completely reversed the habituation memory damage to the open field, both in the number of crosses and in the number of withdrawals, indicating that triple combination was protective against memory damage of habituation to open field (Figure 5).

BDNF data showed a significant reduction in neurotrophin levels [F(4.10)=4.57, p<0.05] in all experimental groups compared to the control group, but treatment with vitamin D + memantine + donepezil could not reverse this effect (Figure 6).

Regarding the cytokine dosage, it was observed that results of TNFα dosage showed no significant differences in the frontal cortex [F(4.16)=1.41, p=0.27] only in the hippocampus [F(4.14)=25.57, p<0.01], where TNFα levels increased in Sham + Aβ1-42 + water, OVX + ACSF + water and OVX + Aβ1-42 + water (Figure 7). In the IL-1β dosage, there was a significant [F(4.17)=5.88, p<0.01] increase in IL-1β levels in the

frontal cortex in all experimental groups compared to the control group, and in the hippocampus showed a significant difference in IL- 1β [$F(4.20)=5.31$, $p<0.01$] with increased levels in Sham + Aβ1-42 + water, OVX + ACSF + water and OVX + Aβ1-42 + water groups (Figure 8). IL-4 levels in frontal cortex were significantly reduced [$F(4.20)=6.67$, $p<0.01$] in Sham + Aβ1-42 + water and OVX + ACSF + water groups. In hippocampus, there was a significant increase [$F(4.17)=7.57$, $p<0.01$] in cytokine levels in OVX + ACSF + water group, and reduction of levels in OVX + Aβ1-42 + water group (Figure 9). The effects on all cytokines were reversed by treatment with vitamin D + memantine + donepezil in the hippocampus resembling the control group results at these dosages.

Finally, to investigate the astrocytes activation in hippocampal sections of experimental groups, we analyzed the immunoreactivity of GFAP. We observed a significant increase on GFAP immunoreactivity in the hippocampi of Sham + Aβ1-42 + water ($p <0.01$), OVX + ACSF + water [$F(1.17)=3.693$, $p=0.0063$] and OVX + Aβ1-42 + water [$F(1.17)=2.537$, $p=0.0406$] groups, when compared to controls animals (Sham + ACSF + water). Notably, the combined therapy with memantine, donepezil and vitamin D attenuated the hippocampal astrogliosis in mice. The OVX + Aβ1-42 + vitamin D + memantine + donepezil groups displayed a reduction in hippocampi GFAP immunoreactivity, when compared to Sham + Aβ1-42 + water [$F(1.17)=5.644$, $p=0.002$], OVX + ACSF + water [$F(1.17)=5.532$, $p=0.0003$] and OVX + Aβ1-42 + water [$F(1.17)=4.603$, $p=0.0014$] groups (Figure 10).

4 Discussion

The hormonal senescence of menopause is a natural part of the aging process. Sometimes this decrease in E2 levels results in physiological changes in the brain (Koebele and Bimonte-Nelson, 2016), especially in the hippocampal region, resulting in loss of synaptic spines and neurodegeneration (Nebel et al, 2018), as well as the conversion of a metabolically active and healthy state to a state of low metabolic and oxidative activity, with an imbalance in amyloid clearance capacity (Zhao, Woody and Chhibber, 2015). This suggests that the once beneficial effects of estrogen are due to a greater predisposition to dementia, such as AD (Bove et al., 2014, Pike, 2016).

These findings corroborate with the present study where animals submitted to OVX and, consequently, with lower levels of E2 presented impairment in short-term memory, an increase in proinflammatory cytokines, a reduction of neurotrophin and antiinflammatory cytokine. In addition, when OVX was associated with administration of the Aβ1-42 peptide, animals had a noticeable increase in IL-1β cytokine (proinflammatory) in the cortex and potentiation of long-term memory damage.

In this study, the evaluation of animals' behavioral parameters and learning capacity demonstrated that Aβ1-42 peptide administration could induce long-term and short-term spatial memory damage and memory of habituation in the open field. Considering that cognitive impairment and spatial memory decline are clinical symptoms of AD and that learning and spatial memory damages have been reported in previous studies with this model (Budni et al, 2017, Garcez et al, 2018), the validity of the dementia model in inducing pathologic-like damage is confirmed.

In general, associative memory tasks may be beneficial in the perception of impairment due to AD, since they are dependent on the integrity of the hippocampus, one of the central regions affected in this pathology (Sperling et al, 2010), and which plays a critical role in spatial orientation and navigation (Lee et al, 2014). Since short-term memory is related more to areas of the prefrontal cortex, whereas long-term memory is more closely related to the hippocampal region (Lee et al, 2014; Djiogue et al, 2018), the results related to memory and behavioral tests can be justified, at least partially, by the fact that vitamin D has a more remarkable performance in the hippocampus (Brown et al., 2003; Dursun, Gezen-Ak and Yilmazer, 2011; Annweiler and Beauchet, 2012), since the associated treatment of memantine + donepezil + vitamin D was effective in the radial maze (long-term memory), but had no beneficial effects on the Y-maze (short-term memory).

The hippocampus is a critical brain region related to memory and learning functions (Şahin et al, 2019), as well as contextual and spatial awareness (Barrientos et al, 2016; Deuker et al, 2016), and is responsible for forming new associations between elements of previously unrelated information, contributing to episodic coding (Sperling et al, 2010, Knierim, 2015, Barrientos et al, 2016, Deuker et al, 2016). The hippocampal complex is one of the first regions affected during AD pathology and its connectivity is altered and is an essential parameter in preclinical AD (Harrison et al, 2015). As for menopause, E2 has a remarkable performance in the hippocampus since it exerts its cerebral effects through ERα and ERβ receptors located in the dendritic spines, dendrites, axons, and nucleus of hippocampal neurons (Djiogue et al, 2018). Its relation with vitamin D stands out for the beneficial effects that this substance exerts in this region, such as: reduction of oxidative stress in hippocampal neurons (Dursun, Gezen-Ak and Yilmazer, 2011; Annweiler and Beauchet, 2012), reconstruction of functional neural networks, increased synthesis of neurotrophic agents and acceleration of neuron growth (Brown et al, 2003), attenuation of Aβ1-42 peptide accumulation and reversal of local inflammatory conditions (Nissou et al 2014) and adjustment in the expression of inflammatory cytokines (Landel et al, 2016). Therefore, an impact on the inflammatory process related to the use of vitamin D should not be considered unexpected (Landel et al, 2016).

In this study, it was observed that concerning the expression of inflammatory cytokines, there was an increase in the levels of TNFα in the hippocampus and IL-1β in the hippocampus and in the frontal cortex of animals submitted to OVX and/or administration of Aβ1-42 peptide, which reinforces the thesis that both, E2 deprivation and AD, present a predisposing profile to an inflammatory environment. Previous studies have demonstrated the relationship between TNFα levels and AD by using antiinflammatory strategies in transgenic mice, resulting in a reduction of pathology related to Aβ1-42 peptide and tau protein and restoring memory deficits after regulating TNFα synthesis (Gabbita et al, 2015) or the performance of its receptor (Detrait et al, 2014). This correlation can be reinforced by observing data from this study about memory damage and proinflammatory cytokine dosage, where the groups of animals that showed an increase of TNFα and IL-1β also showed short and long memory damage duration and habituation in the open field. In addition, the amplified response of cytokines in the hippocampus can be justified by the high expression of IL-1β receptors in neurons and glia in the granular cells of the gyrus

and the pyramidal cell layer of the hippocampus and by the particularly dense microglial population in this region (Barrientos et al, 2016).

However, the associated treatment was able to reverse the increase of cytokines TNFα and IL-1β significantly in animals submitted to OVX associated with AD. Thus, reinforcing the antiinflammatory competence of vitamin D, already reported in previous studies where the effects of this vitamin were able to reduce inflammatory parameters in the hypothalamus (Farhangi et al, 2017), and to reverse the levels of inflammatory cytokines due to the accumulation of Aβ1-42 peptide (Mizwicki et al, 2013).

Cytokines are considered essential mediators in the neuroinflammation process and, although classified as pro and antiinflammatory, their balance is that it stands out as fundamental in the effective response to injuries in the central nervous system (Uchoa, Moser and Pike, 2016). Thus, analysis of IL-4 levels, an antiinflammatory cytokine, was also performed in this study, and it was observed that IL-4 levels were decreased in groups submitted to AD or OVX (isolated) in the frontal cortex and groups exposed to OVX and administration of the Aβ1-42 peptide concomitantly in the hippocampus. Previous studies have shown similar results by reducing IL-4 levels after estrogen deprivation (Yasui et al, 2007; Ma et al, 2007) and AD (Tai et al, 2015; Budni et al, 2017; Garcez et al, 2017). However, the triple treatment of association with vitamin D restored levels of this cytokine in the hippocampus in animals submitted to OVX plus AD, corroborating with previously highlighted antiinflammatory activity (Annweiler, 2016). However, in this study, it cannot be said that this effect was exclusively due to vitamin D administration since no group with exclusive administration of vitamin D or memantine and donepezil were performed.

Furthermore, in this study, the levels of BDNF in the hippocampus were evaluated, and it was demonstrated that both OVX and Aβ1-42 administrations were effective in reducing the levels of this neurotrophin. These results support the theory that decreasing E2 levels, as well as AD, lead to a reduction in neurotrophin levels, including BDNF (Peng et al, 2005; Wu et al, 2015; Zhao, Woody e Chhibber, 2015; Czyzyk et al, 2017). However, treatment with the triple combination was not able to reverse this effect.

It is worth highlighting that the associated treatment of vitamin D, memantine, and donepezil demonstrated positive results since it was able to reverse damages of long-term spatial memory and memory of habituation in the open field and reestablishing the levels of antiinflammatory and proinflammatory cytokines. Indeed, neuroinflammation is an important feature of AD, closely related to neuronal damage and cognitive impairments development in the disease. In this process, activated astrocytes release mediators that contribute and amplify the immune system recruitment and activation (Guzman-Martinez et al., 2019). An essential point of this study is that we observed that combined therapy attenuate astrogliosis in hippocampi of female mice exposed to both Aβ and OVX.

This fact is considerably important since there is currently no cure for AD, and current classical treatments, in addition to having transient effects, are limited in relation to cognition enhancements (Annweiler, 2011; Mielke et al, 2012). Therefore, the association of classical drugs with a previously known and approved substance, such as vitamin D, would facilitate the insertion of a combined

treatment as part of a "multiple drug" regime aimed at different targets, with the capacity to improve already known damages caused by DA.

In addition, it has previously been shown that levels of the VDR receptor in the brain in the presence of the Aβ1-42 peptide have been decreased, even in patients with sufficient levels of vitamin D, indicating that supplementation may be necessary even in patients without hypovitaminosis seeking to stimulate and regulate the mechanisms mediated by vitamin D in the brain, impaired by the pathophysiology of AD (Dursun, Gezen-Ak and Yilmazer, 2011). Thus, the relevance of associating vitamin D with classic drugs present in the therapy of these patients is exposed.

Finally, there is evidence linking female sex to an impaired response to treatment with classic drugs for AD, indicating a slower progression of clinical improvement with the use of cholinesterase inhibitors in women, especially those with the presence of the ApoE4 allele, unlike males, in which clinical progression was faster (Zhao, Woody e Chhibber, 2015), thus justifying the need for continuous studies in search of solutions with a specific action in this target audience. Therefore, according to this study, it is observed that the association of vitamin D with memantine and donepezil may be an important future therapeutic strategy for AD, especially in women.

5 Conclusion

This study is the first to evaluate this associated treatment in a double model, DA, and OVX. The results first demonstrated that the OVX induction model associated with Aβ1-42 peptide administration was effective in estrogen deprivation and induction of memory damage in female mice. On the other hand, our findings pointed out that treatment increased serum levels of vitamin D, reverse long-term spatial memory damage, and habituation memory in the open field, at least in part by modulating the levels of cytokines and decreasing astrogliosis.

Declarations

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References

1. Afzal, S., Bojesen, S. E., & Nordestgaard, B. G. (2014). Reduced 25-hydroxyvitamin D and risk of Alzheimer's disease and vascular dementia. Alzheimer's and Dementia. doi:10.1016/j.jalz.2013.05.1765

2. Alzheimer's, a. (2018). Alzheimer's Disease fact and figures.
3. Annweiler, C. (2016). Vitamin D in dementia prevention. Annals of the New York Academy of Sciences. doi:10.1111/nyas.13058
4. Annweiler, C., & Beauchet, O. (2012). Possibility of a new anti-alzheimers disease pharmaceutical composition combining memantine and vitamin d. Drugs and Aging. doi:10.2165/11597550-000000000-00000
5. Annweiler, C., Dursun, E., Féron, F., Gezen-Ak, D., Kalueff, A. V., Littlejohns, T.,.. . Beauchet, O. (2015). 'Vitamin D and cognition in older adults': Updated international recommendations. Journal of Internal Medicine. doi:10.1111/joim.12279
6. Annweiler, C., Fantino, B., Parot-Schinkel, E., Thiery, S., Gautier, J., & Beauchet, O. (2011). Alzheimer's disease - input of vitamin D with mEmantine assay (AD-IDEA trial): Study protocol for a randomized controlled trial. Trials. doi:10.1186/1745-6215-12-230
7. Balin, B. J., & Hudson, A. P. (2014). Etiology and pathogenesis of late-onset Alzheimer's disease. Current Allergy and Asthma Reports. doi:10.1007/s11882-013-0417-1
8. Barrientos, R. M., Kitt, M. M., Watkins, L. R., & Maier, S. F. (2015). Neuroinflammation in the normal aging hippocampus. Neuroscience. doi:10.1016/j.neuroscience.2015.03.007
9. Bermejo-Pareja, F., Llamas-Velasco, S., & Villarejo-Galende, A. (2016). Prevención de la enfermedad de Alzheimer: un camino a seguir. Revista Clinica Espanola. doi:10.1016/j.rce.2016.05.010
10. Bove, R., Secor, E., Chibnik, L. B., Barnes, L. L., Schneider, J. A., Bennett, D. A., & De Jager, P. L. (2014). Age at surgical menopause influences cognitive decline and Alzheimer pathology in older women. Neurology. doi:10.1212/WNL.0000000000000033
11. Briones, T. L., & Darwish, H. (2012). Vitamin D mitigates age-related cognitive decline through the modulation of pro-inflammatory state and decrease in amyloid burden. Journal of Neuroinflammation. doi:10.1186/1742-2094-9-244
12. Brown, J., Bianco, J. I., McGrath, J. J., & Eyles, D. W. (2003). 1,25-Dihydroxyvitamin D 3 induces nerve growth factor, promotes neurite outgrowth and inhibits mitosis in embryonic rat hippocampal neurons. Neuroscience Letters. doi:10.1016/S0304-3940(03)00303-3
13. Budni, J., Feijó, D. P., Batista-Silva, H., Garcez, M. L., Mina, F., Belletini-Santos, T., Quevedo, J. (2017). Lithium and memantine improve spatial memory impairment and neuroinflammation induced by β-amyloid 1-42 oligomers in rats. Neurobiology of Learning and Memory. doi:10.1016/j.nlm.2017.03.017
14. Cai, Z. L., Wang, C. Y., Gu, X. Y., Wang, N. J., Wang, J. J., Liu, W. X.,.. . Li, C. H. (2013). Tenuigenin ameliorates learning and memory impairments induced by ovariectomy. Physiology and Behavior. doi:10.1016/j.physbeh.2013.05.025
15. Cai, Z., Hussain, M. D., & Yan, L. J. (2014). Microglia, neuroinflammation, and beta-amyloid protein in Alzheimer's disease. nternational Journal of Neuroscience. doi:10.3109/00207454.2013.833510
16. Crous-Bou, M., Minguillón, C., Gramunt, N., & Molinuevo, J. L. (2017). Alzheimer's disease prevention: From risk factors to early intervention. Alzheimer's Research and Therapy. doi:10.1186/s13195-017-

0297-z

17. Czyzyk, A., Filipowicz, D., Podfigurna, A., Ptas, P., Piestrzynska, M., Smolarczyk, R.,.. . Meczekalski, B. (2017). Brain-derived neurotrophic factor (BDNF) plasma concentration in patients diagnosed with premature ovarian insufficiency (POI). Gynecological Endocrinology. doi:10.1080/09513590.2017.1290073
18. Davey, D. A. (2017). Prevention of Alzheimer's disease, cerebrovascular disease and dementia in women: the case for menopause hormone therapy. Neurodegenerative Disease Management. doi:10.2217/nmt-2016-0044
19. de Oliveira J, Moreira EL, dos Santos DB, Piermartiri TC, Dutra RC, Pinton S, Tasca CI, Farina M, Prediger RD, de Bem AF. Increased susceptibility to amyloid-β-induced neurotoxicity in mice lacking the low-density lipoprotein receptor. J Alzheimers Dis. 2014;41(1):43–60. doi: 10.3233/JAD-132228.
20. DELLU, F. et al. A two-trial memory task with automated recording: study in young and aged rats. Brain Res, v. 588, n. 1, p. 132-9, 14 ago. 1992.
21. Detrait, E. R., Danis, B., Lamberty, Y., & Foerch, P. (2014). Peripheral administration of an anti-TNF-α receptor fusion protein counteracts the amyloid induced elevation of hippocampal TNF-α levels and memory deficits in mice. Neurochemistry International. doi:10.1016/j.neuint.2014.04.001
22. Deuker, L., Bellmund, J. L., Navarro Schröder, T., & Doeller, C. F. (2016). An event map of memory space in the hippocampus. eLife. doi:10.7554/eLife.16534
23. Djiogue, S., Djiyou Djeuda, A. B., Seke Etet, P. F., Ketcha Wanda, G. J. M., Djikem Tadah, R. N., & Njamen, D. (2018). Memory and exploratory behavior impairment in ovariectomized Wistar rats. Behavioral and Brain Functions. doi:10.1186/s12993-018-0146-7
24. Durk, M. R., Han, K., Chow, E. C. Y., Ahrens, R., Henderson, J. T., Fraser, P. E., & Pang, K. S. (2014). 1,25-Dihydroxyvitamin D3 Reduces Cerebral Amyloid- Accumulation and Improves Cognition in Mouse Models of Alzheimer's Disease. Journal of Neuroscience. doi:10.1523/jneurosci.2711-13.2014
25. Dursun, E., Gezen-Ak, D., & Yilmazer, S. (2011). A novel perspective for Alzheimer's disease: Vitamin D receptor suppression by amyloid-β and preventing the amyloid-β induced alterations by vitamin D in cortical neurons. Journal of Alzheimer's Disease. doi:10.3233/JAD-2010-101377
26. Farhangi, M. A., Mesgari-Abbasi, M., Nameni, G., Hajiluian, G., & Shahabi, P. (2017). The effects of vitamin D administration on brain inflammatory markers in high fat diet induced obese rats. BMC Neuroscience. doi:10.1186/s12868-017-0400-1
27. Fatemi, I., Heydari, S., Kaeidi, A., Shamsizadeh, A., Hakimizadeh, E., Khaluoi, A., & Allahtavakoli, M. (2018). Metformin ameliorates the age-related changes of d-galactose administration in ovariectomized mice. Fundamental and Clinical Pharmacology. doi:10.1111/fcp.12364
28. Foyet, H.S., Hritcu, L., Ciobica, A., Stefan, M., Kamtchouing, P., Cojocaru, D., 2011. Methanolic extract of Hibiscus asper leaves improves spatial memory deficits in the 6-hydroxydopamine-lesion rodent model of Parkinson's disease. J Ethnopharmacol 133(2), 773–779.
29. Garcez, M. L., Mina, F., Bellettini-Santos, T., Carneiro, F. G., Luz, A. P., Schiavo, G. L.,.. . Budni, J. (2017). Minocycline reduces inflammatory parameters in the brain structures and serum and reverses

memory impairment caused by the administration of amyloid β (1-42) in mice. Progress in Neuro-Psychopharmacology and Biological Psychiatry. doi:10.1016/j.pnpbp.2017.03.010

30. Garcez, M. L., Mina, F., Bellettini-Santos, T., da Luz, A. P., Schiavo, G. L., Macieski, J. M. C.,.. . Budni, J. (2019). The Involvement of NLRP3 on the Effects of Minocycline in an AD-Like Pathology Induced by β-Amyloid Oligomers Administered to Mice. Molecular Neurobiology. doi:10.1007/s12035-018-1211-9
31. Graham, W. V., Bonito-Oliva, A., & Sakmar, T. P. (2017). Update on Alzheimer's Disease Therapy and Prevention Strategies. Annual Review of Medicine. doi:10.1146/annurev-med-042915-103753
32. Guzman-Martinez L, Maccioni RB, Andrade V, Navarrete LP, Pastor MG1, Ramos-Escobar N. Neuroinflammation as a Common Feature of Neurodegenerative Disorders. Front Pharmacol. 2019 Sep 12;10:1008. doi: 10.3389/fphar.2019.01008. eCollection 2019.
33. Hamdan, A. C., & Ramos, A. A. (2016). Avaliação Neuropsicológica na Doença Alzheimer e Demência Frontotemporal: critérios nosológicos. Interação em Psicologia. doi:10.5380/psi.v18i3.47039
34. Hansberg-Pastor, V., González-Arenas, A., Piña-Medina, A. G., & Camacho-Arroyo, I. (2015). Sex hormones regulate cytoskeletal proteins involved in brain plasticity. Frontiers in Psychiatry. doi:10.3389/fpsyt.2015.00165
35. Harrison, T. M., Burggren, A. C., Small, G. W., & Bookheimer, S. Y. (2016). Altered memory-related functional connectivity of the anterior and posterior hippocampus in older adults at increased genetic risk for Alzheimer's disease. Human Brain Mapping. doi:10.1002/hbm.23036
36. Heppner, F. L., Ransohoff, R. M., & Becher, B. (2015). Immune attack: The role of inflammation in Alzheimer disease. Nature Reviews Neuroscience. doi:10.1038/nrn3880
37. Hernando-Requejo, V. (2016). Nutrición y deterioro cognitivo. Nutricion Hospitalaria. doi:10.20960/nh.346
38. Hritcu, L., Cioanca, O., Hancianu, M., 2012. Effects of lavender oil inhalation on improving scopolamine-induced spatial memory impairment in laboratory rats. Phytomedicine 19(6), 529–534.
39. Hung, S. Y., & Fu, W. M. (2017). Drug candidates in clinical trials for Alzheimer's disease. Journal of Biomedical Science. doi:10.1186/s12929-017-0355-7
40. Imtiaz, B., Tuppurainen, M., Tiihonen, M., Kivipelto, M., Soininen, H., Hartikainen, S., & Tolppanen, A. M. (2014). Oophorectomy, hysterectomy, and risk of Alzheimer's disease: A nationwide case-control study. Journal of Alzheimer's Disease. doi:10.3233/JAD-140336
41. Kim, S. H., Kandiah, N., Hsu, J. L., Suthisisang, C., Udommongkol, C., & Dash, A. (2017). Beyond symptomatic effects: potential of donepezil as a neuroprotective agent and disease modifier in Alzheimer's disease. British Journal of Pharmacology. doi:10.1111/bph.14030
42. Knierim, J. J. (2015). The hippocampus. Current Biology. doi:10.1016/j.cub.2015.10.049
43. Koebele, S. V., & Bimonte-Nelson, H. A. (2016). Modeling menopause: The utility of rodents in translational behavioral endocrinology research. Maturitas. doi:10.1016/j.maturitas.2016.01.015

44. Landel, V., Millet, P., Baranger, K., Loriod, B., & Féron, F. (2016). Vitamin D interacts with Esr1 and Igf1 to regulate molecular pathways relevant to Alzheimer's disease. Molecular Neurodegeneration. doi:10.1186/s13024-016-0087-2
45. Lee, J. Y., Kho, S., Yoo, H. B., Park, S., Choi, J. S., Kwon, J. S., Jung, H. Y. (2014). Spatial memory impairments in amnestic mild cognitive impairment in a virtual radial arm maze. Neuropsychiatric Disease and Treatment. doi:10.2147/ndt.s58185
46. Lobo, R. A., Davis, S. R., De Villiers, T. J., Gompel, A., Henderson, V. W., Hodis, H. N.,.. . Baber, R. J. (2014). Prevention of diseases after menopause. Climacteric. doi:10.3109/13697137.2014.933411
47. Ma, L. J., Guzmán, E. A., DeGuzman, A., Muller, H. K., Walker, A. M., & Owen, L. B. (2007). Local cytokine levels associated with delayed-type hypersensitivity responses: Modulation by gender, ovariectomy, and estrogen replacement. Journal of Endocrinology. doi:10.1677/JOE-06-0024
48. Matsuzono, K., Hishikawa, N., Ohta, Y., Yamashita, T., Deguchi, K., Nakano, Y., & Abe, K. (2015). Combination Therapy of Cholinesterase Inhibitor (Donepezil or Galantamine) plus Memantine in the Okayama Memantine Study. Journal of Alzheimer's Disease. doi:10.3233/JAD-143084
49. Mielke, M. M., Leoutsakos, J. M., Corcoran, C. D., Green, R. C., Norton, M. C., Welsh-Bohmer, K. A.,.. . Lyketsos, C. G. (2012). Effects of Food and Drug Administration-approved medications for Alzheimer's disease on clinical progression. Alzheimer's and Dementia. doi:10.1016/j.jalz.2011.02.011
50. Mizwicki, M. T., Liu, G., Fiala, M., Magpantay, L., Sayre, J., Siani, A., Teplow, D. B. (2013). 1α,25-dihydroxyvitamin D 3 and resolvin D1 retune the balance between amyloid-β phagocytosis and inflammation in Alzheimer's disease patients. Journal of Alzheimer's Disease. doi:10.3233/JAD-121735
51. Morley, J. E. (2014). Does vitamin D modulate cognition? Nature Reviews Neurology. doi:10.1038/nrneurol.2014.193
52. Mpandzou, G., Aït Ben Haddou, E., Regragui, W., Benomar, A., & Yahyaoui, M. (2016). Vitamin D deficiency and its role in neurological conditions: A review. Revue Neurologique. doi:10.1016/j.neurol.2015.11.005
53. Nebel, R. A., Aggarwal, N. T., Barnes, L. L., Gallagher, A., Goldstein, J. M., Kantarci, K.,.. . Mielke, M. M. (2018). Understanding the impact of sex and gender in Alzheimer's disease: A call to action. Alzheimer's and Dementia. doi:10.1016/j.jalz.2018.04.008
54. Nissou, M. F., Guttin, A., Zenga, C., Berger, F., Issartel, J. P., & Wion, D. (2014). Additional clues for a protective role of vitamin D in neurodegenerative diseases: 1,25-dihydroxyvitamin D3 triggers an anti-inflammatory response in brain pericytes. Journal of Alzheimer's Disease. doi:10.3233/JAD-140411
55. Peng, S., Wuu, J., Mufson, E. J., & Fahnestock, M. (2005). Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. Journal of Neurochemistry. doi:10.1111/j.1471-4159.2005.03135.x
56. Pike, C. J. (2017). Sex and the development of Alzheimer's disease. Journal of Neuroscience Research. doi:10.1002/jnr.23827

57. Prasad Gabbita, S., Johnson, M. F., Kobritz, N., Eslami, P., Poteshkina, A., Varadarajan, S.,.. . Harris-White, M. E. (2015). Oral TNFα modulation alters neutrophil infiltration, improves cognition and diminishes tau and amyloid pathology in the 3xtgad mouse model. PLoS ONE. doi:10.1371/journal.pone.0137305
58. Reitz, C., & Mayeux, R. (2014). Alzheimer disease: Epidemiology, diagnostic criteria, risk factors and biomarkers. Biochemical Pharmacology. doi:10.1016/j.bcp.2013.12.024
59. Reitz, C., Brayne, C., & Mayeux, R. (2011). Epidemiology of Alzheimer disease. Nature Reviews Neurology. doi:10.1038/nrneurol.2011.2
60. Resende, R., Ferreiro, E., Pereira, C., & Resende de Oliveira, C. (2008). Neurotoxic effect of oligomeric and fibrillar species of amyloid-beta peptide 1-42: Involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. Neuroscience. doi:10.1016/j.neuroscience.2008.06.036
61. Rigalli, A., & Loreto, V. E. (2009). Experimental Surgical Models in the Laboratory Rat. Boca Raton: Taylor & Francis Group.
62. Ruiz-Muñoz, A. M., Nieto-Escamez, F. A., Aznar, S., Colomina, M. T., & Sanchez-Santed, F. (2011). Cognitive and histological disturbances after chlorpyrifos exposure and chronic Aβ(1-42) infusions in Wistar rats. NeuroToxicology. doi:10.1016/j.neuro.2011.05.014
63. Şahin, S., Gürgen, S. G., Yazar, U., İnce, İ., Kamaşak, T., Acar Arslan, E., Cansu, A. (2019). Vitamin D protects against hippocampal apoptosis related with seizures induced by kainic acid and pentylenetetrazol in rats. Epilepsy Research. doi:10.1016/j.eplepsyres.2018.12.005
64. Shen, L., & Ji, H. F. (2015). Vitamin D deficiency is associated with increased risk of Alzheimer's disease and dementia: Evidence from meta-analysis. Nutrition Journal. doi:10.1186/s12937-015-0063-7
65. Siebert, C., Pierozan, P., Kolling, J., dos Santos, T. M., Sebotaio, M. C., Marques, E. P.,.. . Wyse, A. T. S. (2017). Vitamin D3 Reverses the Hippocampal Cytoskeleton Imbalance But Not Memory Deficits Caused by Ovariectomy in Adult Wistar Rats. NeuroMolecular Medicine. doi:10.1007/s12017-017-8449-7
66. Sperling, R. A., Dickerson, B. C., Pihlajamaki, M., Vannini, P., LaViolette, P. S., Vitolo, O. V.,.. . Johnson, K. A. (2010). Functional alterations in memory networks in early alzheimer's disease. NeuroMolecular Medicine. doi:10.1007/s12017-009-8109-7
67. Tai, L. M., Ghura, S., Koster, K. P., Liakaite, V., Maienschein-Cline, M., Kanabar, P.,.. . LaDu, M. J. (2015). APOE-modulated Aβ-induced neuroinflammation in Alzheimer's disease: Current landscape, novel data, and future perspective. Journal of Neurochemistry. doi:10.1111/jnc.13072
68. Takahashi, T. A., & Johnson, K. M. (2015). Menopause. Medical Clinics of North America, 99(3), 521–534. doi:10.1016/j.mcna.2015.01.006
69. Uchoa, M. F., Moser, V. A., & Pike, C. J. (2016). Interactions between inflammation, sex steroids, and Alzheimer's disease risk factors. Frontiers in Neuroendocrinology. doi:10.1016/j.yfrne.2016.09.001
70. Ueda, K., Fukui, Y., & Kageyama, H. (1994). Amyloid β protein-induced neuronal cell death: neurotoxic properties of aggregated amyloid β protein. Brain Research. doi:10.1016/0006-8993(94)91736-1

71. Vest, R. S., & Pike, C. J. (2013). Gender, sex steroid hormones, and Alzheimer's disease. Hormones and Behavior. doi:10.1016/j.yhbeh.2012.04.006
72. VIANNA, M. R.M.. Role of Hippocampal Signaling Pathways in Long-Term Memory Formation of a Nonassociative Learning Task in the Rat. Learning & Memory, [s.l.], v. 7, n. 5, p.333-340, 1 set. 2000.
73. Wu, Y. W. C., Du, X., Van Den Buuse, M., & Hill, R. A. (2015). Analyzing the influence of BDNF heterozygosity on spatial memory response to 17β-estradiol. Translational Psychiatry. doi:10.1038/tp.2014.143
74. Yasui, T., Maegawa, M., Tomita, J., Miyatani, Y., Yamada, M., Uemura, H., Irahara, M. (2007). Changes in serum cytokine concentrations during the menopausal transition. Maturitas. doi:10.1016/j.maturitas.2006.11.002
75. Zhao, L., Woody, S. K., & Chhibber, A. (2015). Estrogen receptor β in Alzheimer's disease: From mechanisms to therapeutics. Ageing Research Reviews. doi:10.1016/j.arr.2015.08.001

Figures

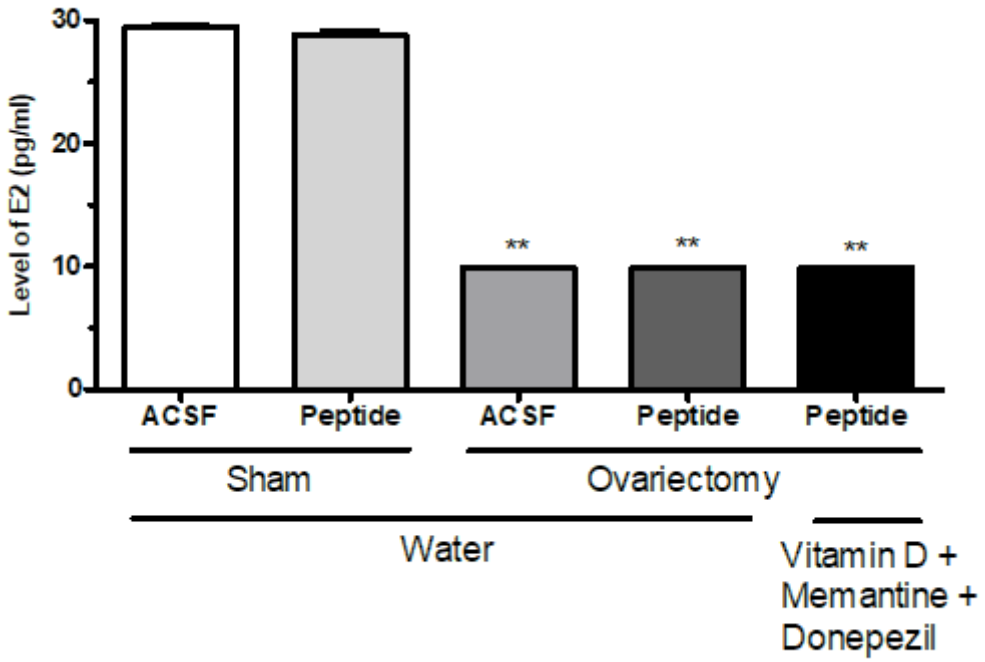

Figure 1

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on serum E2 levels of female mice exposed to Aβ and OVX. Data are expressed as mean ± S.E.M. (6 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF + Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p <0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Duncan's post-hoc test).

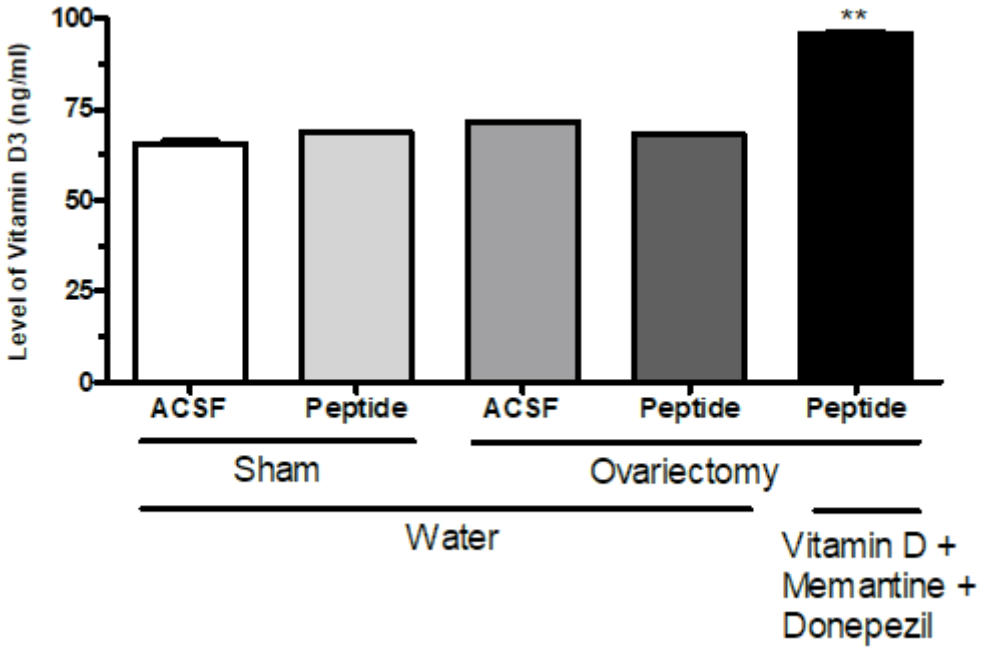

Figure 2

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on serum vitamin D levels of female mice exposed to Aβ and OVX. Data are expressed as mean ± S.E.M. (4-6 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF + Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p <0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Duncan's post-hoc test).

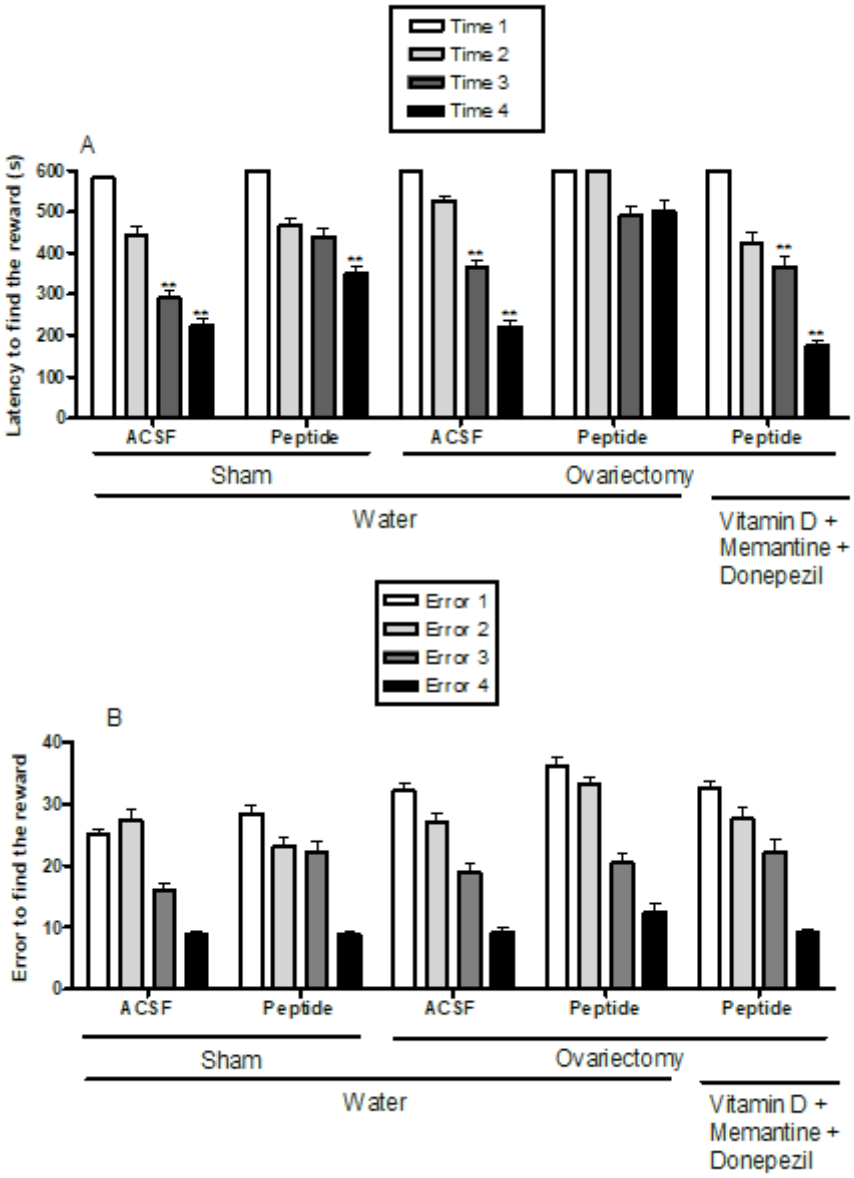

Figure 3

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on the radial maze test of female mice exposed to Aβ and OVX. (A) Time until the animals find the cereal or until 10min (B) Errors committed until the animals find the cereal or until 10min. Data are expressed as mean ± S.E.M. (8-11 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF + Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p <0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Duncan's post-hoc test).

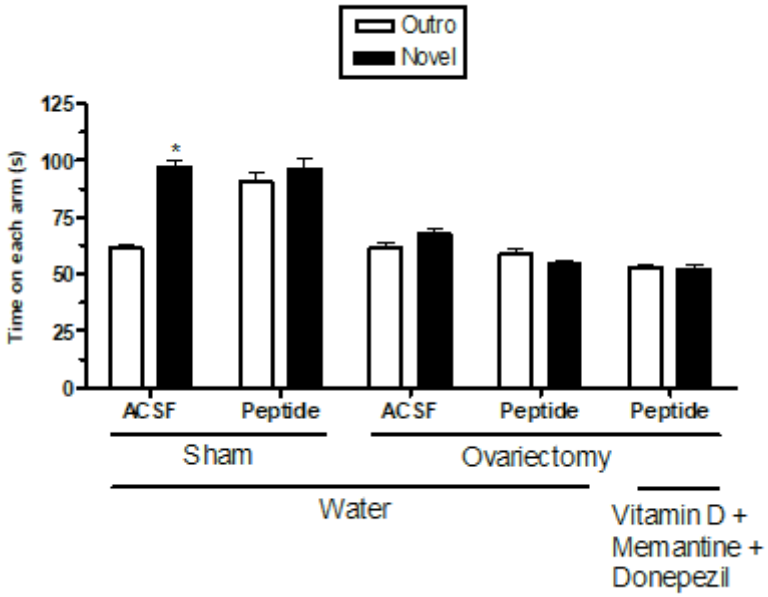

Figure 4

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on the Y-maze test of female mice exposed to Aβ and OVX. The figure shows the time explored in arms “other” and “novel”. Data are expressed as mean ± S.E.M. (9-13 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF + Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p <0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Duncan’s post-hoc test).

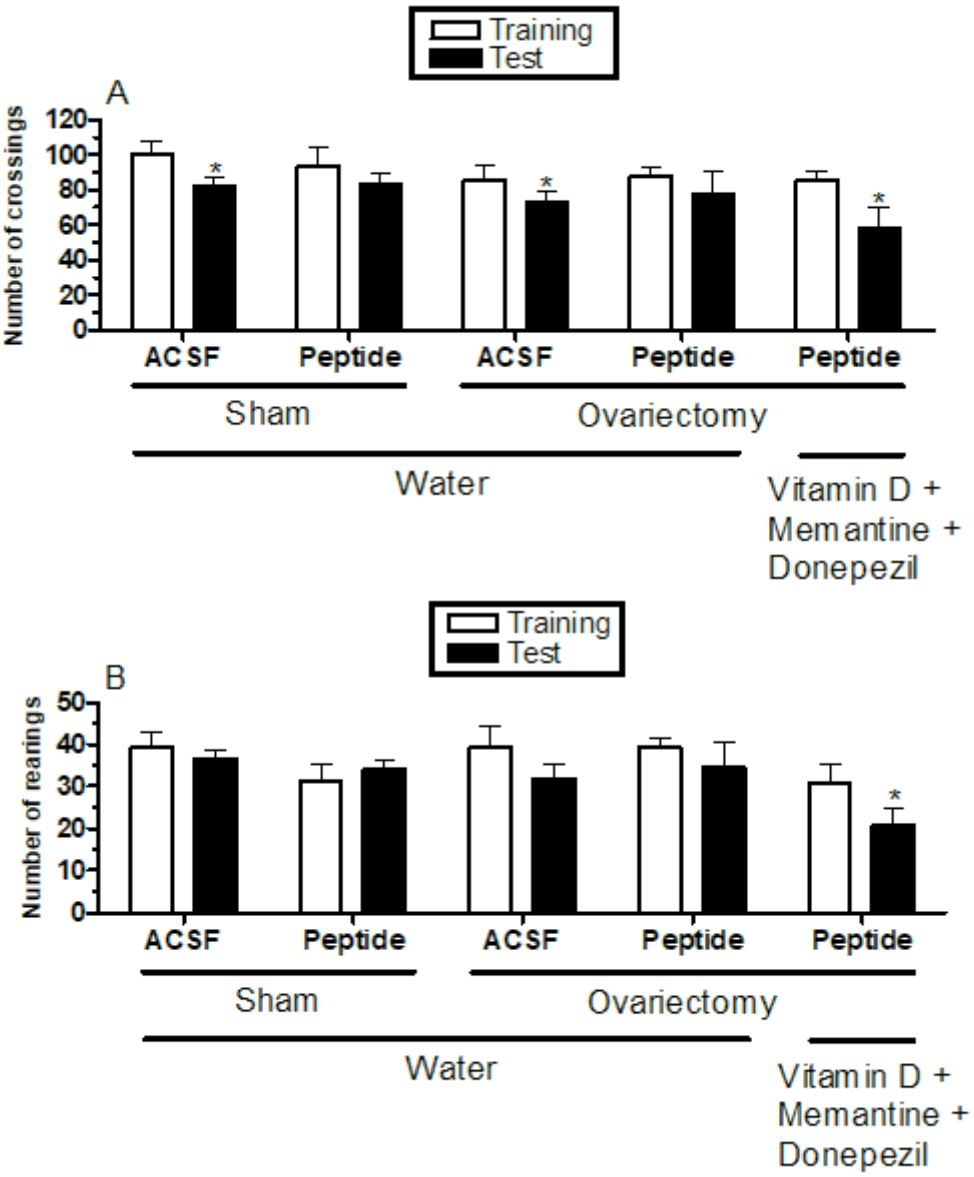

Figure 5

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on the habituation to open field test of female mice exposed to Aβ and OVX. (A) Crossings (B) Rearings. Data are expressed as mean ± S.E.M. (8-11 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF + Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p <0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Duncan's post-hoc test).

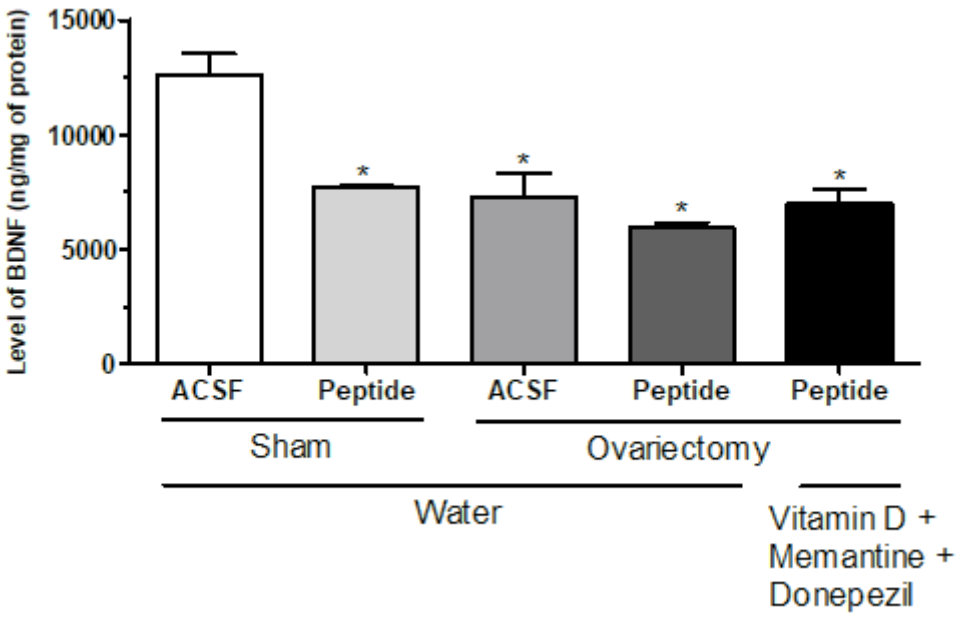

Figure 6

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on hippocampus BDNF levels of female mice exposed to Aβ and OVX. Data are expressed as mean ± S.E.M. (3 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF + Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p <0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Duncan's post-hoc test).

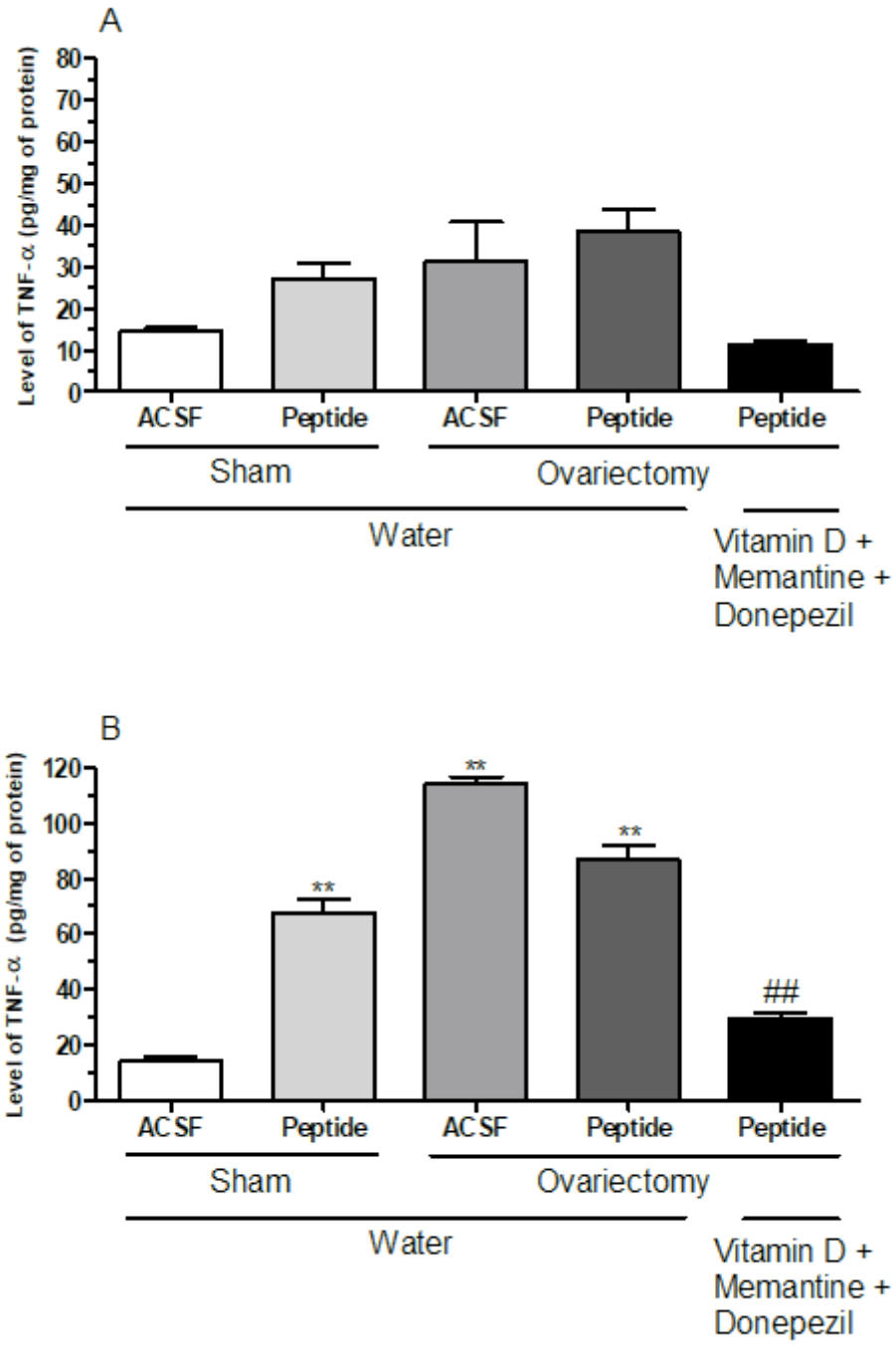

Figure 7

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on TNF-α levels of female mice exposed to Aβ and OVX. (A) TNF-α levels in frontal cortex of female mice exposed to Aβ and OVX and treated with vitamin D + memantine + donezepil. (B) TNF-α levels in hippocampus of female mice exposed to Aβ and OVX and treated with vitamin D + memantine + donezepil. Data are expressed as mean ± S.E.M. (3-5 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF + Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p

<0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Duncan's post-hoc test).

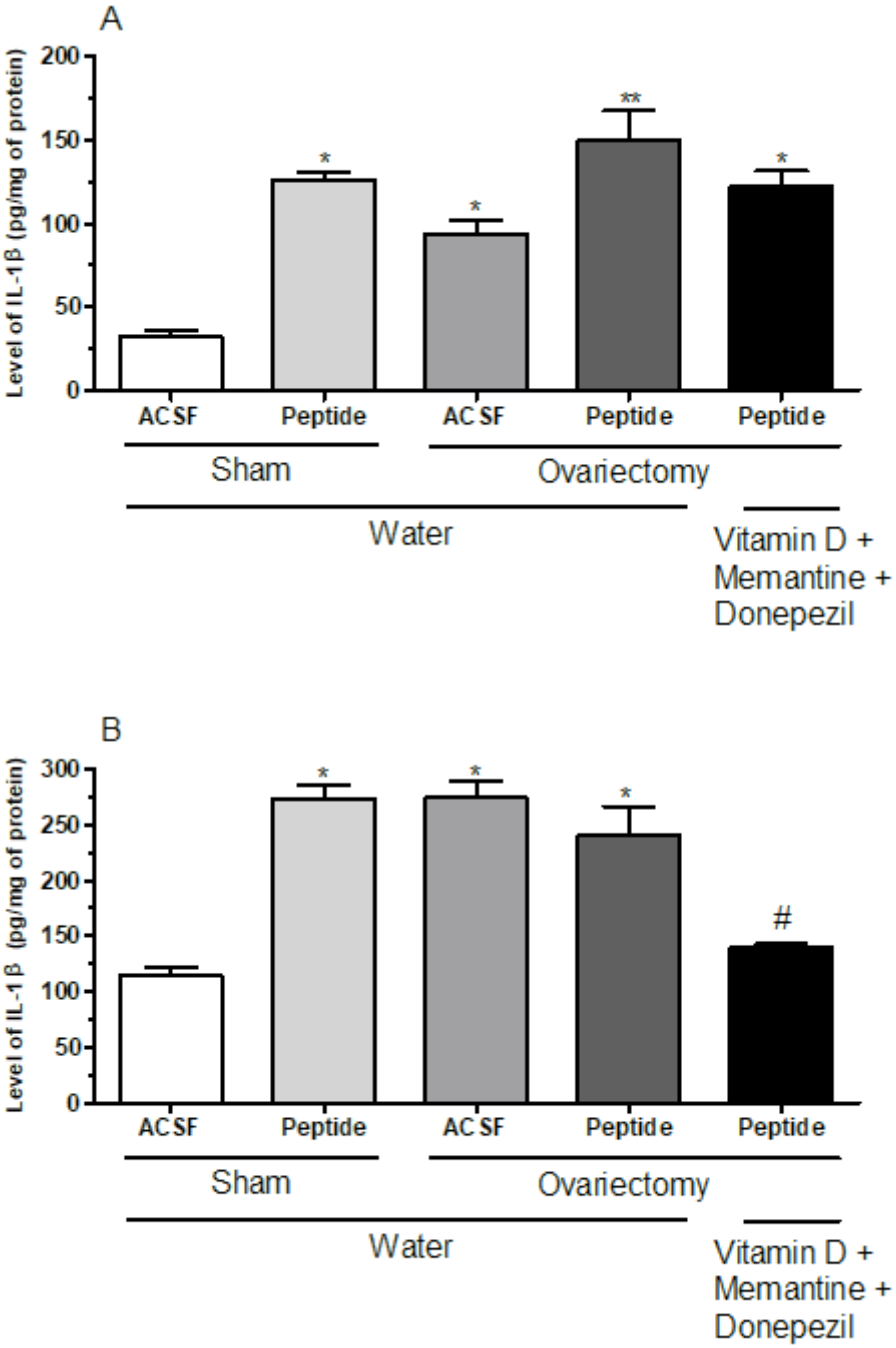

Figure 8

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on IL-1β levels of female mice exposed to Aβ and OVX. (A) IL-1β levels in frontal cortex of female mice exposed to Aβ and OVX and treated with vitamin D + memantine + donezepil. (B) IL-1β levels in hippocampus of female mice exposed to Aβ and OVX and treated with vitamin D + memantine + donezepil. Data are expressed as mean ± S.E.M. (3-5 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF

+ Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p <0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Duncan's post-hoc test).

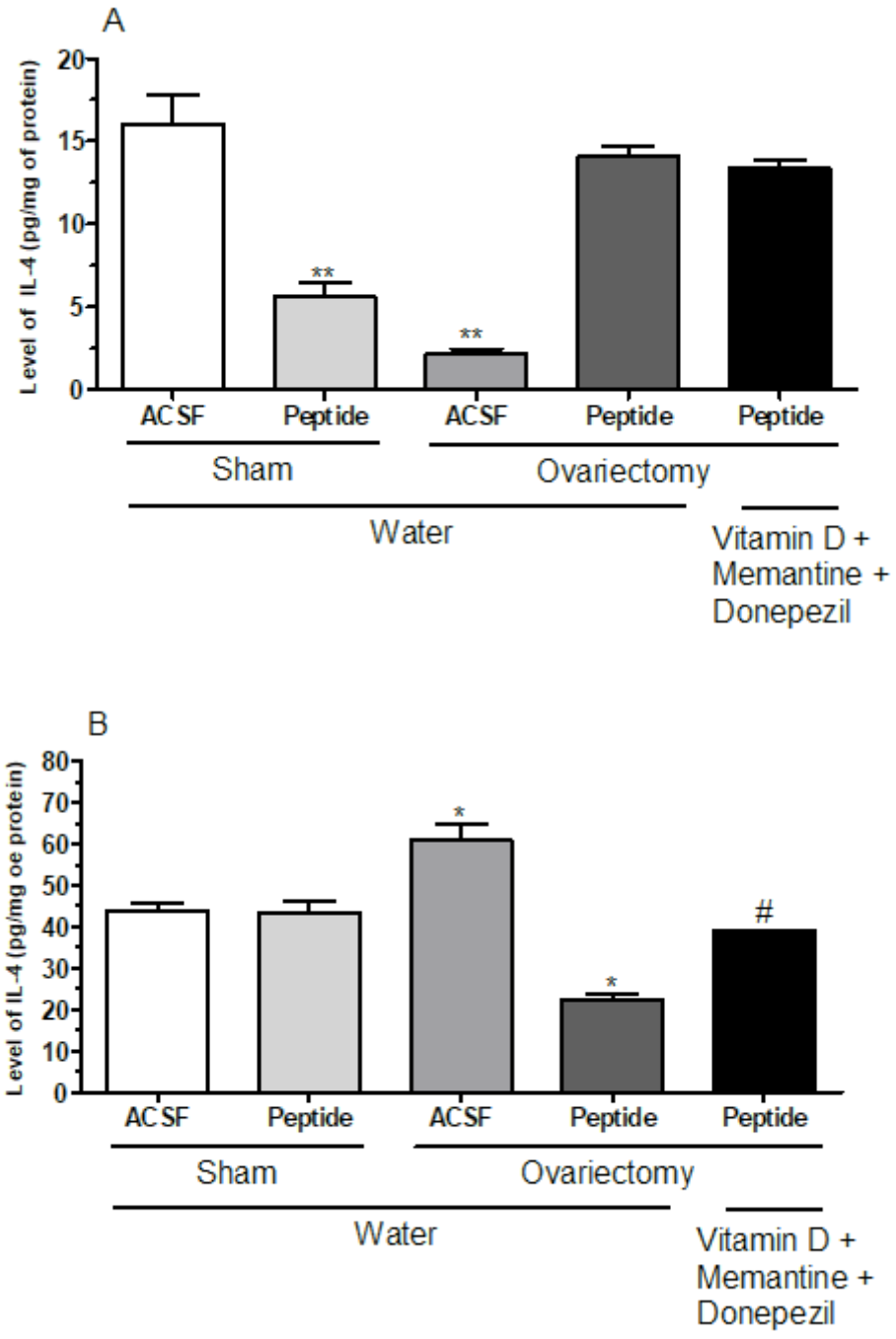

Figure 9

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on IL-4 levels of female mice exposed to Aβ and OVX. (A) IL-4 levels in frontal cortex of female mice exposed to Aβ and OVX and treated with vitamin D + memantine + donezepil. (B) IL-4 levels in hippocampus of

female mice exposed to Aβ and OVX and treated with vitamin D + memantine + donezepil. Data are expressed as mean ± S.E.M. (3-5 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF + Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p <0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Duncan's post-hoc test).

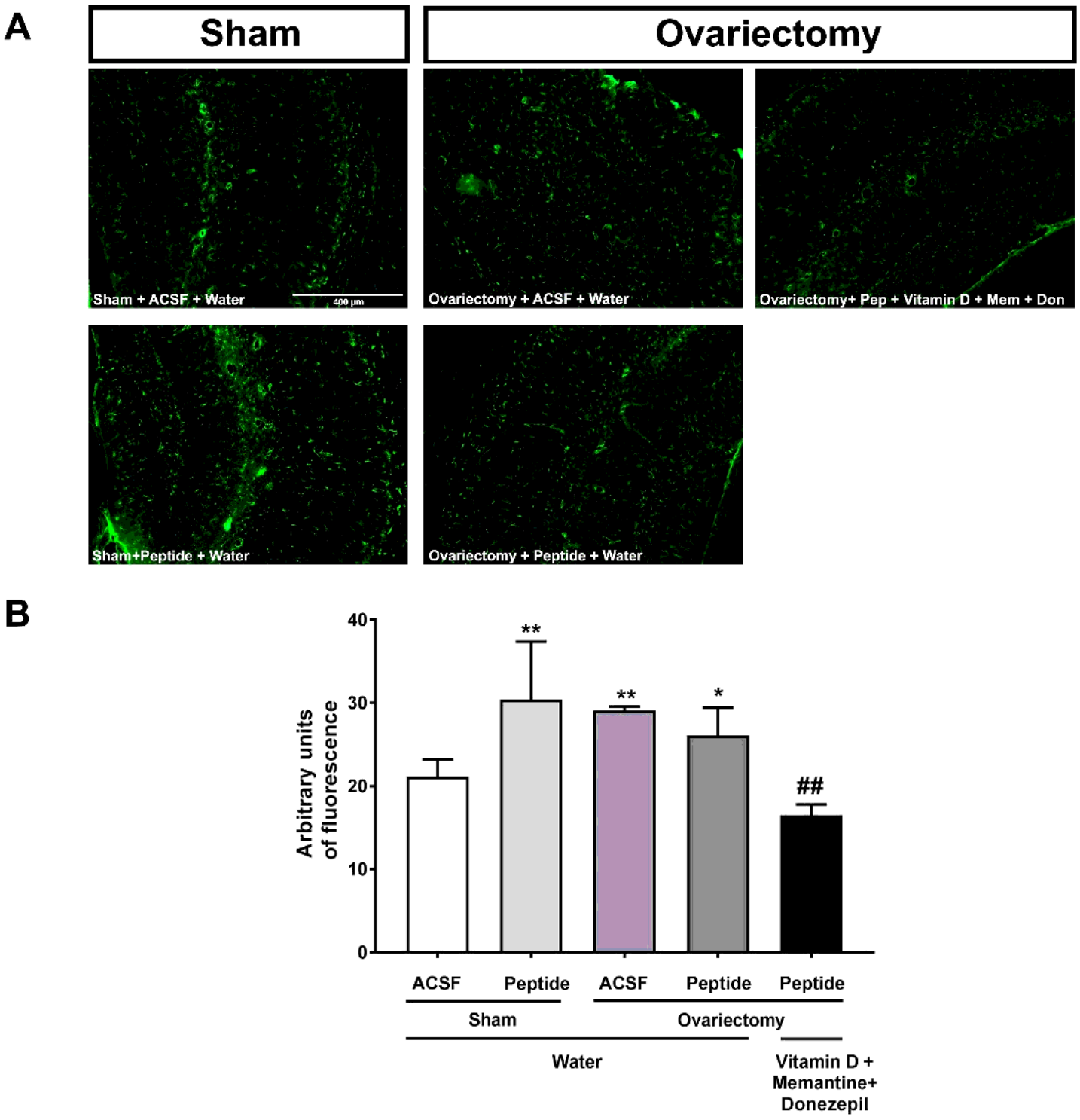

Figure 10

Effects of treatment with vitamin D (420 U/kg) + memantine (5 mg/kg) + donezepil (1 mg/kg) on astrogliosis in hippocampi of female mice exposed to Aβ and OVX. The images of immunofluorescence staining were obtained with a Microscopy EVOS ®FL Auto Imaging System. (A) Representative images of immunofluorescence assay for GFAP (astrocytes marker) in hippocampi slices of female mice exposed to Aβ and OVX and treated with vitamin D + memantine + donezepil. Scale bars, 400μm. (B) Quantification in arbitrary units of fluorescence for GFAP in total hippocampus in the different experimental groups. Data are expressed as mean ± S.E.M. (4-6 animals per group). *p <0.05 and **p <0.01 compared to Sham + ACSF + Water group, ## p <0.01 compared to Sham + Aβ1-42 + water (p <0.001), OVX + ACSF + water (p <0.001) and OVX + Aβ1-42 + water (p <0.01) groups (One-way ANOVA followed by Newman-Keuls post-hoc test).