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Original article

Ascorbic acid attenuates cognitive impairment and brain oxidative stress in ovariectomized mice

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ABSTRACT

Background: Menopause is associated with increased oxidative stress and memory impairment. Based on the antioxidant property of ascorbic acid (AA), It's effect on cognitive function, the serum level of the brain-derived neurotrophic factor (BDNF) and the activity of antioxidant enzymes within the brain in ovariectomized (OVX) mice was investigated.
Methods: AA (100, 300 and 500 mg/kg), was orally administrated per day in OVX mice for 30 days. Tactile learning and working memory were evaluated by the novel object recognition task and T-maze continuous alternation task, respectively. The levels of serum BDNF were measured and animals' brains were analyzed for the superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity.
Results: AA prevented from the deleterious effects of ovariectomy on learning memory (300 and 500 mg/kg) and working memory (100 and 500 mg/kg). The serum BDNF level was also increased in OVX animals treated with AA (100 and 500 mg/kg). Furthermore, AA (500 mg/kg) increased the SOD and GPx activity in the brain of OVX animals.
Conclusions: Collectively, the results of the present study suggest that AA might be an appropriate choice in loss or reduction of estradiol for the amelioration of cognitive impairment.

Introduction

Menopause is a part of women's life which influences their psychological, physiological or sociological status and impairs their quality of life [1]. The level of sex hormones such as estrogens and progesterone decrease in this period. One of the most common symptoms of menopause is cognitive dysfunction and memory disorders [2]. Estrogen effects are mediated through the formation of new synaptic connections in the central nervous system (CNS) [3]. Estrogen also increases the activity of the cholinergic system [4]. By inducing ovariectomy (OVX) learning and memory are affected due to estrogen deficiency [5]. Estrogen also increases the level of the brain-derived neurotrophic factor (BDNF) in some brain areas, especially hippocampus [6]. Estrogen has neuro-protective and neurotrophic properties and is essential for the preservation of learning and memory [7]. Previous studies have shown that the risk of Alzheimer and memory impairment increases following the low level of estrogen after menopause [1]. Oxidative stress occurs during menopause because of the progressive loss of estrogen and consequently attenuation of its protective roles as well as deficient endogenous antioxidant activity. It has been demonstrated that the antioxidant defense systems including superoxide dismutase (SOD) and glutathione peroxidase (GPx) as well as the level of other antioxidants such as ascorbic acid (AA) and vitamin E, are decreased during menopause [1]. The antioxidant effects of estrogen inhibit oxidative stress. Estrogen restricts cell death in the nervous system tissue by inhibition of elevated intracellular free Ca^{2+} which is an important element in the development of ischemic injury induced by reactive oxygen species (ROS) [8]. Estrogen inhibits neurodegeneration especially in the hippocampus *via* various mechanisms such as antioxidant effects [8] and increasing the expression of genes which have a role in BDNF and nerve growth factor (NGF) production [7]. This may explain how estrogen improves cognitive functions such as learning and memory [9].

AA or vitamin C is a water-soluble vitamin which has a high concentration in the brain tissue under normal conditions. In the recent studies, it has been demonstrated that low level of AA is correlated with cognitive impairments [10,11]. Also, it is well established that AA protects from memory disorders, especially

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during aging *via* inducing antioxidant effect [12]. Moreover, it has been shown that AA increases the level of antioxidants such as SOD and GPx as well as CAT activity [13,14]. Furthermore, AA is an essential cofactor for some enzymes in the brain such as ten-eleven translocase-2 which elevates the production of BDNF [15].

Regarding the destructive effects of menopause on cognitive function and the antioxidant defense system and on the other hand, the considerable antioxidant property of AA and its positive effects on CNS, the present study aimed to evaluate the effects of AA on learning and memory, activity of the antioxidant system and serum BDNF level in ovariectomized mice.

Materials and methods

Animals

The total number of 40 female NMRI mice weighing 20 ± 5 g (mean age: 4–6 weeks) were used in this study. Animals were kept at 4 per cage ($27 \times 22 \times 18$). All animals maintained under a 12 h/12 h dark/light (lights on 08:00–20:00) by the unlimited availability of food and water and temperature of 23 ± 2.0 °C. All experimental procedures were done according to the guidelines for the care and the European Communities Council Directive 22 September 2010 (2010/63/EU) (ethical code: IR.RUMS.REC.1394.104).

Ovariectomy procedure

Mice were kept under anesthetize by intraperitoneal (*ip*) administration of ketamine (90 mg/kg) and xylazine (4.5 mg/kg). A ventral incision between 2 and 3 nipples on each side was made and then ovaries were removed slowly by a cautery device and after closing the wound, 22,000 iu/kg penicillin was injected. The sham group underwent the same procedure without removal of the ovaries. The vaginal observed under the light microscope for five days and if the cornified epithelial cells did not observe in the vaginal smear, the castration was confirmed [16].

Experimental groups

Mice were randomly divided into five groups as follows: sham (surgery without removing ovaries), ovariectomized group (OVX), AA100, AA 300 and AA 500 (ovariectomized mice treated with AA 100, 300 and 500 mg/kg orally for 30 days, respectively). Six days after surgery and confirmation of castration the treatment was started. The dosages and route used for AA administration were selected based on previous studies [17,18].

Behavioral assessment

Novel object recognition task (NORT)

The NORT was used to assess the tactile learning and carried out in a $50 \times 50 \times 50$ cm (length × width × height) Plexiglas box equipped with a video-based Ethovision system (Nodulus, Wageningen, The Netherlands). In the familiar phase, mice were placed in the box to get familiar with the environment for 5 min. During the training phases, two identical objects were placed in the box, and the mouse was allowed to explore the box and the objects freely for 5 min. The test phase was carried out 4 h later. One of the familiar objects was replaced by a novel object. The exploration time of the novel and familiar objects were recorded. Exploration was considered when the nose of the mouse was oriented toward the objects (within 2 cm of the object). Results were expressed as discrimination ratio (the difference between exploration time of novel and familiar objects in the test phase divided by the total time spent exploring the objects in the test phase) [19].

T-maze continuous alternation task (T-CAT)

T-maze apparatus was used to evaluate the working memory, as it is one of the most wildly used technique in such memory evaluation [20]. Briefly, the animal was placed in the start box of T-maze and a forced trial was done with a goal arm blocked. Then, the blockade of the arm was opened. After that, the mice were tested for 13 choice trials and the spontaneous alternations were recorded. The highest latency to fill out all 13 trials is 900 s. Working memory index was calculated according to the following formula: [(number of alternating entries made/the total number of free choice entries made) × 100].

Sample collection

Animals were initially anesthetized with ether and blood samples were collected from retro-orbital sinus. The blood samples were centrifuged at $6000 \times g$ for 10 min and the obtained serum was stored at −80 °C for measurement of BDNF. Then mice were sacrificed with a guillotine and the brains were immediately removed from the skull. The brains were then weighted and placed in 2 ml microtubes. Samples were first frozen in liquid nitrogen and then transferred to −80 °C freezer.

Serum BDNF level measurement

Serum BDNF levels were determined by ELISA kit (Cusabio, China) according to the manufacturer's instructions. Serum BDNF levels were expressed as pg/ml and the minimum detectable level of BDNF was typically 2 pg/ml.

SOD and GPx activity evaluating

We measured the activity of enzymes GPx and SOD as indicators of oxidative stress. This was done in accordance with the protocol of company (Zell Bio GmbH Ulm. Deutschland, Germany). The brain samples were homogenized in ice-cold phosphate buffered saline (PBS, pH 7.4) (100 mg tissue per 1 mL PBS). Suspension from each sample was centrifuged at 4,000–6,000 RPM for 20 min at 4 °C and the supernatant was collected. The concentration of protein was measured by Bradford method.

For GPx activity, according to the instructions in the kit, the required value of the supernatant solution was incubated with reagents for 5 min at 37 °C. Then, mix well and centrifuged at 4000 RPM for 10 min. Again, incubated for 5 min at room temperature. The light absorbance was read with microplate reader/ELISA reader (DRG, USA) at 412 nm. The enzyme activity was shown in units per milligram of tissue protein (Unit/mg protein).

For SOD activity, according to the instructions in the kit, the required value of the supernatant solution was added to the chromogen. Then, shake the samples for homogenization well. The light absorbance was read with microplate reader/ELISA reader (DRG, USA) at 420 nm. The enzyme activity was shown in units per milligram of tissue protein (Unit/mg protein).

Data analysis

SPSS20 was used for data analysis. Data are presented as mean ± SEM. The significance of difference was assessed by one-way ANOVA followed by Bonferroni's *post hoc* tests. Differences were reported significant when $p < 0.05$.

Results

The effect of AA on the NORT

A significant difference was found for the discrimination ratio between sham and OVX groups [$F(4, 42) = 8.297$, $p < 0.001$]. Administration of AA at the doses of 300 mg/kg ($p = 0.009$) and 500

mg/kg ($p = 0.027$) increased the discrimination ratio compared to the OVX group (Fig. 1).

The effect of AA on the T-CAT

T-CAT was used to evaluate the effect of the AA on working memory in ovariectomized mice. Animals in the OVX group showed significantly less spontaneous alternations than sham group [F (4, 40) = 4.136, $p = 0.037$]. AA at the dose 100 mg/kg ($p = 0.041$) and 500 mg/kg ($p < 0.001$) increased the mean percent alternations compared to the OVX group (Fig. 2).

The effect of AA on the serum BDNF level

Our results showed that the serum level of BDNF increased in mice treated with AA at the doses of 100 and 500 mg/kg [F (4, 27) = 1.973, $p = 0.020$ and 0.044, respectively] compared to the OVX group (Fig. 3).

The effect of AA on the brain GPx activity

As shown in Fig. 4, our results showed that the activity of GPx decreased in OVX group in comparison with the sham group [F (4, 27) = 3.625, $p = 0.047$]. Compared to the OVX group, mice treated with AA at the dose of 500 mg/kg showed a significant increase in the activity level of GPx ($p = 0.025$) (Fig. 4).

The effect of AA on the brain SOD activity

The activity of SOD increased significantly [F (4, 27) = 6.929, $p < 0.001$] in mice treated with AA at the dose of 500 mg/kg compared to the OVX group. This factor was not significantly affected when AA was administered at the doses of 100 and 300 mg/kg (Fig. 5).

Discussion

In the present study we showed that AA has protective effects on tactile learning and working memory. AA also increased the activity level of antioxidant enzymes such as SOD and GPx in OVX mice. Also, we found that administration of AA (100 and 500 mg/kg) for 30 days increases the serum level of BDNF.

AA is a water-soluble vitamin and unlike humans, mice internally produce the vitamin C [21]. Our results showed that OVX disrupts the working memory and tactile learning in mice which could be improved by administration of AA. Memory and learning impairments are among the most common complications of menopause that are associated with disruption of the hippocampus function as a result of neuronal loss in this brain region. Many studies have demonstrated that memory disorders and hippocampal dysfunction occur in the absence of estrogen [22,23]. It has been well documented that estrogen replacement therapy, improves the cognitive function [24]. On the other hand, we observed that treatment with AA at the doses 300 and 500 mg/kg for 30 days improves tactile learning and working memory of OVX mice. Our results confirm previous findings, for example, Tomé et al., have shown that treatment with AA could prevent the cognitive dysfunction and decrease the seizure-induced neuronal damage in the hippocampus. They found that AA has neuroprotective effects *via* scavenging of free radicals and inhibiting the lipid peroxidation [25]. AA deficiency causes cognitive dysfunction which probably occurs due to the activation of oxidative stress pathways in the brain [26]. In another study, Kumar et al. have concluded that AA treatment in stress-induced disorders, results in a significant improvement in learning and memory [27]. Also, Moretti et al., showed that treatment of mice with low doses of AA (0.1 and 1 mg/kg) for 21 days did not alter the BDNF levels in cerebral cortex and hippocampus, but showed that AA was able to modulate cell survival related enzymes, such as Protein kinase B (Akt) in the cerebral cortex and p38 mitogen-activated protein kinases (p38 MAPK) in the hippocampus [28]. It is possible that AA improves tactile learning and working memory through the antioxidant property. According to the previous studies, it has been reported that the loss or reduction of estradiol due to OVX causes a significant reduction in BDNF level [29]. But in our study, we did not observe any significant reduction in BDNF level of serum in the OVX group. Such discrepancies might be due to the different duration of the experiments (1 month *vs.* 2 month). It is well established that mRNA expression of BDNF increases during the estrous cycle and in response to estradiol [3]. Consistent with our results, reduction in serum BDNF level probably is one of the causes of impaired cognitive function and memory in postmenopausal women [30]. Our results also showed that 30 days administration of AA supplementation (at the dose 100 and 500 mg/kg) increases the level of BDNF in serum. AA is an essential cofactor for several enzymes in the brain which regulate the production of the protective molecules such as BDNF [15]. Therefore, AA may be neuroprotective through increasing the level of BDNF in absence of estrogen.

Our results showed that OVX decreases the activity level of GPx in mice brains. In postmenopause, deficiency of estrogen and consequently increased free oxygen radicals can impress the

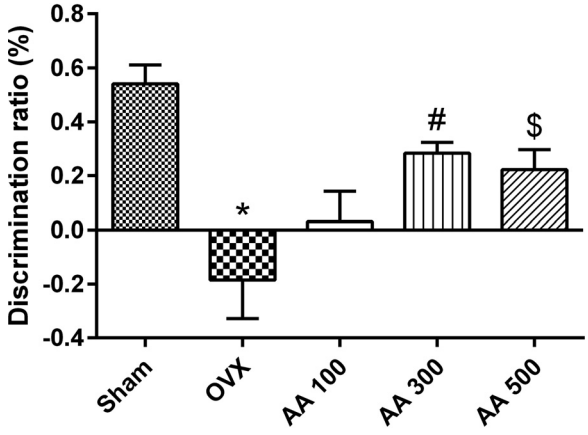

Fig. 1. The effect of AA on tactile learning in OVX mice. Values are expressed as Mean ± SEM (n = 8 in each group). $^{*}p < 0.001$ compared to the sham group, $^{\#}p = 0.009$ and $^{\$}p = 0.027$ compared to the OVX group.

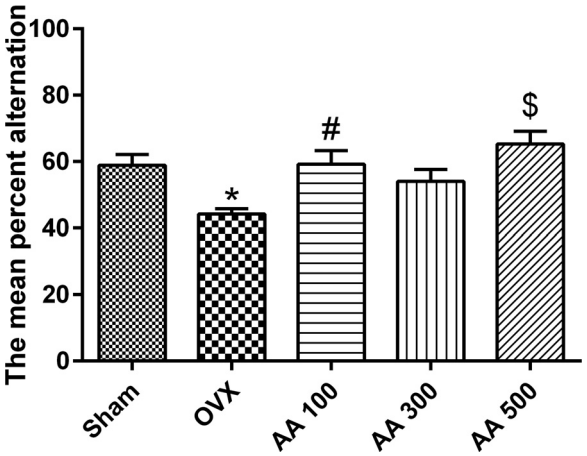

Fig. 2. The effect of AA on working memory in OVX mice. Values are expressed as Mean ± SEM (n = 8 in each group). $^{*}p = 0.037$ compared to the sham group, $^{\#}p = 0.041$ and $^{\$}p < 0.001$ compared to OVX group.

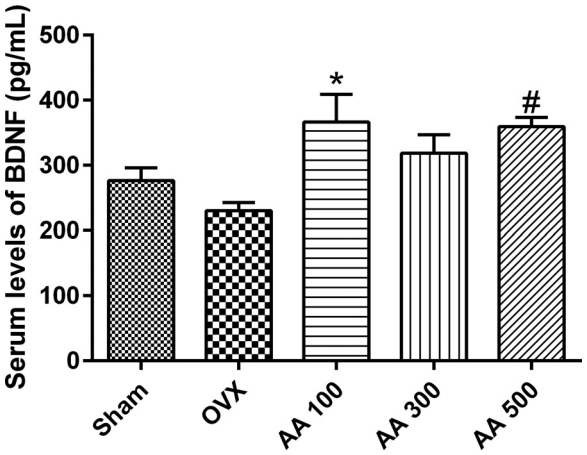

Fig. 3. The effect of AA on the serum level of BDNF. Values are expressed as Mean ± SEM (n = 8 in each group). $^{*}p = 0.020$ and $^{\#}p = 0.044$ compared to the OVX group.

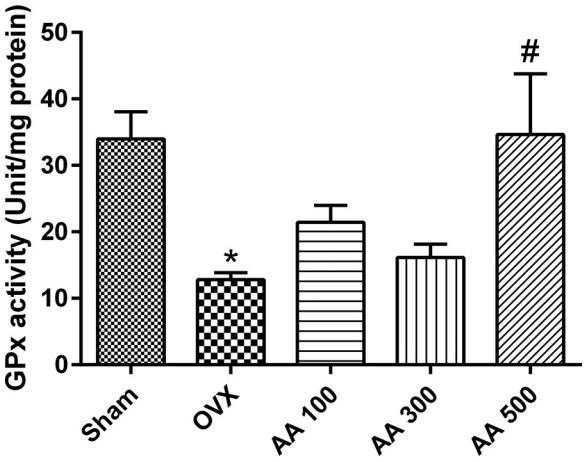

Fig. 4. The effect of AA on activity level of GPx. Values are expressed as Mean ± SEM (n = 8 in each group). $^{*}p = 0.047$ compared to the sham group, $^{\#}p = 0.025$ compared to the OVX group.

antioxidant enzymes and decrease the activity of the antioxidant defense system [31]. SOD acts as the most important enzyme in all aerobic organisms which catalyzes the dismutation of superoxide (O_2-) into oxygen and hydrogen peroxide (H_2O_2). GP_X degrades the H_2O_2 and hydroperoxides with expending glutathione (GSH) [32]. The antioxidant system inhibits lipid peroxidation *via* antioxidants such as GPx and SOD [1]. By decreasing the level of estrogen in OVX condition, production of free oxygen radicals reduces the level of antioxidant enzymes such as SOD and GPx [33]. Also, it has been shown that AA level is reduced during menopause due to its

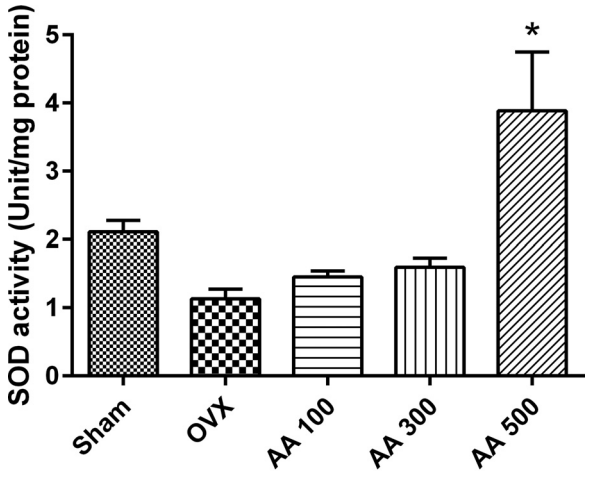

Fig. 5. The effect of AA on activity level of SOD. Values are expressed as Mean ± SEM (n = 8 in each group). $^{*}p < 0.001$ compared to OVX group.

increased consumption to counteract the elevated oxidative stress [1]. We also found that 30 days administration of AA supplementation (at the dose 500 mg/kg) causes an increase in SOD and GPx activities in the brain of OVX mice. Previous studies have suggested that a chronic lack of AA may enhance the development of oxidative stress in the brain during normal aging and increase amyloid production [12]. Many studies have investigated the effect of AA on oxidative stress and concluded that this vitamin can protect the body from oxidative injury *via* increasing the activity and/or level of antioxidant enzymes [11,25,27]. Therefore, it seems that the AA effects observed in this study might be due to the increased the activity of the antioxidant defense system.

Conclusion

In the current study, we showed that AA ameliorates cognitive dysfunctions associated with ovariectomy in mice. It seems that the mechanism of this improvement in memory performance might be mediated by (i) the antioxidant effects of AA, (ii) increasing the level of neurotrophin BDNF and (iii) increasing the activity of SOD and GPx in the brain. However, further studies are necessary to precisely reveal the underlying cellular signaling.

Conflict of interest

The authors declare no conflict of interest relevant to this study.

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