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Neuroprotective effect of *Asparagus racemosus* root extract via the enhancement of brain-derived neurotrophic factor and estrogen receptor in ovariectomized rats

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ABSTRACT

Ethnopharmacological relevance: *Asparagus racemosus* (AR) is well known as an Ayurvedic rasayana which used traditionally by Ayurvedic practitioners for nervous disorders and prevent aging. In our previous study it was found that ethanol AR root extract can improve learning and memory impairment, induced by an ovariectomy, but the extract's mechanisms as a neuroprotective property are still unknown.

Aim of the study: This study aimed to examine the effects and mechanisms of ethanol AR root extract on the alteration of brain-derived neurotrophic factor (BDNF) and estrogen receptor (ER) subtypes in ovariectomized (OVX) rats.

Materials and methods: Adult female Wistar rats were divided into five groups, 4 groups underwent ovariectomy, and one group was designed to be the sham control group. Two groups were gavaged with propylene glycol for sham, and a second group similarly prepared for OVX. Two further groups of OVX rats were gavaged once daily, one group with 100 mg/kg b.w. of ethanol AR root extract and the second group with 1000 mg/kg b.w. of ethanol AR root extract. The fifth group was gavaged once daily with 0.1 mg/kg b.w. of 17α-ethynylestradiol (EE). BDNF, ERα and ERβ expression were evaluated by western blot analysis.

Results: The western blot analysis revealed that the OVX rats showed a significant decrease in BDNF and a down-regulation of ERα and ERβ in the frontal cortex and hippocampus. It was also demonstrated that EE and AR root extract increased BDNF, ERα and ERβ in the frontal cortex and hippocampus of ovariectomized rats.

Conclusions: Based on these results, the enhancement of BDNF and ERs up-regulation may be involved in the neuroprotective effects of ethanol AR root extract in ovariectomized rat.

1. Introduction

Asparagus racemosus (AR), also known as shatavari (family Asparagaceae), is an important medicinal plant endemic to tropical India and Thailand. It is well known drug in Ayurvedic rasayana that prevent aging, increase longevity and improve mental function. In addition, it is recommended in Ayurvedic texts for the prevention and treatment of gastric ulcers, dyspepsia and as a galactagogue (Alok et al., 2013). The major active compounds in the root of AR are steroidal saponins such as asparacoside, shatavarin IV, V and XI (Onlom et al., 2017a, 2017b, 2017c) and other constituents such as racemosol and asparagamine (Bopana and Saxena, 2007). The phytoestrogenic properties of AR are widely known and used as a hormonal modulator in a stimulant health tonic for women (Joseph, 1998), which has effects similar to endogenous estrogen (Saxena et al., 2010). The neuroprotective properties of AR have also been documented. The oral administration with AR root methanolic extract (50, 100 and 200 mg/kg b.w.) for 7 days could enhance the memory and protect against scopolamine-induced amnesia in rodents (Ojha et al., 2010). It has also previously been reported that the mice pre-treated with AR root acetone extract at

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the dose of 18 mg/kg b.w mixed with diet daily for 2 weeks could prevent the neuronal damage induced by kainic acid (Parihar and Hemnani, 2004). In addition, Saxena and co-workers demonstrated that *Asparagus racemosus* root extract (100 mg/kg b.w.) for 30 days could increase the percentage of normal cell in stress group stress in experimental animals as well as show a significant increase in test score of extract treated patients as compared to stress group patients (Saxena et al., 2007). Furthermore, AR root extract has been demonstrated for its adaptogenic activity against different kinds of stressors in animals (Rege et al., 1999).

Menopause is defined as the cessation of the menstrual cycle in women along with a decrease in ovarian steroidal hormones such as estrogen and progesterone. The deprivation of estrogen in menopausal women is considered not only to disturb reproductive functions but also produces many psychological symptoms such as anxiety, difficulty concentrating, depression and forgetfulness (Miquel et al., 2006). Clinical study has suggested that ovarian hormone withdrawal after menopause could increase the risk of Alzheimer's disease (AD), a neurodegenerative disorder (Gao et al., 1998). Several lines of evidence have suggested that estrogen may exert its neuroprotective effect by maintaining brain homeostasis through regulation of neuroinflammation, oxidative stress, cerebral blood flow, extracellular glutamate levels and neuroprotective signaling (Acaz-Fonseca et al., 2014; Arevalo et al., 2015a). Moreover, some mechanisms of estrogen are involved in the regulation of plasticity of synaptic circuits in key cognitive brain regions, such as the somatosensory cortex, the prefrontal cortex and the hippocampus (Arevalo et al., 2015b). Decrement of brain-derived neurotrophic factor (BDNF) levels is associated with neurodegenerative disorder relating to learning and memory impairment (Lindsay et al., 1991; Sohrabji and Lewis, 2006). Many studies indicated that estrogen modulates BDNF expression in the hippocampus and cerebral cortex (Spencer et al., 2008; Luine and Frankfurt, 2013). Estrogen exerts its effects by binding to classical ER subtypes, ERα and ERβ. These receptors distribute in several brain areas including the frontal cortex and hippocampus where specifically associated with recognition memory. Previous studies have shown that following long-term ovariectomized (OVX) in rats, the expression of ERα showed the down-regulation in the telencephalon and hippocampus (Navarro et al., 2013) while shot-term OVX in rats found the up-regulation of ERα in the hippocampus (Cardoso et al., 2010). However, these effects of OVX were reversed by exogenous estradiol administration. Furthermore, the expression of ERβ was found to be decreased in the rat brain of three-month OVX (Rose'Meyer et al., 2003). The estrogen replacement therapy (ERT) relieves menopausal symptoms as well as prevents cardiovascular disease and osteoporosis including decrease the risk for neurodegenerative disorder. However, there are several concerns over the ERT because it produces serious side-effects such increased risk for endometrial cancer, breast cancer and venous thromboembolic events (Barrett-Connor and Grady, 1998).

The results obtained from our previous study have revealed that the administration of AR root extract for three months could reverse the learning and memory impairment induced by ovariectomy, relating to diminished neuronal damage in medial prefrontal cortex and hippocampus without effect on circulating estradiol concentration (Lalert et al., 2013). These suggested that AR root extract might bind directly to the estrogen receptors without enhancing the endogenous estrogen levels to improve memory dysfunction induced by OVX. Although AR is well known for its phytoestrogenic and neuroprotective properties, the beneficial effects and mechanisms of AR on learning and memory impairment induced by OVX rat are not clearly understood. Based on our previous results, the present study aims to investigate the effects of the chronic administration of AR root extract on the alterations of BDNF and ER subtypes on the brain regions associated with learning and memory process, the frontal cortex and hippocampus, in an animal model of estrogen deficit, OVX rats.

2. Materials and methods

2.1. Plant material and preparation of crude extract

The AR roots were collected from Ampur Muang, Rayong, Thailand. The voucher specimen of the plant was kept at the Pharmaceutical Botany Mahidol (PBM) herbarium, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand (Collection no. RKT 0005). The main chemical components of this plant sample have been reported previously as saponin glycosides (Onlom et al., 2017a, 2017b, 2017c). The roots of AR were dried by hot air oven at 45 °C for 24 h before milled into coarse powder. After that the dried powdered roots of AR (5 kg) was macerated at room temperature with hexane for 3 days. Then residue was macerated with 95% ethanol for 3 days, and was filtered and extracted again to provide the crude AR ethanolic extract with 10.94% yield. The extract contained 7.4% of saponin glycosides equivalent to shatavarin IV determined by ELISA using monocolonal antibody against shatavarin IV (Onlom et al., 2017b). Moreover, HPLC-Q-TOF-MS/MS analysis as described in the previous study (Onlom et al., 2017a) indicated that the major saponins in the extract was shatavarin IX (5.94 ± 0.03 mg/g) (Supplementary data). The extract was mixed with 20% propylene glycol to stock suspension in a dose of 100 and 1000 mg/kg b.w. Doses were chosen on the basis of prior studies demonstrating pro-cognitive effects and absence of toxicity (Lalert et al., 2013; Kumar et al., 2010). The suspension was administered by gavage once daily.

2.2. Animals

Eight-weeks-old (260–310 g) female Wister rats were obtained from the National Laboratory Animal Center Mahidol University, Nakhon Pathom, Thailand. The rats were acclimatized for at least one week before starting the experiment. They were housed two-three per cage under a standard 12 h dark/light at constant temperature of 24 ± 1 °C. The animals were allowed free access to food (C.P.082, S.W.T. Co. Ltd, Thailand) and tab water ad libitum. The experiments were comply with standard of animal care and use established under the ethical guidelines and policies of Naresuan University, and all protocols were approved by the Ethical committee for the Use of Animal, Naresuan University, Thailand (No.56040051).

2.3. Surgery and treatments

Before the experiments begun, all animals were examined for estrous cycles by using vaginal cornification assay for three consecutive cycles to select the rats with normal estrus cycle (Everett, 1948). Bilaterally ovariectomy or sham-operating was performed under the anesthetized by an intraperitoneal injection of sodium pentobarbital 50 mg/kg b.w. during a diestrus phase. The ovariectomy was performed by making a small 1 cm dorsolateral incision. The ovaries surrounded by fat were exposed and the vessels supplying to the ovaries were ligated and ovaries were removed. Sham surgery was performed in the same manner without removing the ovaries. (Sayed et al., 2013). After the operation, all animals were leaved in home cage for 15 days to recover from the surgery. During the recovery period, vaginal smear were taken once daily to confirm that all animal were an anestrus. After that the animals were randomly divided into following five groups (n = 6 in each group) that included group 1 (sham); sham-operated rats with vehicle administration (propylene glycol; PG), group 2 (OVX); OVX rats with vehicle administration, group 3 (OVX + AR100); OVX rats with 100 mg/kg b.w. of AR root extract administration, group 4 (OVX +;AR1000); OVX rats with 1000 mg/kg b.w. of AR root extract administration and group 5 (OVX + EE); OVX rats with 0.1 mg/kg b.w. of 17α-ethynylestradiol administration (EE) as a positive control. All experimental groups were administrated by gavage once daily for 90 days. At the end of the experimental periods, all rats were euthanized

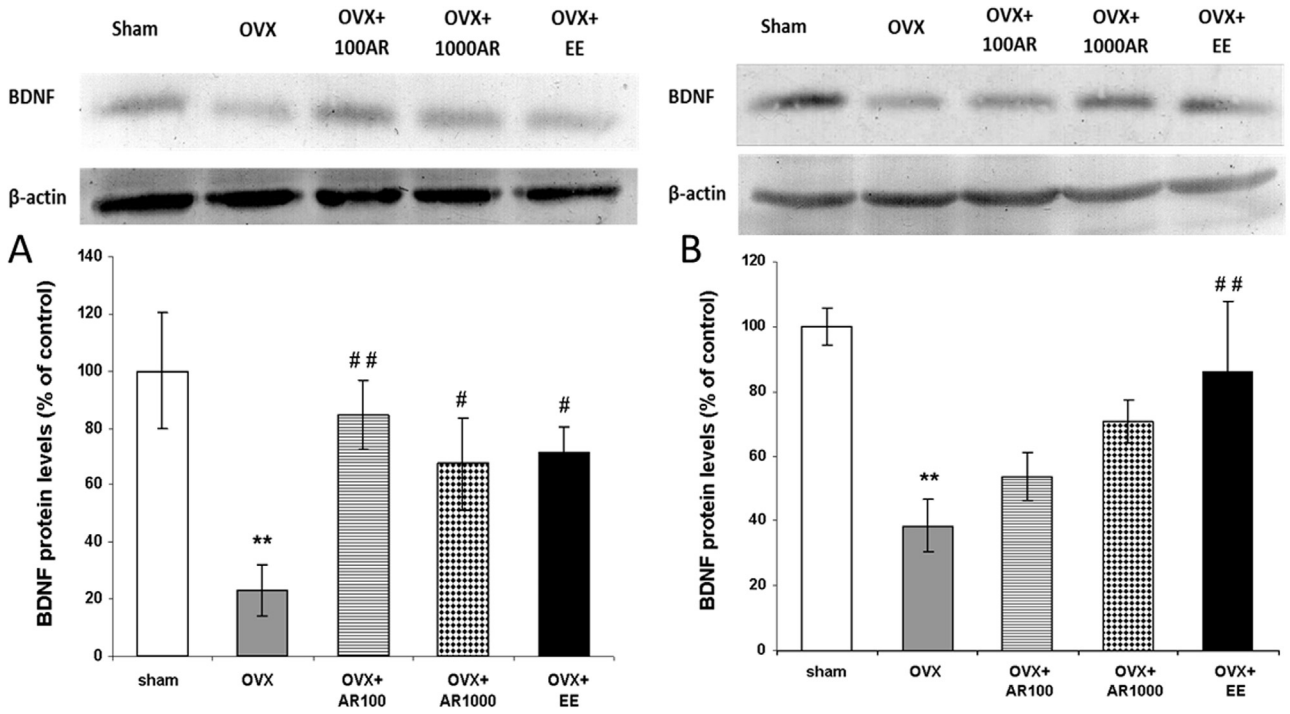

Fig. 1. Effect of the AR root extract on the expression of BDNF protein in the hippocampus (A) and frontal cortex (B). In the AR-treated groups, the OVX rats were received with AR root extract at the dose of 100 mg/kg b.w. (OVX + AR100) or 1000 mg/kg b.w. (OVX + AR1000). In the EE-treated group, the OVX rats were given with 17α-ethynylestradiol at the dose of 0.1 mg/kg b.w. (OVX + EE), while the sham and OVX groups were received with vehicle at the same volume. All experimental groups were administrated by gavage once daily for 90 days. The protein levels of BDNF were detected by Western blot analysis. The quantitative data are expressed as percentage value taking the same control group as 100%. Each histogram bar is represented as mean ± S.E.M. ($n = 6$), $^{**}P < 0.01$ compared to the sham, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to OVX group.

with an excessive dose of sodium pentobarbital followed by decapitation. The brains were rapidly removed out of the skull. The frontal cortex and hippocampus were rapidly dissected on ice and maintained at −80 °C until further processing.

2.4. Western blot analyses

The frozen frontal cortex and hippocampus including rat uterus tissue, a positive tissue for ER subtypes detection, were homogenized in tissue extraction reagent I (Invitrogen Corporation, Camarillo, USA) with protease inhibitor cocktail (Sigma-Aldrich Inc, Louis, USA). The homogenates were then centrifuged at 10,000 × *g* at 4 °C for 10 min. For BDNF detection, 50 μg of protein sample was separated by 12.5% SDS polyacrylamide gels and the expression of ER subtypes evaluation, 70 μg of proteins were loaded on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels. The proteins were then electrophoretic transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corporation, BioTrace) at 20 V for 2 h using TE 77 PWR Semi-Dry Transfer Unit (Healthcare Life Sciences, New Jersey, USA). The blotted membranes were blocked nonspecific background with 5% non-fat dry milk in 0.1% tween-20 Tris-buffered saline pH 7.4 (TBS-T) for 1 h at room temperature followed by incubation with diluted 1:200 polyclonal rabbit anti-BDNF in 5% non-fat dry milk in TBS-T (SantaCruz Biotechnology, California, USA) or 1:400 diluted rabbit polyclonal anti- ERα antibody in TBS-T and 1:500 diluted rabbit polyclonal anti-ERβ antibody with 5% non-fat dry milk in TBS-T overnight at 4 °C. After washing three times for 10 min each in TBS-T, the blotted membranes were incubated 30 min at room temperature with avidin-biotinylated horseradish peroxidase complexes (Vector Laboratories Inc, Burlingame, USA) to detect the expressions of BDNF protein and incubated with 1:1000 diluted goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Vector Laboratories Inc, Burlingame, USA) with 5% non-fat dry milk in TBS-T for 2 h at room temperature to detect the expressions of ER subtypes protein followed by washing. Immunoreactive bands were visualized using ImmPACT™ diaminobenzidine (Vector Laboratories Inc, Burlingame, USA) and followed by rinsing in distilled water to terminate the reaction. The intensities of the immunoreactive bands were analyzed with Image J software (National Institutes of health, Bethesda, Maryland, USA) and normalized the visualized bands to their respective internal loading control protein, β-actin. The results were reported when the control level of protein expression was considered as 100% and the treated levels were calculated as relative percentages for each experiment.

2.5. Statistical analysis

The data were expressed as the mean ± standard error of the mean and analyzed using a Statistical Package for the Social Sciences (SPSS) software. Multiple comparisons among experimental groups were performed by one-way ANOVA with post hoc LSD test. The statistical significance was determined as *P-value* less than 0.05.

3. Results

3.1. Effect of the AR root extract on the expressions of BDNF protein in OVX rats

Using western blot analysis, the expressions of BDNF protein in frontal cortex and hippocampus were evaluated. The results revealed that the expressions of BDNF protein in OVX group were significantly decreased in both the frontal cortex and hippocampus when compared to sham control group ($P < 0.05$). Interestingly, our results showed that the expressions of BDNF protein in the hippocampus in the OVX + AR100 and OVX + AR1000 groups were significantly higher than that observed in the OVX group ($P < 0.01$ and $P < 0.05$, respectively). The reverse disastrous effect of OVX was also observed in

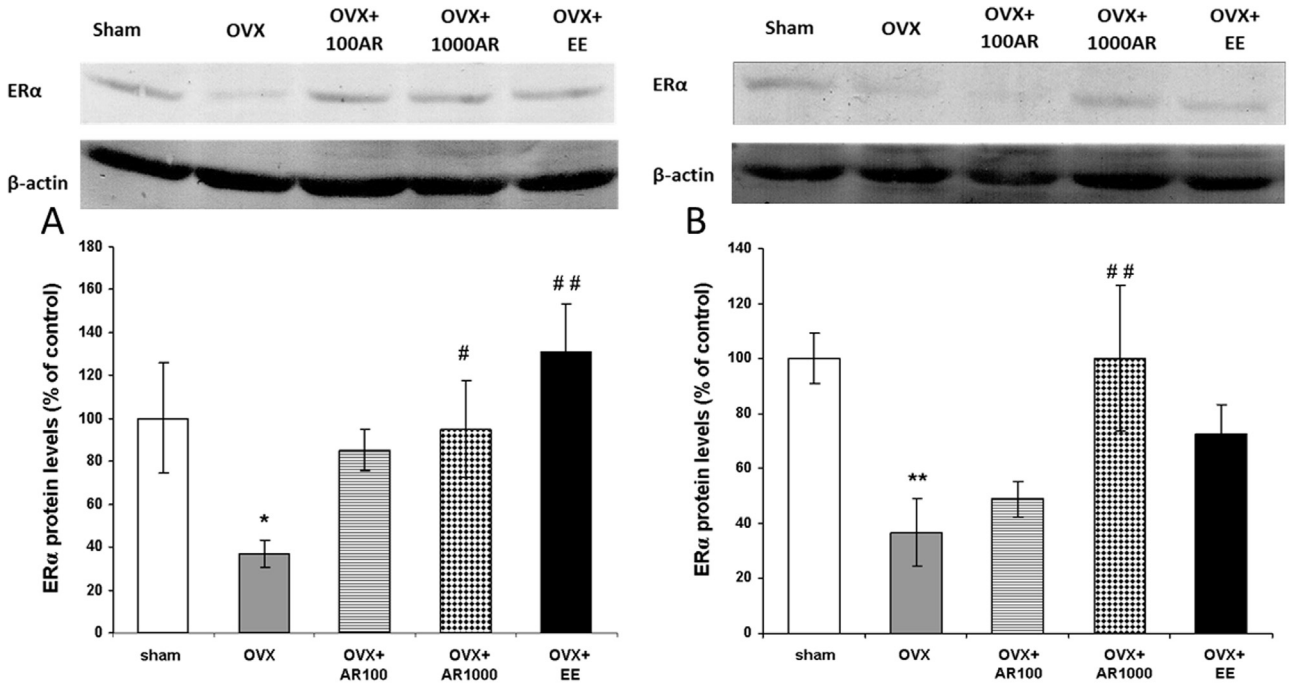

Fig. 2. Effect of the AR root extract on the expression of ERα protein in the hippocampus (A) and frontal cortex (B). In the AR-treated groups, the OVX rats were received with AR root extract at the dose of 100 mg/kg b.w. (OVX + AR100) or 1000 mg/kg b.w. (OVX + AR1000). In the EE-treated group, the OVX rats were given with 17α-ethynylestradiol at the dose of 0.1 mg/kg b.w. (OVX + EE), while the sham and OVX control group were received with vehicle at the same volume. All experimental groups were administrated by gavage once daily for 90 days. The protein levels of ERα were detected by Western blot analysis. The quantitative data are expressed as percentage value taking the same control group as 100%. Each histogram bar is represented as mean ± S.E.M. ($n = 6$), $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to the sham, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to OVX group.

the OVX received 0.1 mg/kg b.w. of EE (OVX + EE) ($P < 0.05$). These data are shown in the Fig. 1A. Additionally, we found that the level of BDNF protein expression in the frontal cortex of OVX received EE was significantly higher than that in the OVX group ($P < 0.01$). The data are shown in the Fig. 1B.

3.2. Effect of the AR root extract on the expressions of ER subtypes protein in OVX rats

To determine the expressions of ER subtypes in the frontal cortex and hippocampus, the results were revealed that the expression of ERα in the hippocampus was significantly decreased in OVX group ($P < 0.05$) when compared to sham control group (Fig. 2A). Additionally, we found that the level of ERα protein expression in the frontal cortex was also significantly lower than that ($P < 0.05$) in the sham control group (Fig. 2B). However, when comparison with those from the OVX group we found that the expression of ERα in only OVX received high dose of AR root extract (1000 mg/kg b.w.) and EE were significantly increased in the hippocampus ($P < 0.05$ and $P < 0.01$, respectively). Moreover, high dose of AR root extract was also significantly increased the levels of ERα in the frontal cortex when compared to OVX group ($P < 0.01$) which the effect was not observed in the OVX received AR 100 mg/kg b.w. or EE.

For the expressions of ERβ in the frontal cortex and hippocampus, we observed that there was significantly reduced in the levels of ERβ in OVX group when compared to the sham control group ($P < 0.01$ and $P < 0.05$, respectively). Interestingly, the results obtained were revealed that the levels of ERβ protein expression in OVX rats received AR root extract 100 and 1000 mg/kg b.w. were significantly increased when compared to those in the OVX group ($P < 0.01$ and $P < 0.05$, respectively). The OVX administrated with EE was also presented the higher of ERβ protein level ($P < 0.05$) than that in the OVX group (Fig. 3A). Moreover, a significant increasing in the levels of ERβ protein was also observed in the frontal cortex of OVX that received AR root extract 100 and 1000 mg/kg b.w. and EE ($P < 0.05$) comparing with the OVX group (Fig. 3B).

4. Discussion

In this study, our results revealed that long-term estrogen deprivation in OVX rats decreased BDNF protein expression and also down-regulated the expression of ER subtypes in the frontal cortex and hippocampus. However, AR could reverse the reduction of BDNF and ER induced by OVX in the hippocampus and frontal cortex. It is known that estrogen and phytoestrogen are able to mediate morphology and physiology of the cortex and hippocampus, enhance cognitive function, and protect neurons from various kinds of brain insults (Takuma et al., 2007; Pompili et al., 2012). Estrogen has also been recognized for its role in modulating BDNF (Scharfman and MacLusky, 2006; Pluchino et al., 2013). Binding of BDNF to tyrosine kinase receptor B (TrkB) promotes neuronal plasticity and neuronal survival essential to learning and memory (Driscoll et al., 2012; Li et al., 2012). The major active compound present in the AR root is steroidal saponin namely shatavarins (Hayes et al., 2008) which have phytoestrogenic effects (Bopana and Saxena, 2007). Previous studies have shown that ginsenoside Rg1, high steroidal saponins, can reverse the corticosterone-induced changes in mRNA levels of BDNF in hippocampal mice (Chan et al., 2002; Lee et al., 2003; Chen et al., 2014).

Both alpha and beta ERs are important for hippocampal function, as knockout of either receptor in mice impairs hippocampal-dependent learning (Fugger et al., 2000; Rissman et al., 2002; Day et al., 2005). Various studies have shown that BDNF and TrkB including PSD-95 mRNA were decreased in ERα and ERβ knockout mice (Spencer-Segal et al., 2012). Romeo and co-workers showed that ERα expression of rat hippocampal dendritic spines in the proestrus was higher than in the diestrus phase (Romeo et al., 2005). In addition, study of the alteration of ER expression in rat brain has considered that ERβ in the cortex significantly declined with aging (Wilson et al., 2002). Our results

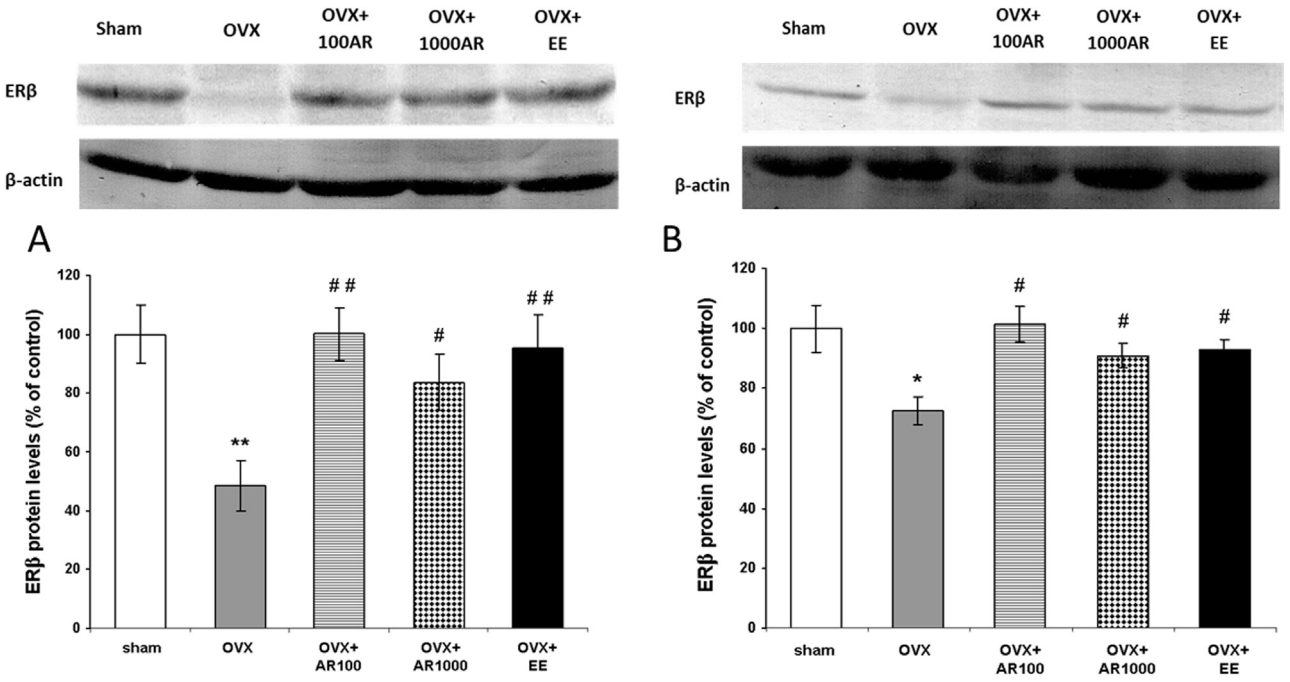

Fig. 3. Effect of the AR root extract on the expression of ERβ protein in the hippocampus (A) and frontal cortex (B). In the AR-treated groups, the OVX rats were received with AR root extract at the dose of 100 mg/kg b.w. (OVX + 100AR) or 1000 mg/kg b.w. (OVX + 1000AR). In the EE-treated group, the OVX rats were given with 17α-ethynylestradiol at the dose of 0.1 mg/kg b.w. (OVX + EE), while the sham and OVX control group were received with vehicle at the same volume. All experimental groups were administrated by gavage once daily for 90 days. The protein levels of ERβ were detected by Western blot analysis. The quantitative data are expressed as percentage value taking the same control group as 100%. Each histogram bar is represented as mean ± S.E.M. ($n = 6$), $^{**}P < 0.05$ and $^{**}P < 0.01$ compared to the sham, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to OVX group.

identified a down-regulation of ER protein expression in both the frontal cortex and hippocampus after 90 days of OVX. Various studies have indicated that estrogen affects ER expression. Although our result of down-regulation of ER proteins in OVX is not consistent with the study of Cardoso and colleagues identifying how OVX after 15 days produced ERα up-regulation in the rat hippocampus (Cardoso et al., 2010), our finding is in accordance with a recent longer-term study. This demonstrated a decrement of ERα expression in the rat cortex and hippocampus after 6–24 months of ovariectomy (Navarro et al., 2013) and an amelioration of ERβ in the brain of 3-month OVX rats (Rose'Meyer et al., 2003). These results may indicate that short-term ovariectomy causes a short period in which a lack of circulating estrogen results in a compensatory up-regulation of ERs for physiological responses. On the other hand, the lack of estrogen for 3 months has supported the idea that long-term estrogen insufficiency leads to down-regulation of its receptors in the brain. It is reasonable to suggest that estrogen plays an important role in ER down-regulation in brain. In the present study, AR root extract could restore a down-regulation of ERα and ERβ in OVX, as can EE, in the frontal cortex and hippocampus. Phytoestrogenic properties of AR are widely known and used as a hormonal modulator in a herbal stimulant health tonic for women (Saxena et al., 2010). Although the effects of phytoestrogen are poorly understood, it could act through estrogen receptors. Previous study has reported that coumestrol, one class of phytoestrogens, could mediate the expression of ERβ in the paraventicular nuclease of hypothalamus (Patisaul et al., 1999). In addition, previous study showed that dietary intake of phytoestrogens up-regulated ERα in breast tumor cells in premenopausal women (Touillaud et al., 2005). Thus AR may mediate its activities through ER binding and trigger ER expression to initiate the neuroprotective effects in the OVX rats.

Previously, our group has demonstrated that chronic treatments with AR root extract had no effect on estradiol levels but could improve learning and memory performance and reverse neuronal injury in the medial-prefrontal cortex and CA1 and CA3 sub-fields of the hippocampus (Lalert et al., 2013). Furthermore we have found that the high dose of AR (1000 mg/kg) for 90 days had no effect on wet weight of peripheral tissue (kidney, liver, spleen and uterus) while others have found no toxicity at this and higher doses (Kumar et al., 2010). The data obtained from this study indicate that estrogen insufficiency produced a decrease of BDNF and ER protein in the hippocampus and frontal cortex. Similar to EE, AR root extract could prevent the deteriorating effects of estrogen decline. These results suggest that the neuroprotective effects of AR root extract relate to its ability to increase the expression of ER and BDNF proteins which are diminished by estrogen deprivation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2018.07.014

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