Neuroprotective Effects of the *Asparagus racemosus* Root Extract on Ovariectomized Rats

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Abstract

Estrogen decline in menopausal women may impair the cognitive function. This change can be ameliorated by estrogen replacement therapy (ERT). However, ERT is often overshadowed by the serious side effects of estrogen use in menopause women. *Asparagus racemosus* (AR) is well known for its phytoestrogenic properties while neuroprotective effects of AR in ovariectomized model are unknown. This study aimed to investigate effects of AR root extract on serum estradiol level, learning and memory, and neuronal viability in hippocampus and medial prefrontal cortex (mPFC) of ovariectomized rats by using electrochemiluminescence immunoassay, the novel object recognition test and histological analysis, respectively. Adult female Wistar rats were divided into five groups and gavaged for 90 days with vehicle (propylene glycol) for sham and OVX groups and another 3 groups of OVX rat were gavaged with 100 or 1000 mg/kg B.W./day of AR root extract or 0.1 mg/kg B.W./day of 17α-ethynylestradiol (EE), respectively. There was a significant decrease in recognition index of the OVX rats and AR root extract could reverse this effect. The serum estradiol level was significantly decreased in OVX group whereas AR root extract did not statistically change from that demonstrated by OVX group. The OVX rats showed a marked decrease in the number of neurons in hippcampus and mPFC. AR root extract and EE could reduce the neuronal loss in hippocampus and mPFC. The results obtained suggest that AR could improve cognitive ability in ovariectomized model which associated with increase neuronal viability of hippocampus and mPFC. AR may be a beneficial agent for prevention cognitive decline induced by ovariectomy.

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Menopause is characterized by deprivation of ovarian steroid hormones such as estrogen. Many studies have shown that menopause results in behavioral, neurochemical, and molecular alterations[1,2] leading to cognitive dysfunction.[3,4] Estrogen widely influences on brain function. It has been shown to promote neuronal growth, enhanced long-term potentiation[5] and reduced neuronal death from apoptosis.[6] Many evidences suggested that estrogen could improve cognitive impairment. Wallace (2006) reported that rats and humans with suppressed ovarian function exhibited performance impairment on tasks of hippocampal- and prefrontal-cortical-dependent memory that could be corrected by exogenous estrogen treatment.[7] Hormone replacement therapy has been shown to improve cognitive function in postmenopausal women[8] whereas long-term treatment of estrogen for menopausal women has been criminated by the serious side effects. Therefore, the development of safer and more effective drug therapy would benefit for menopausal women with memory deficits.

Phytoestrogens are non-steroidal plant compounds that are able to exert estrogenic effects. Many studies reported that phytoestrogen treatment could improve cognitive decline in postmenopausal women and in ovariectomized rodents.[9-11] These are attracted interest as a potential alternation to the estrogen supplements.

Asparagus racemosus (AR) or Shatavari is a species of asparagus which is widely throughout Sri Lanka, India, and Himalayas. AR is called “Samsip” in Thailand. It is known for its phytoestrogenic properties to be useful for female rejuvenation.[12] Previous study, it was reported that the major active constituents of AR root extracts are steroidal saponins namely shatavarins.[13] These saponins enhanced memory and protected scopolamine-induced amnesia in rodents.[14] Moreover, methanolic extract of AR roots showed adaptogenic activity in animals exposed to different kinds of stressors[15] and against kainic acid (KA)-induced hippocampal and striatal neuronal damage in mice.[16] However, the effects of AR root extracts on learning and memory impairment in estrogen deprived rats are unknown.

Therefore, this study aimed to investigate effects of AR root extract on serum estradiol level, learning and memory and neuronal viability in hippocampus and medial prefrontal cortex (mPFC) of

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ovariectomized rats by using electrochemiluminescence immunoassay, the novel object recognition test and histological analysis, respectively.

Materials and Methods

Plant preparation and extraction

The AR roots were collected from Rayong, Thailand. The voucher specimen of plant was kept at Faculty of Pharmaceutical Science, Naresuan University. AR roots were dried by hot air oven at 45 ^{o}C for 24 h before milled into coarse powder. Thereafter, the dried powdered AR roots were macerated at room temperature with hexane for 3 days. Then residue were macerated with 95% ethanol for 3 day which were filtered and residue were extracted again by same procedure were used. The crude of AR root ethanolic extract was mixed with propylene glycol (PG) to stock suspension in a dose of 100 and 1000 mg/kg B.W. The suspension was orally administered to the rats during 7:00-8:00 a.m.

Animals and treatments

Eight–weeks-old female Wistar rats were purchased from the National Laboratory Animal Center Mahidol University, Nakhon Prathom, Thailand. The rats were acclimatized for at least two weeks before starting the experiments. They were housed in group (2-3 rats) under a standard light/dark (l2:12 h) at constant temperature of 24±1 ^{o}C. The animals had free access to food (C.P.082, S.W.T. Co.Ltd, Thailand) and water *ad libitum*. The experiment protocol was approved by the Ethical committee for the Use of Animal, Naresuan University.

The rats were checked for estrous cycle by using vaginal cornification assay for three consecutive cycles before treating. Animals were anesthetized with pentobarbital (50 mg/kg B.W., i.p.) and bilaterally ovariectomized (OVX) or sham-operated using dorsolateral approach under aseptic conditions during a diestrus phase. After surgery, they were housed for 15 days and vaginal smears were taken daily to confirm that the animals were anestrus. After that, the animals were randomly grouped as follows: sham group (n=7) as sham-operated rat were given vehicle (PG), ovariectomized group (n=7) as OVX rat were given to vehicle, ovariectomized with AR 100 (n=6) or AR1000 (n=6) group as OVX rat were treated with AR root extract 100 mg/kg B.W. or 1000 mg/kg B.W., respectively. 17α- ethynylestradiol (EE) was given 0.1 mg/kg B.W. in OVX rats as a positive control (n=6). All experimental groups were administrated once daily by oral gavages for 90 days.

Novel object recognition (NOR) test

After the completed treatment for 90 days, rats underwent the novel object recognition test. Animals were tested in a black open field (100 cm x 100 cm with 50 cm high walls). This test used two of three identically objects, where different of shapes but similar size. Object A, B and C was the cylinder, pyramidal and cuboidal shape, respectively. The position of two objects was in the center of the arena, 20 cm from each other. For the experimental protocol, before training the rat was placed in the arena to habituation for 5 min. After 24- h to habituation, rat was training. The training session, object A and B were placed in a symmetric position. Each rat was allowed to explore in the arena for a total of 10 min. The amount of time spent exploring object A and B (T_A and T_B, respectively) were considered as a preference index [$(T_A \times 100)/(T_A + T_B)$]. After completed training session for 10 min, the testing session was initiated. This session, rat was placed in arena for 10 min for exploring object, where one of the original object (B) was replaced by novel object (C). The amount of the time spent exploring the object A and C (T_A and T_C, respectively) were calculated as the recognition index [$(T_C \times 100)/(T_A + T_C)$].

Serum estradiol levels assay

After evaluation of learning and memory abilities, all experimental animals were sacrificed using pentobarbital (50 mg/kg B.W.). Then, their blood was collected by cardiac puncture method. Serum for determine to estradiol level was separated by centrifugation at 1000 rpm for 10 minutes at room temperature and kept at -80 ^{o}C until analysis.

Serum estradiol levels were measured by using eletrochemiluminescence immunoassay (ECLIA) with Elecsys 2010 automate analyzer (Roche Diagnostics GM6H, Mannhein, Germany) at Faculty of Medicine, Chiang Mai University Hospital

Histological analysis

All experimental animals were sacrificed after blood collection. Their brains were rapidly removed and fixed with 10% formalin. For paraffin sections, formalin fixed brain were dehydrated in an ethanol series and embedded in paraffin. Each block was coronal sectioned at 5 µm thickness and stained with hematoxylin and eosin (H&E).

All slides were analyzed by light microscope (Nikon Eclipse 80i, Nikon) and all images were photographed and captured with a digital photo camera attached to the microscope for determine the histological changes. The neuronal viability was determined using 5 sections from each animal. The viable neurons in the dentate gyrus, CA1, CA2 and CA3 sub-regions of hippocampus were counted at the transverse levels of bregma -3.14 to 3.30 mm[17] within a unit area (80 x 80 µm^2)[18] and neuron in mPFC was counted at the levels of bregma 2.70 to 3.20 mm[17] in a unit area (500 x 500 µm^2).[19] Quantitative evaluation of neuron was carried out using image analysis software (Image-Pro® Plus, Media Cybernetics, Silver Spring, USA). The viable cells were defined as round-shaped and cytoplasmic membrane intact cell without any nuclear condensation or distorted aspect.[20]

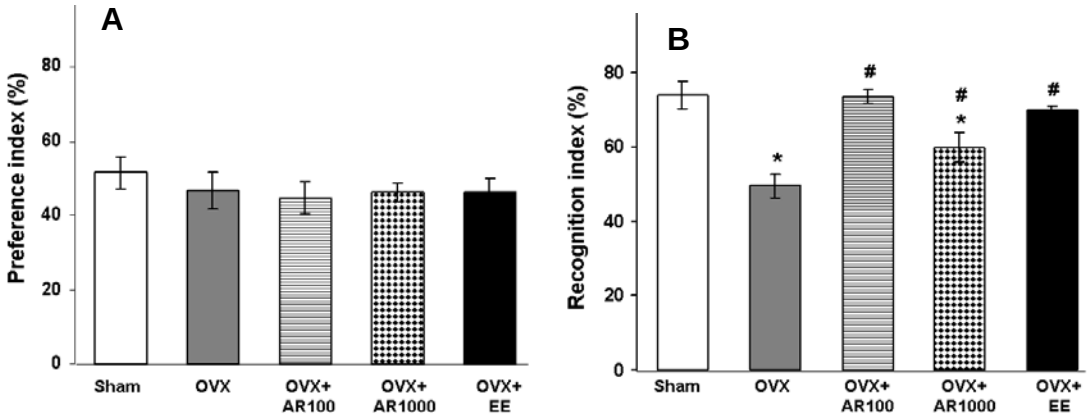

Figure 1. Effect of AR root extract on recognition memory of OVX rats were assessed by novel object recognition (NOR) test after 90 days of treatment. Results are expressed as percentage of preference index (**A**) and recognition index (**B**). Each histogram bar is expressed as mean ± SEM (sham-operated control group (Sham), OVX group (OVX), OVX treated with AR root extract 100 mg/kg group (OVX+AR100), OVX treated with AR root extract 1000 mg/kg group (OVX+AR1000), OVX treated with EE 0.1 mg/kg group (OVX+EE). $^{*}P < 0.05$, *vs* Sham, $^{\#}P < 0.05$, *vs* OVX (one-way ANOVA followed by LSD test).

Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). Comparison among experimental groups was performed by using one-way analysis of variance (ANOVA) followed by LSD test. $P < 0.05$ was taken as significant difference.

Results

Effects of AR root extract on NOR test

The statistical analysis revealed that there was not significantly different in the preference index ($F_{4, 26} = 0.398$, $P = 0.808$) (Figure 1 A) while there was significantly different in the recognition index ($F_{4, 26} = 13.251$, $P < 0.001$) (Figure 1 B). OVX group was significantly lower recognition index than the sham-operated group. In contrast, the OVX+AR100, OVX+AR1000 or EE group showed significantly higher recognition index than that OVX group (Figure 1 B).

Effect of AR root extract on serum estradiol levels

The serum estradiol level ($F_{4, 25} = 1.849$, $P = 0.043$) was significantly decreased in OVX group compared to sham group. There was not significantly different between OVX and OVX-AR 100, 1000 and EE (Figure 2).

Effect of AR root extract on histological changes

OVX group was significantly decreased the number of neurons in the CA1 region. AR 100 mg/kg or 0.1 mg/kg of EE could reverse this effect ($F_{4, 13} = 2.864$, $P = 0.03$) (Figure 3 A). Figure 3 (B) showed that the number of neurons in the CA3 region of OVX group decreased significantly compared with those in the sham ($F_{4, 14} = 3.994$, $P = 0.023$). In contrast, AR root extract 100 and 1000 mg/kg or 0.1 mg/kg of EE could significantly increase the number of neurons in the CA3 region compared to OVX group. However, there was no significant difference in the number of neurons in the dentate gyrus (the data was not shown). The OVX group exhibited significantly decreased the number of neurons in mPFC ($F_{4, 13} = 11.824$, $P < 0.001$) while OVX-treated with 100 and 1000 mg/kg of AR root extract or 0.1 mg/kg of EE showed significantly increased the number of neurons in this region (Figure 3 C).

Discussion

This study demonstrated the neuroprotective role of the ethanol AR root extract on learning and memory impairment in association with protecting the neuronal loss of hippocampus and mPFC in ovariectomized rats.

It has been recognized that 90 days after ovariectomy in young rats (10 weeks) resulted in learning and memory impairment in the Morris water maze task.[21] Some study revealed that memory performance in NOR test was impaired and spine densities in the pyramidal neurons of the mPFC and the CA1 were reduced (17-53%) seven weeks after ovariectomy. These results suggested that cognitive

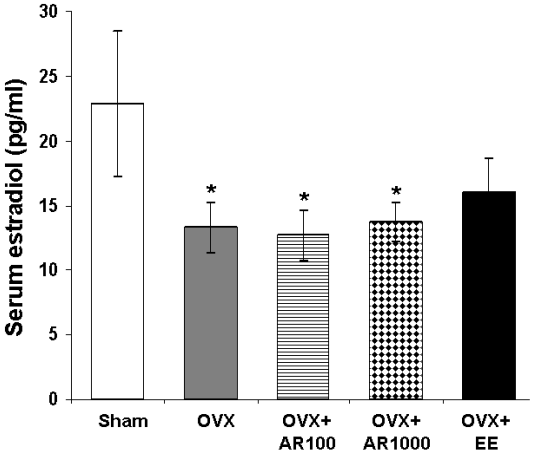

Figure 2. Effect of AR root extract on serum estradiol levels were assessed by ECLIA system after 90 days of treatment. Each histogram bar is expressed as mean ± SEM $^{*}P < 0.05$, *vs* Sham (one-way ANOVA followed by LSD test).

impairments observed in OVX rats might be associated with morphological changes in brain areas mediating memory.[7]

In this study, NOR was used to evaluate the recognition memory that consisted with familiarity and recollection component. Familiarity involves the conscious awareness without an ability to recall anything while recollection involves remembering discrete details about an experience to which the learner has been previously exposed.[22] We found that the OVX induced a decrease the percentage of recognition index and level of estradiol. These result indicated that estrogen insufficiency impaired learning and memory abilities. On the contrary, the ethanol root extract of AR could reverse the impairment of learning and memory induced by ovariectomy but it could not increase estrogen levels. A phytoestrogenic property of AR was wildly known whereas changes in serum estradiol level of OVX-treated AR were not observed in our study. A previous study demonstrated that treatment with menosan, polyherbal formulation comprising many herbals including AR, increased in uterine weight and uterine glycogen levels as compared to the OVX controls without altering the hormone level. It is suggested that menosan may binds directly to the estrogen receptors without enhancing the endogenous estrogen levels.[23] These results considered that AR root extract might be another mechanism to improve memory dysfunction induced by OVX.

AR root extract increased neuronal viability in the CA1 and CA3 sub-region of hippocampus and the mPFC area. Both hippocampus and mPFC are implicated in recognition memory.[24] AR-treated rats learned better than OVX rats in NOR test and increased neuronal viability in the CA1 and CA3 sub-region of hippocampus and mPFC. It demonstrated that the ability of AR root extract to reduced neuronal injury induced by OVX may improve the cognitive impairment. These result suggested that AR may be a beneficial agent for prevention cognitive decline induced by ovariectomy.

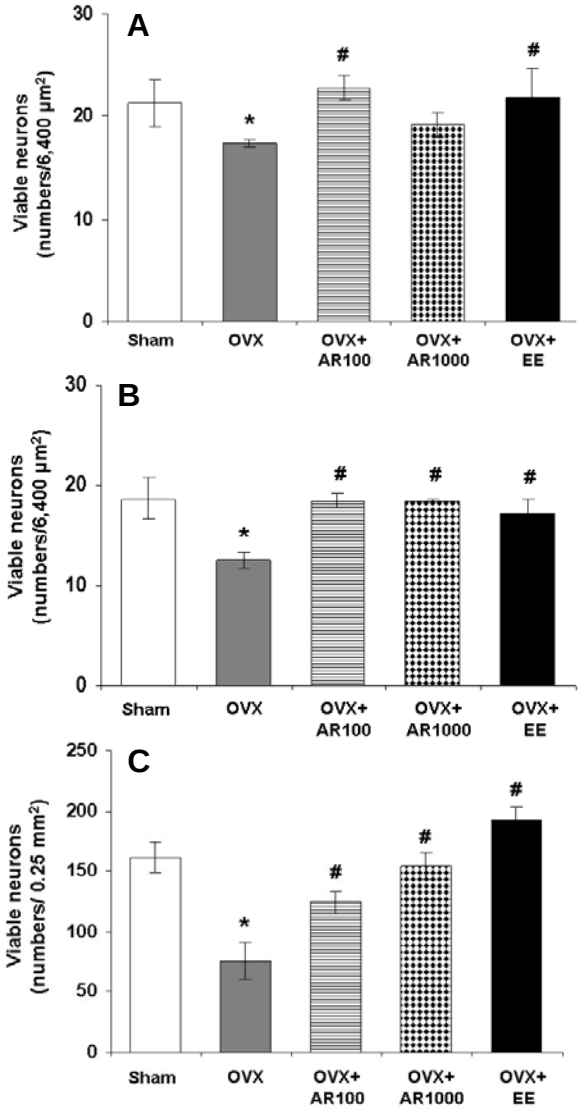

Figure 3 Effect of AR root extract on histological changes was determined by histological analysis after 90 days of treatment. The number of viable neurons was shown in the hippocampal CA1 (**A**) and CA3 (**B**) region and mPFC (**C**). Each histogram bar is expressed as mean ± SEM *P < 0.05, *vs* Sham, #P < 0.05, *vs* OVX (one-way ANOVA followed by LSD test).

Conclusion

AR could improve cognitive ability in ovariectomized model which associated with increase neuronal viability of mPFC and hippocampus. Future studies are needed to identify the active substance(s) of AR and to examine their pharmacological properties.

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Conflict of Interest

None to declare.

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