

The Effects of *Puerariae Flos* on Stress-induced Deficits of Learning and Memory in Ovariectomized Female Rats

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Puerariae flos (PF) is a traditional oriental medicinal plant and has clinically been prescribed for a long time. The purpose of the present study was to examine the effect of PF on repeated stress-induced alterations of learning and memory on a Morris water maze (MWM) test in ovariectomized (OVX) female rats. The changes in the reactivity of the cholinergic system were assessed by measuring the immunoreactive neurons of choline acetyltransferase (ChAT) in the hippocampus after behavioral testing. The female rats were randomly divided into four groups: the nonoperated and nonstressed group (normal), the sham-operated and stressed group (control), the ovariectomized and stressed group (OS), and the ovariectomized, stressed and PF treated group (OSF). Rats were exposed to immobilization stress (IMO) for 14 d (2 h/d), and PF (400 mg/kg, p.o.) was administered 30 min before IMO stress. Results showed that treatments with PF caused significant reversals of the stress-induced deficits in learning and memory on a spatial memory task, and also increased the ChAT immunoreactivities. In conclusion, administration of PF improved spatial learning and memory in OVX rats, and PF may be useful for the treatment of postmenopausal-related dementia.

Key Words: *Puerariae flos*, Ovariectomy, Morris water maze, Choline acetyltransferase, Immobilization stress

INTRODUCTION

Gender-related differences in stress response and susceptibility to stress-associated disorders are consistently observed, and are proposed to at least partially depend on sex hormonal status (Barrett and Bush, 1991; Baum and Grunberg, 1991; Saunders et al., 1999; McEwen, 2001a; McEwen, 2001b). Women have a higher incidence of some stress-related disorders, such as depression, while men are generally at a greater risk for cardiovascular diseases, such as myocardial infraction and hypertension, than age-matched, premenopausal women (Reckelhoff, 2001). The higher incidence and severity of depression are associated with the presence or absence of ovarian hormones (Shors et al., 2003).

The ovarian hormones which are related to the menopause-induced depression influence the hippocampal anatomy and physiology; therefore, they affect behavior in adult female rats (Nancy and William, 1997; Wolly, 2000). The hippocampus plays an important role in the negative feedback mechanism of the limbic-hypothalamic-pituitary adrenal (LHPA) axis. It expresses high levels of adrenal steroid receptors and is susceptible especially to damage as a result of stress (Kellendonk et al., 2002). In the hippocampus,

stress hormones induce regression of the dendritic processes and cell death, inhibit neurogenesis, and impair the ability of neurons to survive noxious stimuli such as seizure, hypoxia-ischemia, metabolic poisons, hypoglycemia and oxygen radical generators (Sapolsky, 2000). The damage caused by a heightened stress response which results from dysregulation of the LHPA axis has been suggested to play a role in the reduced hippocampal volume which is often associated with major depression (Bremner et al., 2000; Sheline, 2000). The atrophy of hippocampus induces the dysfunction of memory-related hippocampal neurons (Fibiger et al., 1991; Janowsky and Overstreet, 1995).

Puerariae flos (PF) is one of the earliest medicinal plants to be used in Asia. PF is known to have hypoglycemic, antioxidant, antimutagenic, hypolipidemic, antidiabetic, antithrombotic, and antiallergic activities (Yamazaki et al., 2005). It has also been reported that it is useful in treatment of alcoholism. For example, decreased blood alcohol level, hepatoprotection and elimination of acetaldehyde of the blood were reported after treatment of PF (Bammer and Chesher, 1982; Matsunaga and Mukasa, 1986; Yamazaki et al., 2002). However, a question of whether repeated-treatment of PF improves a deficit of learning and memory in ovariectomized and stressed rats or not has hardly been examined.

The aim of the present study was to explore the behavioral and the neurobiological effects of PF on ovariectom-

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ABBREVIATIONS: LHPA, limbic-hypothalamic-pituitary adrenal; PF, *Puerariae flos*; ChAT, choline acetyltransferase; IMO, immobilization stress; OVX, ovariectomy.

ized female rats and to form a basis for clinical treatment. Memory improving effect of PF was tested *via* a Morris water maze (MWM) and immunohistochemical changes of choline acetyltransferase in the hippocampus.

METHODS

Subjects and stress procedure

Sprague Dawley female rats at the age of 8 weeks (Samtaco, Inc. Korea) were used for the study. The rats were housed under a controlled temperature (22~24°C) with a 12 h light/dark cycle. The lights were on from 8:00 to 20:00. Food and water were available *ad libitum*. They were allowed at least 1 week to adapt to their environment before the experiments. The animal experiments were carried out in accordance with the Prevention of Cruelty to Animals Act 1986 and NIH guidance for the care and use of laboratory animals for experimental procedures, and were approved by local committee review.

The female rats were randomly divided into four groups (n=6 per group): the nonoperated and nonstressed group (normal), the sham-operated and stressed group (control), the ovariectomized and stressed group (OS), and the ovariectomized, stressed and PF treated group (OSF). Using aseptic conditions, bilateral ovariectomy was performed under general anesthesia with pentobarbital sodium (50 mg/kg, i.p.). After postoperative recovery for 7 days, the ovariectomized rats were stressed daily. Stress was produced by forcing the animals into an immobilizer device (a disposable rodent restraint cone, Havard Instrument, U.S.A.) for 2 h (10:00~12:00 a.m.) for 14 days. From the 8th day after the first immobilization, PF group was treated daily with the *Pueraria flos* extract (400 mg/kg, p.o.) for 2 weeks, and other groups were given sterile saline. Immobilization began 30 min after the treatments.

Preparation of herbal extracts

PF was purchased from an oriental drug store (Jungdo Inc. Seoul, Korea). The boucher specimens are deposited at the herbarium located in the College of Oriental Medicine, Kyung Hee University. The dried PF samples (200 g) were immersed in a 10-fold volume of distilled water, boiled at 80°C for 1 h, and then the water extract was collected. The process was repeated once more, and the extracts were combined and concentrated with a rotary evaporator and vacuum-dried to yield 9.1% (w/w) of the extract.

Morris water maze (MWM) test

After 7 days of immobilized stress, all the animals started training on a MWM task in a swimming pool (1.8 m diameter and 0.5 m high, filled with milky water at a temperature in the 22±2°C range) for 7 days. A 12 cm diameter round platform was hidden in a constant location (the quadrant center) within the pool with its top surface submerged 1.5 cm below the water level. The rats were trained to locate the hidden island in four trials per day for 6 days. On the 7th day, they started in the quadrant opposite to the target and were forced to swim for 60 s in the pool without a platform. The spatial memory of the rats was assessed as the latency time. The time spent searching for the platform in the training quadrant, *i.e.*, the previous location of the

platform, was recorded and used as a measure of memory retention. A video camera was mounted on the ceiling above the pool and was connected to a video-recorder and tracking device (S-MART; Pan-Lab, Barcelona, Spain), which permitted on-line and off-line automated tracking of the path taken by the animal.

ChAT Immunohistochemistry

At the end of the behavioral observation, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and then perfused transcardially with 100 ml of saline, followed by 500 ml of a 4% solution of formaldehyde prepared in phosphate buffer. The brains were then removed, postfixed in the same fixative for two to three hours at 4°C and then placed overnight at 4°C in PBS containing 20% sucrose. On the following day, the brain was cut into coronal sections that were sliced to 30 µm-thicknesses. Sections were processed for choline acetyltransferase (ChAT) immunoreactivity by using sheep anti-ChAT polyclonal antibody (Chemicon international, Temecula, CA). The primary antibody was prepared at a dilution of 2000× in 0.3% PBST, 2% normal rabbit serum and 0.001% kehole limpit hemocyanin (Sigma, USA). The sections were incubated in the primary antibody for 72 h at 4°C. Following rinsing in PBST, the sections were incubated for 2 h at room temperature in biotinylated rabbit anti-sheep secondary antibody (Vector Laboratories, Burlingame, CA) that was diluted 200× in PBST containing 2% normal rabbit serum. After three more rinses in PBST, the sections were placed in Vectastain Elite ABC reagent (Vector laboratories, Burlingame, CA) for 2 h at room temperature. Following a further rinsing in PBS, the tissue was developed using diaminobenzadine (sigma, USA) as the chromogen. Images were captured using an Axio Vision 3.0 imaging systems (Zeiss, Oberkochen, Germany) and processed in Adobe Photoshop. For measuring cells of ChAT, the grid was placed on CA1 and CA3 in the hippocampus areas according to the method of Paxinos G. *et al* (Paxinos et al., 1985). The number of cells was counted at 100x magnification using a microscope rectangle grid measuring 200×200 µm.

Statistical analysis

Statistical comparisons for the behavioral and histochemical studies were done using the one-way ANOVA, respectively, followed by LSD *post hoc* test. All results are presented as means±S.E.M., and we used SPSS 15.0 for Windows for analysis of the statistics. The significance level was set at $p < 0.05$.

RESULTS

Morris water maze test (MWM)

Fig. 1 shows mean group latencies to reach the hidden platform in the MWM for all groups for 6 days. There were no statistically significant differences among the groups.

To examine the spatial memory on the 7th day of retention test, the time spent swimming to the platform was compared among the groups as seen in Fig. 2. The times spent around the platform were significantly different among the groups ($F_{3,24}=4.859$, $p < 0.01$); the normal and

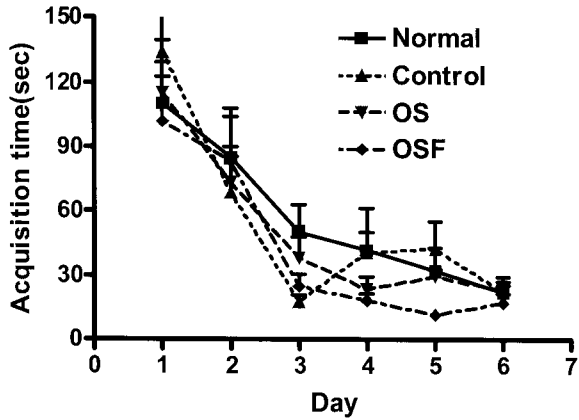


Fig. 1. Changes of the latency time during 6 d of the acquisition test in the Morris water maze test. Repeated measures of ANOVA of swimming time among the groups, followed by LSD test. Each value represents mean±S.E.M.

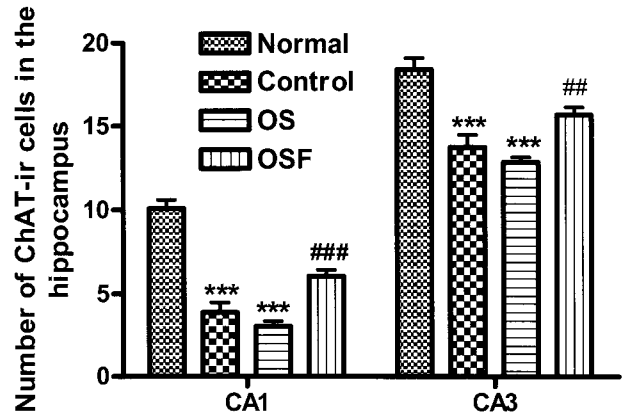


Fig. 3. The number of choline acetyltransferase (ChAT) immunostained nuclei in the different hippocampal areas of the experimental groups after 7 d of the water maze test. The results of ChAT-reactivity were analyzed by performing separate one-way ANOVA of neurons among the groups, followed by LSD test. Each value represents mean±S.E.M. ***p<0.01 compared to normal and #p<0.01, ###p<0.001 compared to OS.

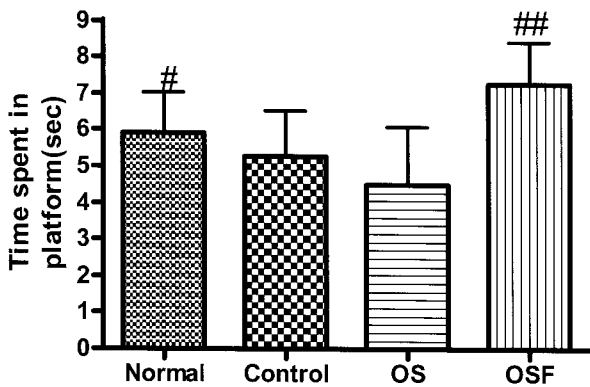
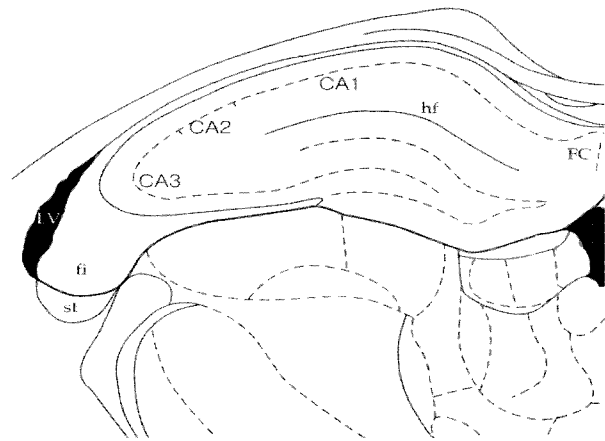


Fig. 2. The latency time on the 7th day of the retention test in the Morris water maze test. The results of retention test were analyzed by performing separate measures of one-way ANOVA of swimming time among the groups, followed by LSD test. Each value represents mean±S.E.M. #p<0.05 and ##p<0.01 compared to OS.



OSF groups spent more time around the platform than the OS group ($p<0.05$ for the normal group, $p<0.01$ for the OSF group). PF treatment attenuated the repeated stress and ovariectomy-induced deficit of learning and memory on the water maze.

Immunohistochemistry of ChAT

The results on the ChAT immunoreactive cells per section from different hippocampal formations are shown in Fig. 3 and 4. The number of ChAT neurons in the CA1 area was 10.1 ± 0.6 in the normal group, 3.9 ± 0.6 in the control group, 3.0 ± 0.3 in the OS group and 6.0 ± 0.4 in the OSF group ($F_{3,62}=43.3$, $p<0.001$), thus indicating two-fold increases in the number of ChAT neurons in the OSF group compared to that of the OS ($p<0.001$). The ChAT immunoreactive cells in the CA3 area were 18.4 ± 0.6 in the normal group, 13.7 ± 0.8 in the control group, 12.9 ± 0.3 in the OS group and 15.7 ± 0.5 in the OSF group [$F_{3,62}=20.1$, $p<0.01$]. Thus, the number of ChAT positive neurons in the OSF group was

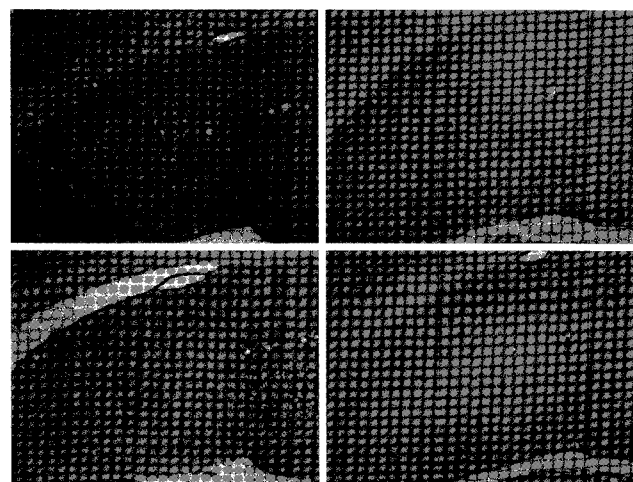


Fig. 4. Photographs showing the distribution of ChAT-immunoreactive cells in the hippocampus of Normal group (A), Control group (B), OS group (C), OSF group (D). Sections were cut coronally at 30 μm and the scale bar represents 200 μm (200×200).

increased to 121.7% of the OS ($p < 0.01$).

DISCUSSION

In this study, we investigated whether treatment with PF can improve impairment of memory and affect cholinergic cells (ChAT) in the hippocampus of ovariectomized rats. Results showed that both PF ameliorated stress-induced deficits of memory measured by in the MWM, and displayed an increased ChAT immunoreactivity in the hippocampal CA1 and CA3 areas of ovariectomized rats.

Ovariectomy in female Sprague-Dawley rats, which has been a widely used in vivo model, is characterized by progressive memory deficits, degeneration of central cholinergic nerve system and differentiation imbalance (Gur et al., 1991; Ohkura et al., 1994; Yamazaki et al., 2002).

The Morris water maze is well-established paradigm for evaluating deficits in hippocampal-dependent memory, and the MWM spatial learning task has been used in the validation of rodent models for neurocognitive disorders and for the evaluation of possible neurocognitive treatments (Mcgonigal et al., 1974–1988; Matsunaga and Mukasa, 1986; Hooge and De deyn, 2001; Luine et al., 2003; Sato et al., 2003). In this study, spatial memory tended to be improved in OSF group during the training days, but not in the OS group. Our results of retention test revealed that PF treatment significantly increased the time spent in platform as compared with the OS rats, suggesting that PF ameliorated disordered learning of OS rat. The data of spatial probe trial demonstrated that PF protects the animals from the OVX-induced decrease of the spatial retention, especially long-term memory.

The degeneration of the cholinergic innervation from the basal forebrain to the hippocampal formation in the temporal lobe is thought to be one of the factors to determine the progression of memory decay, both during normal aging and Alzheimer's diseases (Wu et al., 1999). The best available marker for cholinergic neurons in the basal forebrain is ChAT activity. ChAT synthesizes the neurotransmitter acetylcholine in the basal forebrain, cortex, hippocampus, and amygdala. A significant reduction in ChAT activity has been reported in the postmortem brains of demented patients. In addition, there is a 20~50% decrease in ChAT activity in the hippocampus of the OVX rats (Rabbani et al., 1997). However, in the present study, treatment with PF prevented stress-induced loss of ChAT-immunoreactive neurons, suggesting that PF exerts beneficial effects on cholinergic neurotransmission in the brain by increasing the hippocampus ChAT activities. These behavioral and neurochemical results indicate that the memory-enhancing potentials of PF may be mediated by cholinergic mechanisms in the rat brain.

PF is known to be a rich source of isoflavonoids and triterpenoid saponins, and isoflavonoid and triterpenoid saponins have been shown to have an ameliorating effect on memory (Parkard, 1998; Yamazaki et al., 2002). Isoflavonoid phytoestrogens have weak agonist activity at estrogen receptors, and Packard (1998) reported that estrogen selectively influences memory storage independent of its effect on non-mnemonic processes, and that estrogen interacts with cholinergic systems in memory modulation. Also, the genistin has antioxidant properties similar to estradiol and could be used as a substitute for estradiol to prevent or treat central neurodegeneration in postmenopausal

woman (Xu J et al., 2007). Based on the above evidence that isoflavonoid phytoestrogens may enhance memory through cholinergic neuronal functions, it is not, therefore surprising that PF, which is rich in isoflavonoids such as tectridin, has an ameliorating effect on impairment of learning and memory.

The above observations indicate that administration of PF accelerates the synthesis of acetylcholine in the hippocampus, thereby may improving the memory-related behavior in ovariectomized and immobilization stressed rats. Further studies are necessary to deepen our understanding of the pharmacology of PF and to elucidate the mechanism of action of these effects by using purified PF constituents for the treatment of postmenopausal related dementia.

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