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The effects of an herbal medicine Bu-Wang-San on learning and memory of ovariectomized female rat

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ABSTRACT

Ethnopharmacological significance: Bu-Wang-San (BWS) is a traditional Chinese herbal medicine for the treatment of learning and memory impairment. The effect of BWS on neuroprotection and how BWS increases CA1 dendritic spine synapse density in menopaused women was investigated in the model of ovariectomized (OVX) rats.

Materials and methods: Sixteen OVX rats were divided into two groups, the OVX group and OVX + BWS group. After 3 months, Morris water maze was used to assess spatial acquisition and spatial retention. Swim time, swim distance, swim speed, quadrant time and platform crossing were recorded. The ultrastructure of the pyramidal cell and spine synapse density were examined by transmission electron microscopy (TEM).

Results: In the spatial acquisition and spatial retention phase of testing, BWS group functioned significantly better than control group. Ultrastructural observation of the hippocampal CA1 region of OVX group showed swelling of mitochondria, the broken and reduced cristas and even crista dissolution; however, the mitochondria were protected well in BWS group. In addition, BWS significantly increased spine synapse density.

Conclusions: These results suggested that BWS could improve cognitive ability of menopause-induced learning and memory impairment. The positive effect of BWS on rat learning and memory was associated with increase of spinal synapse density and protection of mitochondrial function of the pyramidal cell in hippocampal CA1 region from menopause-induced injury.

1. Introduction

Menopause marks the end of reproductive capacity of women and results from the permanent cessation of ovarian function. Natural or surgical menopause is confirmed by absence of menstrual periods for 12 consecutive months, excluding other obvious pathologic or physiologic causes (Notelovitz, 1989). Some symptoms such as hot flashes, tiredness, irritability, insomnia, palpitations, memory or concentration difficulties, and mood swings or depression begin in the peri-menopause and increase as women progress through the menopause (Hardy and Kuh, 2002). Many studies have shown that learning and memory may be negatively affected altered by the loss of estrogen after menopause (Sherwin, 1988). These changes can be ameliorated by estrogen replacement therapy (ERT) (Ditkoff et al., 1991; Sherwin, 1994; Kimura, 1995). As a neuroprotective and neurotrophic factor, estrogen (E_2) helps maintain memory and cognition (Sughrue and Merchenthaler, 2000; Wise et al., 2001), decreases the risk and delays the onset of neurological disorders, e.g. Alzheimer's disease (AD). Indeed, estrogen has been shown to increase cerebral blood flow, to act as an anti-inflammatory agent and enhance neural synapse activity (Toran-Allerand et al., 1999; Roof and Hall, 2000). Numerous studies indicate that estrogen is essential for optimal brain function (Toran-Allerand et al., 1999; Roof and Hall, 2000; Sughrue and Merchenthaler, 2000; Wise and Dubal, 2000; Wise et al., 2001). However, the above-mentioned health benefits of ERT were often overshadowed by the serious side-effects of estrogen use in menopaused women. Specifically, long-term use of estrogen in postmenopausal women may lead to the increased risk of endometrial and breast cancer (Hammond, 1994; Grady et al., 1995; Anonymous, 1997). Accordingly, there has been a growing interest in alternative therapies. One alternative employs phytoestrogens, the other alternative employs Chinese herbal medicine.

Phytoestrogens as nonsteroidal plant compounds that are structurally or functionally similar to estrogens and may have similar beneficial effects. These substances have a 2-phenylnaphthaline-type chemical structure and bind to estrogen receptors in vitro and in vivo (Welshons et al., 1987; Lephart et al., 1996; Pan et al.,

Abbreviations: OVX, ovariectomized; E_2, estrogen; TEM, transmission electron microscopy; ERT, estrogen replacement therapy; BWS, Bu-Wang-San.

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1999a,b; Patisaul et al., 1999; Jacob et al., 2001; Kim et al., 2001). Specifically, dietary supplementation with isoflavones or lignans had an estrogenic effect, as shown by the maturation of vaginal epithelium on cytology (Wilcox et al., 1990).

Chinese herbal medicine has been used for thousands of years in China and other Asian countries. In clinical practice of traditional Chinese medicine (TCM), it is common to modify original formula by adding or substituting herbs in accordance with a patient's condition to enhance the efficacy of the original formula. Most of the regular use of traditional Chinese medicine is not associated with serious side effects. Specific formula of traditional Chinese herbal medicines has been reported to be effective against cognitive disorder (Junping et al., 2005; Hai-Fa et al., 2006; Young-Ju et al., 2007). However, there was little information available in literature about whether herbal medicines with neuroprotective effect could affect postmenopausal cognitive disorder. Bu-Wang-San (不忘散, BWS), a classical Chinese herbal formula, has been used to treat the postmenopausal cognitive disorder for years in clinic. The mechanism as to how BWS protects learning and memory has not been studied. Pyramidal neurons in the hippocampal CA1 are particularly vulnerable from impairment. We have used ovariectomized rat as the estrogen-depleted, postmenopausal model (Kalu, 1991) to examine the effect of BWS on postmenopausal cognitive disorder. In addition, we have studied the effect of BWS, for the first time, on the change of ultrastructure of CA1 region and spinal synapse density with electron microscope.

2. Materials and methods

2.1. Composition and preparation of Bu-Wang-San

BWS consists of four medicinal compositions as shown in Table 1. All of these herbs were purchased from Jian-lian Company of Traditional Crude Drugs (Jinan, China), and carefully authenticated by Dr. Li-Hong Zhong, Pharmaceutical Preparation Section, Qilu Hospital Affiliated to Shandong University, Jinan, China. Voucher specimens (numbers were listed in Table 1) were deposited at the Herbarium of Shandong University (Jinan, China). After drying, these herbs were mixed in proportion. Forty-eight grams of the mixed material was mixed with 300 ml of distilled water and boiled for 1 h at 100 °C. The extract was filtered, and the residual medicine was boiled in water following the same procedure once more. Finally, the pool of the extracts from two boiling and filtering was lyophilized to form a dried powder. The yield of BWS extract was 25% (w/w) of the original herbs. The resulting lyophilized powder, stored at 4 °C, was diluted to the appropriate concentrations with distilled water and filtered before use.

2.2. Animals and treatment

2.2.1. Animals

Ten-week-old virgin female Wistar rats weighing 260–300 g were purchased from Shandong University Laboratory Animal Shelter (Shandong, China). Rats were housed in individual cages under controlled environmental conditions (22 ± 2 °C relative humidity 40–60%, 12-h dark/light cycles, food and water *ad libitum*). All rats were treated parallelly in terms of daily manipulation.

Table 1
Recipe of Bu-Wang-San (BWS) formulation

Components	Voucher specimens	Part used	Amount used (g)
Panax ginseng C.A. Mey	SDU0810	Root	6
Acorus gramineus Soland	SDU0156	Stem and root	12
Poria cocos (Schw.) Wolf	SDU0123	Dried sclerote	20
Polygala tenuifolia Willd	SDU0241	Root	10

2.2.2. Ovariectomy and medical treatment

The rats were divided randomly into three groups; the first group was given a sham operation (Sham group), the others were ovariectomized (OVX) (EI-Bakri et al., 2004). They were either bilaterally ovariectomized or sham-operated through dorsal incision under anaesthesia with 3% sodium pentobarbital (30 mg/kg, i.p.). The next day, ALL post-operative rats were injected with penicillin (22,000 u.i./kg) for 3 days, and the vaginal smear was taken from each rats for 4 days. Rats of the Sham group with a classic estrous cycle were selected ($n = 8$). Success of the ovariectomization was confirmed by demonstration of predominantly leukocytes with few epithelial cells in vaginal smears over at least 4 days. The successful OVX rats were further randomly divided into two groups ($n = 8$ equally): OVX group, OVX + BWS group. The next day, the Sham group and the OVX group received distilled water; and the OVX + BWS group received Bu-Wang-San (2.4 g/(kg day)) by the oral gavage daily for 3 months. The dose of BWS was determined by conversion of regular dose for human to that for rat and also by a pilot experiment with different doses of BWS for rat. All surgical procedures and protocols used were in accordance with the Guidelines for Ethical Care of Experimental Animals, which was approved by the Shandong University Animal Care and Use Committee.

2.3. Morris water maze

All rats were put into the Morris water maze to assess learning and memory performance on a spatial orientation task (Morris, 1981). A circular 180-cm diameter swimming pool made of black polyethylene was filled 32-cm deep with 25 ± 2 °C water. The water was made opaque by the addition of pure milk powder (Inner Mongolia Yili Industrial Group Co., Ltd., China). A platform, which consisted of a round transparent lucite platform (10 cm diameter and 30 cm high) invisible to the rat, was hidden below the surface of water in one of the four quadrants of the pool. Conspicuous visual cues outside the pool were provided for orientation. A video camera suspended above the pool was connected to a video tracking system (MI-200, Chengdu Taimeng Technology & Market Co., Ltd., China) that recorded that recorded the swimming pattern including the length of the swim path on each trial.

2.4. Behavioral test

2.4.1. Spatial acquisition

Testing was conducted between 8:30 to 11:30 a.m. and 1:30 to 4:30 p.m.; rats were trained throughout 4 days, with eight trials per day, and there was a 2-min break between each trial. The first day, rats were initially placed on the platform and allowed to stay there for 30 s. They were then placed in the pool at the edge of the platform with their front paws touching it and were allowed to climb out of the water onto the platform and stand for 30 s. This was repeated three additional times. Finally, they were placed at the edge of the pool and allowed to swim to the cued platform and climb onto it. Animals that failed to locate the platform within 120 s were manually guided to it. From the second day to the fourth day, the test was carried out in following way: rats were placed at the edge of the pool. The point of placing rats was each alternative midpoint of the four quadrants. Rats were allowed to swim to the submerged platform and climb onto it and stayed there for 30 s. Rats that failed to locate the platform within 120 s were manually

guided to it. We recorded the swim time (s), swim distance (cm) and swim speed (cm/s) on each trial.

2.4.2. Spatial retention

Spatial probe trial was conducted 1 and 24 h after the spatial acquisition phase to determine the short-/long-term memory. Each rat was allowed to swim for 60 s. During this trial, the platform was removed from the pool. We measured the parameters including quadrant time (percentage of time spent in the quadrant in which the platform was located in the spatial acquisition phase) and platform crossings (the number of times the rat crossed the exact location of the platform).

2.5. Ultrastructural observation and spine synapse density

Rats were deeply anesthetized with 3% sodium pentobarbital (30 mg/kg, i.p.) and perfused through the ascending aorta sequentially with 100 ml of physiological saline (0.9% NaCl) and 60 ml of 3% glutaraldehyde. The region of the forebrain containing the hippocampal formation was removed and cut into blocks. Blocks were then retrimmed into 1 mm^3 and further fixed in 3% glutaraldehyde overnight. The next day, the blocks were washed three times with 0.2 mol/l phosphate buffer and were fixed with 1% osmium tetraoxide, washed with 0.2 mol/l phosphate buffer again, and dehydrated by different concentrations of ethanol. The sections were immersed in fresh Spon812 resin/acetone (1:1) for 30 min, and embedded and convergenced overnight at 70 °C. Semi-thin sections were obtained and stained with toluidine blue for light microscopic examination to locate the pyramidal cell. Thin sections (50 nm) were made with an ultramicrotome and stained with 2% uranyl acetate and lead citrate. The ultrastructure of the pyramidal cell in CA1 region and was observed using H-7000FA transmission electron microscopy (TEM) (Hitachi Co., Ltd., Tokyo, Japan). The photos were taken with magnifying times of 5000–10,000. Analysis of synaptic density was performed by counting all synapses in each photo. We used an indirect counting technique based on geometrical assumptions. N_v are based on the relationship between the number of profiles of objects per unit area of the section, Q_A (total number synapses/photo), the caliper height of the objects in a direction normal to the plane of the section, H (rate of synapses/photo), and the thickness of the sections, h (50 nm): $N_v = Q_A/(H + h)$ (Cruz-Orive, 1987). The number of synapses (N) was also counted. Synapse was defined as having both a postsynaptic density and at least two vesicles in the presynaptic density and at least two vesicles in the presynaptic terminal no more than 0.2 μm from the synaptic cleft.

2.6. Statistical analysis

All statistical analysis was performed using SPSS software (Version 10.0, SPSS Inc., Chicago, IL, USA). Data was expressed as mean ± S.E.M. Statistical comparisons were performed by one-way analysis of variance (ANOVA). When one or more of the groups were found to follow a non-normal distribution, nonparametric statistics were used for analysis. The level of significance was accepted at $P < 0.05$.

3. Result

3.1. Spatial acquisition

In the spatial acquisition phase of testing, the effect of testing was significant for both swim time and swim distance, and there was a significant treatment by block interaction. On this trial, OVX groups had significantly longer swim time than Sham and BWS groups, which was confirmed by conducting a separated repeated measure ANOVA for each group. Animals in Sham and BWS groups did achieve the shorter swim time at the end of the whole trial. Comparison of the three groups at the end of the experiment showed that untreated OVX rats had significantly longer swim time than did both two other groups, whereas OVX rats treated with BWS demonstrated reduced swim time, which was close to swim time of Sham rats. Moreover, there was a significantly decreased trend for swim time both in Sham group and BWS group, Block one of Sham and BWS groups had longer swim times than Block six of Sham and BWS group ($P < 0.01$), but there was no differ between Block one and Block six of OVX group ($P > 0.05$) (Fig. 1A).

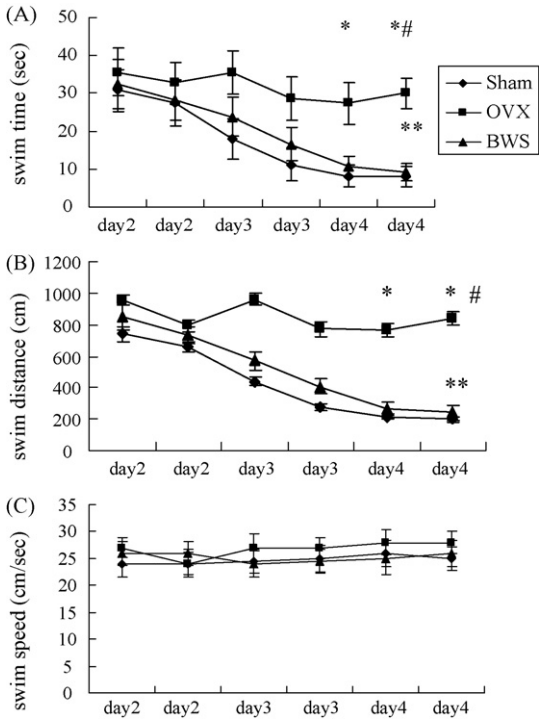

Fig. 1. Performance in the spatial acquisition task as illustrated by (A) swim time, (B) swim distance and (C) swim speed. BWS-treated rats displayed significantly short swim times and swim distances than OVX rats during day 3 (*$P < 0.01$). Swim times and swim distances in Block one of Sham and BWS group were shorter than these in Block six (**$P < 0.01$), there was no significant differ between Block one to Block six of OVX group (#$P > 0.05$). OVX and BWS treatment did not affect swim speeds. Each symbol represents the mean ± S.E.M. OVX, ovariectomized; BWS, Bu-Wang-San.

As was done for swim time, there was a significant of block on swim distance. BWS again showed a dramatic positive effect when swim distance is used for analysis. OVX rats had significantly longer swim distances than did both two other groups. These animals in BWS group required significantly less swim distance during the trial. Swim distance of BWS group is close to that of Sham group. And there was a decreased trend for swim distance both in Sham

Table 2
Group mean (±S.E.M.) for the quadrant time and platform crossings

Group	Quadrant time (%)		Platform crossings (n)	
	Short-term	Long-term	Short-term	Long-term
Sham	38.80 ± 4.29	31.70 ± 4.58	4.13 ± 0.83	3.75 ± 1.04
OVX	24.60 ± 4.67*	18.40 ± 4.71*	2.88 ± 0.83	2.25 ± 0.71
BWS	35.90 ± 4.32**	29.30 ± 5.02**	3.75 ± 0.71#	3.63 ± 0.92##

Both 1 and 24 h spatial retention trial OVX rats caused a reduction in the target quadrant relative to Sham rats (*$P < 0.01$), but BWS treatment prevented this decline for OVX rats relative to Sham rats (**$P > 0.05$). Rats in BWS group had more significantly platform crossings than those in OVX group (#$P = 0.01$, ##$P < 0.01$).

and BWS group ($P < 0.01$), but no significantly decreased trend in OVX group ($P > 0.05$) (Fig. 1B).

There was no effect of block or treatment on swim speed, and no treatment by block interaction (Fig. 1C).

3.2. Spatial retention

In the spatial retention trial, both quadrant time and platform crossings were significantly affected by BWS (Table 2). BWS treatment protected against the OVX-induced decrease in the retention for the target quadrant during the trial. In both 1 and 24 h spatial retention trial, quadrant time in OVX rats (24.60 ± 4.67, 18.40 ± 4.71) are significantly reduced in comparison to that in Sham rats (38.80 ± 4.29, 31.70 ± 4.58), but BWS treatment prevented this decline in OVX rats, especially during the 24 h spatial retention trial. Rats in BWS group had more significantly platform crossings than those in OVX group.

3.3. Ultrastructural observation

Ultrastructural observation in the hippocampal CA1 region of the OVX group showed that the pyramidal cells were severely edematous with nucleus membranes crimpled or ruptured, chromatin aggregated, and the mitochondria swollen like empty-bubble, and the cristas broken and reduced or largely dissoluted. In contrast, the mitochondria were protected well, and the changes were less prominent in BWS groups. In fact, the ultramorphology in BWS group resembles that in Sham group (Fig. 2).

3.4. Spinal synapse density

The average spinal density per 10 μm is shown in Fig. 3. Spine synapse density in the CA1 region of the hippocampus was significantly increased by BWS treatment (78.40 ± 2.14) in comparison to that the OVX group (60.30 ± 3.57). The BWS group had no significant difference from the Sham group (80.30 ± 3.48).

4. Discussion

The present study clearly showed a neuroprotective role of BWS in ovariectomized rat in association with an increase of CA1 dendritic spinal synapse density. It has been recognized that OVX for 6 days in young animals results in synaptic loss between noradrenergic terminals and gonadotropin hormone releasing hormone (GnRH) neurons, long-term OVX, hypothesized to protect against neuroendocrine aging, failed to guard against any age-related changes (Miller et al., 1998). Interactions of the estrogen receptor system with various growth factors were important for the neurite growth and differentiation (Singh et al., 1999). Some factors can ameliorate the memory disorder of ovariectomized rat by increasing synaptic sprouting, increasing cholinergic activity in the hippocampal formation, protecting neurons against amyloid-induced toxicity or other excitotoxic events (Gibbs, 1994; Morrison and Hof, 1997; Stone et al., 1998).

The Morris water maze is a well-established paradigm for evaluating deficits in hippocampal-dependent memory. In particular, learning and memory deficit is demonstrated by the extended time in acquisition and retention. The Morris water maze was used to test spatial memory in many studies, e.g. a study of exogenous estrogen replacement found that estrogen given to ovariectomized female rats can improve spatial memory (Markham et al., 2002). Others have found that chronic exogenous estrogen can impair spatial memory in the water maze in both rats and mice (Fugger et al., 1998; Holmes et al., 2002). In this study, the fact that spatial memory continued to improve significantly in BWS group during the three training days, but not in the control OVX group, suggests that BWS ameliorated disordered learning of OVX rat. The data of spatial probe trial demonstrated that BWS protects against the OVX-induced decrease of the spatial retention, especially long-term memory.

Estrogen-induced increases in hippocampal plasticity leads to enhanced memory function. Estrogen may prevent impairment of transport systems that maintain ion homeostasis and energy metabolism, and thereby forestall excitotoxic synaptic degeneration and neuronal loss in disorders such as AD and ischemic

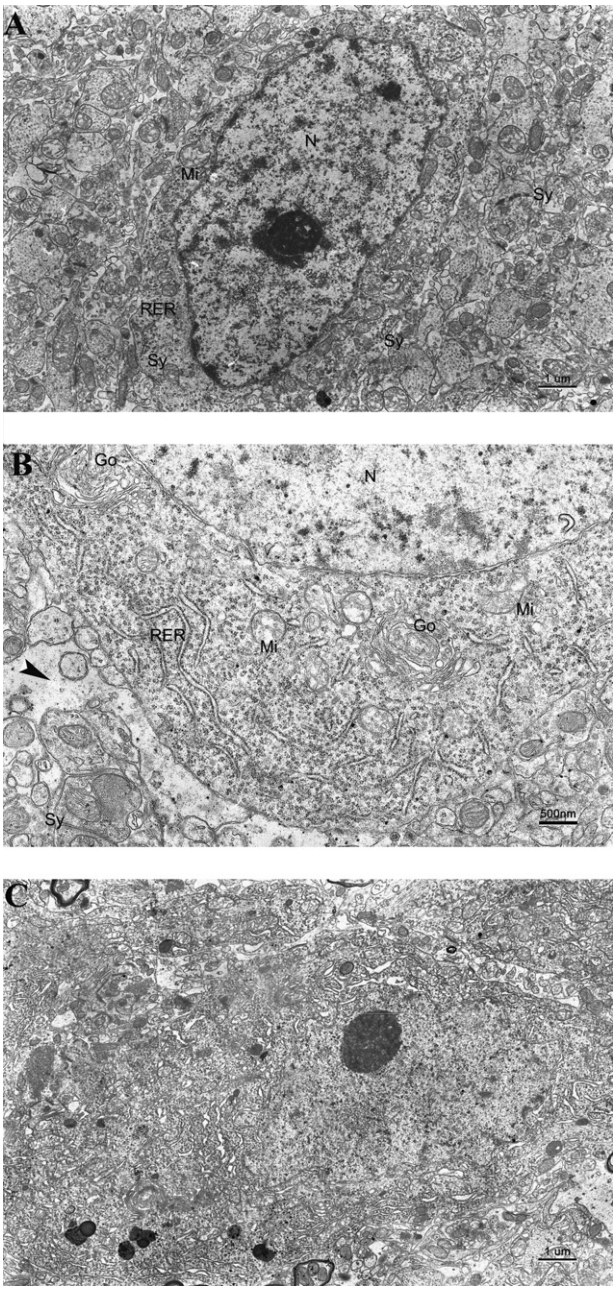

Fig. 2. Ultrastructural observation in the hippocampal CA1 region in three groups. (A) The hippocampal CA1 region of the Sham group was observed that nucleus membranes, chromatin and the mitochondria of pyramidal cells were normal. (B) The hippocampal CA1 region of the OVX group showed that the pyramidal cells were edematus severely, nucleus membranes crimpled or ruptured, chromatin aggregated, and the mitochondria swelled obviously, like empty-bubble, and the cristas broke and reduced or disappeared largely. (C) The mitochondria were protected well, and the changes were less prominent in BWS groups like those of Sham group. Bar = 1 μm in (A) and (C); Bar = 0.5 μm in (B). Mi, mitochondrion; Go, Golgi apparatus; RER, endocytoplasmic reticulum; N, nucleus.

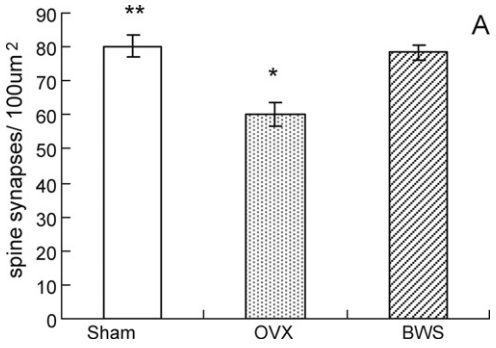

Fig. 3. OVX rats significantly reduced spine synapse density ($^{*}P < 0.01$), but spine synapse density did not differ between Sham- and BWS-treated rats ($^{**}P > 0.05$). Each bar represents the mean ± S.E.M.

stroke (Keller et al., 1997). The ability of estrogen to preserve mitochondrial function, suppress oxidative stress, and counteract the pro-apoptotic actions of mutant presenilin-1 (PS-1) suggests a generalized neuroprotective action of estrogens in both sporadic and inherited forms of AD (Mattson et al., 1997). In this study, BWS increased spine synapse density. In light of the behavioral findings, BWS-treated rats learned better than OVX. The result of ultrastructural observation in the hippocampal CA1 region suggested that BWS is capable of protecting neurons from ovariectomized injury and that the protective effect is associated with protection of mitochondrial function. More work will be needed to further elucidate the effect of BWS on memory in OVX rats, including non-water maze spatial memory tasks and other memory tasks (e.g. inhibitory avoidance), expression of synaptophysin P38.

In summary, our present study suggested that BWS could improve cognitive ability and memory in a model of neuronal impairment induced by estrogen depletion. The mechanisms were possibly associated with increase of spinal synapse density and protection of mitochondrial function of the pyramidal cell in hippocampal CA1 region from estrogen-induced injury. BWS may be a beneficial agent for patients with postmenopausal memory disorder.

Disclosure statement

All surgical procedures and protocols used were in accordance with the Guidelines for Ethical Care of Experimental Animals, which was approved by the Shandong University Animal Care and Use Committee. It is the first report about the effect of Bu-Wang-San on study and memory of menopause. Each author has read and approved the submitted manuscript.

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References

Anonymous, 1997. Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer [see comments] [published erratum appears in Lancet 350 (9089) 1484]. Lancet 350, 1047–1059.

Cruz-Orive, L.M., 1987. Particle number can be estimated using a dissector of unknown thickness of the selector. Journal of microscopy 145, 121–142.

Ditkoff, E.C., Crary, W.G., Cristo, M., Lobo, R.A., 1991. Estrogen improves psychological function in asymptomatic postmenopausal women. Obstetrics and gynecology 78, 991–995.

El-Bakri, N.K., Islam, A., Zhu, S., Elhassan, A., Mohammed, A., Winblad, B., 2004. Effects of estrogen and progesterone treatment on rat hippocampal NMDA receptors: relationship to Morris water maze performance. Journal of cellular and molecular medicine 8, 537–544.

Fugger, H.N., Cunningham, S.G., Rissman, E.F., Foster, T.C., 1998. Sex differences in the activational effect of ERα on spatial learning. Hormones and behavior 34, 163–170.

Gibbs, R.B., 1994. Estrogen and nerve growth factor-related systems in brain. Effects on basal forebrain cholinergic neurons and implications for learning and memory processes and aging. Annals of the New York Academy of Sciences 743, 165–199.

Grady, D., Gebretsadik, T., Kerlikowske, K., Ernster, V., Petitti, D., 1995. Hormone replacement therapy and endometrial cancer risk: a metaanalysis. Obstetrics and gynecology 85, 304–313.

Hai-Fa, Qiao, Sheng, Yang, Wen-Xia, Zhou, Yong-Xiang, Zhang, 2006. NT-1, an active constituent extracted from Tiao Xin Recipe, enhances long-term potentiation of CA1 subfield in rat hippocampal slices. Life Sciences 79, 8–15.

Hammond, C.B., 1994. Women's concerns with hormone replacement therapy—compliance issues. Fertility and sterility 62, 157–160.

Hardy, R., Kuh, D., 2002. Change in psychological and vasomotor symptom reporting during the menopause. Social science & medicine 55, 1975–1988.

Holmes, M.M., Wide, J.K., Galea, L.A.M., 2002. Low levels of estradiol facilitate, whereas high levels of estradiol impair, working memory performance on the radial arm maze. Behavioral neuroscience 116, 928–934.

Jacob, D.A., Temple, J.L., Patisaul, H.B., Young, L.J., Rissman, E.F., 2001. Coumestrol antagonizes neuroendocrine actions of estrogen via the estrogen receptor alpha. Experimental biology and medicine 226, 301–306.

Junping, Kou, Danni, Zhu, Yongqing, Yan, 2005. Neuroprotective effects of the aqueous extract of the Chinese medicine Danggui-Shaoyao-san on aged mice. Journal of Ethnopharmacology 97, 313–318.

Kalu, D.N., 1991. The ovariectomized rat model of postmenopausal bone loss. Bone and mineral 15, 175–192.

Keller, J.N., Germever, A., Begley, J.G., Mattson, M.P., 1997. 17Beta-estradiol attenuates oxidative impairment of synaptic Na^{+}/K^{+}-ATPase activity, glucose transport, and glutamate transport induced by amyloid beta-peptide and iron. The Journal of neuroscience: the official journal of the Society for Neuroscience 50, 522–530.

Kim H., Xia H., Li L., Gewin J., 2001. Actions of dietary soy vs. Premarin in mammalian brain, Annu. Meet. Am. Journal of the Chemical Society 221, AGFD-79, p. 79.

Kimura, D., 1995. Estrogen replacement therapy may protect against intellectual decline in postmenopausal women. Hormones and behavior 29, 312–321.

Lephart, E.D., Ladle, D.R., Jacobson, N.A., Rhees, R.W., 1996. Inhibition of brain 5a-reductase in pregnant rats. Effects on enzymatic and behavioral activity. Brain research 739, 356–360.

Markham, J.A., Pych, J.C., Juraska, J.M., 2002. Ovarian hormone replacement to aged ovariectomized female rats benefits acquisition of the Morris water maze. Hormones and Behavior 42, 284–293.

Mattson, M.P., Robinson, N., Guo, Q., 1997. Estrogens stabilize mitochondrial function and function and protect neural cells against the pro-apoptotic action of mutant presenilin-1. Neuroreport 8, 3817–3821.

Miller, M.M., Bennett, H.P., Billiar, R.B., Franklin, K.B., Joshi, D., 1998. Estrogen, the ovary, and neutotransmitters: factors associated with aging. Experimental gerontology 33, 729–757.

Morris, R., 1981. Spatial localization does not require the presence of local cues. Learning and motivation 12, 239–260.

Morrison, J.H., Hof, P.H., 1997. Life and death of neurons in the aging brain. Science 278, 412.

Notelovitz, M., 1989. Is routine use of estrogen indicated in postmenopausal women? An opposing view. The Journal of family practice 29, 410–415.

Pan, Y., Anthony, M., Clarkson, T.B., 1999a. Effect of estradiol and soy phytoestrogens on choline acetyltransferase and nerve growth factor mRNAs in the frontal cortex and hippocampus of female rats. Proceedings of the Society for Experimental Biology and Medicine 221, 118–125.

Pan, Y., Anthony, M., Clarkson, T.B., 1999b. Evidence for up-regulation of brain-derived neurotrophic factor mRNA by soy phytoestrogens in the frontal cortex of retired breeder female rats. Neuroscience letters 261, 17–20.

Patisaul, H.B., Whitten, P.L., Young, L.J., 1999. Regulation of estrogen receptor beta mRNA in the brain: opposite effects of 17beta-estradiol and the phytoestrogen, coumestrol. Molecular brain research 67, 165–171.

Roof, R.L., Hall, E.D., 2000. Gender differences in acute CNS trauma and stroke: neuroprotective effects of estrogen and progesterone. J. Neurotrauma 17, 367–388.

Sherwin, B.B., 1988. Estrogen and/or androgen replacement therapy and cognitive functioning in surgically menopausal women. Psychoneuroendocrinology 13, 345–357.

Sherwin, B.B., 1994. Estrogenic effects on memory in women. Annals of the New York Academy of Sciences 743, 213–230.

Singh, M., Setalo Jr., G., Guan, X., Warren, M., Toran-Allerand, C.D., 1999. Estrogen-induced activation of the mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. The Journal of neuroscience: the official journal of the Society for Neuroscience 19, 1179–1188.

Stone, D.J., Rozovsky, I., Morgan, T.E., Anderson, C.P., Finch, C.E., 1998. Increased synaptic sprouting in response to estrogen via an apolipoprotein E-dependent mechanism: implication for Alzheimer's disease. The Journal of neuroscience: the official journal of the Society for Neuroscience 18, 3180–3185.

Sughrue, P.J., Merchenthaler, I., 2000. Estrogen is more than just a "sex hormone": novel sites for estrogen action in the hippocampus and cerebral cortex. Frontiers in neuroendocrinology 21, 95–101.
Toran-Allerand, C.D., Singh, M., Setalo, G., 1999. Novel mechanisms of estrogen action in the brain: new players in an old story. Frontiers in neuroendocrinology 20, 97–121.
Welshons, W.V., Murphy, C.S., Koch, R., Calaf, G., Jordan, V.C., 1987. Stimulation of breast cancer cells *in vitro* by the environmental estrogen enterolactone and the phytoestrogen equol. Breast cancer research and treatment 10, 169–175.
Wilcox, G., Wahlqvist, M.L., Burger, H.G., Medley, G., 1990. Oestrogenic effects of plant foods in postmenopausal women. BMJ 301, 905–906.
Wise, P.M., Dubal, D.B., 2000. Estradiol protects against ischemic brain injury in middle-aged rats. Biology of reproduction 63, 982–985.
Wise, P.M., Dubal, D.B., Wilson, M.E., Rau, S.W., Böttner, M., 2001. Minireview: neuroprotective effects of estrogen-new insights into mechanisms and action. Endocrinology 142, 969–973.
Young-Ju, Yun, Bombi, Lee, Dae-Hyun, Hahm, Sung-Keel, Kang, Seung-Moo, Han, Hye-Jung, Lee, et al., 2007. Neuroprotective effect of Palmul-Chongmyeong-Tang on ischemia-induced learning and memory deficits in the rat. Biological & pharmaceutical bulletin 30, 337–342.