Effects of miroestrol on cognitive learning and depressive behaviors and related gene expression in ovariectomized ICR mice.

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Abstract

Miroestrol (MR) is a phytoestrogen isolated from *Pueraria candollei* var. *mirifica* (KwaoKrueaKhao), a Thai medicinal plant used for rejuvenation. We examined the effects of MR on cognitive function, oxidative brain damage, and expression of genes coding brain-derived neurotrophic factor (BDNF) and cyclic AMP-responsive element-binding protein (CREB), factors implicated in neurogenesis and synaptic plasticity, in ovariectomized (OVX) mice. OVX decreased a serum 17β-estradiol level and uterine weight. OVX also impaired object recognition performance in the novel object recognition test and spatial cognitive performance in the Y-maze test and the water maze test. Daily treatment of MR dose-dependently attenuated OXV-induced cognitive dysfunction. Moreover, OVX mice had a significantly increased level of thiobarbituric acid reactive substances, an index of oxidative membrane, and down-regulated the expression levels of BDNF and CREB mRNAs in the hippocampus and frontal cortex. MR treatment as well as hormone replacement therapy with 17β-estradiol, significantly reversed these neurochemical alteration caused by OVX. These results suggest that MR ameliorates cognitive deficits in OCX animals via attenuating OVX-induced oxidative stress and down-regulation of BDNF and CREB mRNAs transcription in the brain Our findings raise the possibility that MR and *Pueraria candollei* var. *mirifica*, an original plant containing MR, may have a beneficial effect on cognitive deficits like AD in which menopause/ovariectomy is implicated as risk factors.

1) **Effects of miroestrol on cognitive learning and depressive behaviors* and related gene expression in ovariectomized ICR mice.**

(*The data on depressive behaviors in this program are not shown in this report because the experiments are still ongoing.)

2) **Introduction**

Ovarian hormone 17β-estradiol has a wide variety of function in the central nervous system, especially in cognition, learning, and memory and exerts a protective effect against oxidative stress-mediated degenerative conditions. Therefore, the decrease in the 17B-estradiol level after menopause or ovariectomy is known to enhance incidence of inflammatory pathology involving oxidative stress [1] and can be a risk factor for neurodegenerative diseases such as Alzheimer's disease as well as cardiovascular dysfunction. Recent studies have suggested the preventive effects of hormone replacement therapy (HRT) or phytoestrogen supplement therapy on oxidative stress-mediated neurodegenerative disorders. However, it has been demonstrated that HRT in postmenopausal women is likely to be relevant to the development of breast, cervix and endometrium cancer [2]. Thus, the alternative phytoestrogen might be of potential benefit compared with conventional HRT with a poor safety profile or side effects.

Miroestrol (Fig. 1), a potent phytoestrogenic compound, is a chromene derivative with the chemical structure similar to estradiol. However, unlike 17β-estradiol, miroestrol is not a steroidal compound. Miroestrol is isolated from the tuberous root of *Pueraria candollei* var. *mirifica* (KwaoKrueaKhao) which belongs to the family Leguminosae. This plant has long been used in Thai traditional medicine for rejuvenation in aged people. The other active compounds from the tuberous roots of *P. candollei* are isoflavonoids, such as peurarin, daidzin, genistin, daidzein, and genistein. Recent evidence showed that miroestrol binds with higher affinity to the estrogen receptor α than other isoflavonoids isolated from this plant [3]. Moreover, it increased the levels of glutathione and the activity of antioxidant enzymes in the liver and uteri of β-naphthoflavone-treated mice [4] and exhibited the preventive effect on bone loss in ovariectomized mice [5]. However, the effects of miroestrol on the cognitive function and the oxidative stress in the brain in ovariectomized mice have not been investigated.

The main objectives in this study were to investigate the effect of miroestrol on ovariectomy-induced cognitive dysfunction and oxidative brain damages in mice and to compare the effects of miroestrol with those of 17β-estradiol. This study also aimed to examine the possible involvement of gene expression coding brain-derived neurotrophic factor (BDNF) and cAMP-response element-binding protein (CREB) mRNA expression in the effect of miroestrol since translated forms of these genes play important roles in learning and memory via neurogenesis and synaptic plasticity.

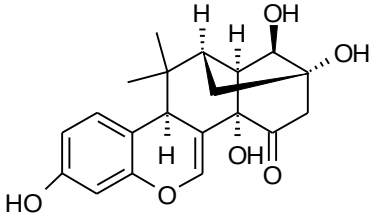

Fig. 1 Chemical structure of miroestrol

4) **Materials and methods**

4.1. Plant materials and isolation of miroestrol

The tuberous root bark of *P. candollei* var.*mirifica* were collected in UbonRatchathani, Thailand, in March 2010 and were identified by Dr. Thaweesak Juengwatanatrakul, Faculty of Pharmaceutical Sciences, UbonRatchathani University, UbonRatchathani, Thailand. The reference specimen (NI-PSKKU 007–010) was deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, KhonKaen University, Thailand.

Dried tuberous root bark of *P. candollei* var.*mirifica* was powdered and extracted three times with hexane, and the maceration was extracted three times with ethyl acetate. The ethyl acetate crude extracts were combined, evaporated, and fractionated by column chromatography (Silica gel 60 with hexane: ethyl acetate 3:1, v/v). The fractionated samples were combined and evaporated at 60 °C. The miroestrol-rich fraction was continued to be purified using high-performance liquid chromatography (HPLC). The HPLC separation was performed using a TSK gel C18 reverse phase column (5 μm, 2 x 60 x 2 cm) and mobile phase consisting of 16 % acetonitrile with a flow rate at 45.0 ml/min. The UV detection wavelength was set at 205 nm for obtaining chromatograms. Miroestrol was identified using ^{1}H-NMR and ^{13}C-NMR spectrum.

4.2. Animals

Fifty-two 5-week-old female mice were obtained from the National Laboratory Animal Center (Mahidol University, NakhonPathom, Thailand). The animals were housed in a light-controlled room with a 12h light/dark cycle and were allowed free access to food and water in the Laboratory Animal Unit of the Faculty of Pharmaceutical Sciences (KhonKaen University, KhonKaen, Thailand). All animal research procedures used in the present study were in accordance with the Guiding Principles for the Care and Use of Animals (NIH Publications No. 80-23, revised in 1996). The present study was also performed in accordance with the Animal Ethics Committee for Use and Care, KhonKaen University, KhonKaen, Thailand (Approval No. AEKKU 01/2555).

4.3. Surgical procedure

To mimic the estrogen-deprived condition in animals, OVX mice were used in this study. The animals underwent bilateral ovariectomy via dorsolateral incision under pentobarbital anesthesia (Nembutal: 60 mg/kg; Ceva Sante Anamale, France). The exposed ovary and associated oviduct were removed and then, the skin incisions were closed. The sham-operated group received the same procedure without removing the ovaries. After a 3-day recovery period, the animals were divided into five groups: (1) sham, (2) ovariectomy (OVX), (3) ovariectomy + 1 μg/kg 17β-estradiol (OVX+ E2), (4) ovariectomy + 0.1 mg/kg miroestrol (OVX + MR 0.1) and (5) ovariectomy + 1 mg/kg miroestrol (OVX + MR 1). The sham and OVX control groups were intraperitoneally administered corn oil. 17β-estradiol and miroestrol were intraperitoneally administered once daily for 8 weeks. To assess the effects of 17β-estradiol and miroestrol in OVX mice, the drugs were administered 1h before the behavioral test.

4.4. Behavioral test

4.4.1. Y-maze test

The Y-maze was used to elucidate the hippocampus-dependent spatial working memory of the animals. The Y-maze consisted of three arms 40 cm long, 18 cm high, 3 cm wide at the bottom and 12 cm wide at the top, which were positioned at equal angles. The maze floor and walls were constructed from dark opaque polyvinyl plastic. One hour after the drug administration, the Y-maze test was conducted. The animals were individually placed on one arm, and the sequence of arm entries were recorded manually over a 8-minutes period. An actual alternation was defined as entries into all three arms on consecutive choices (i.e. 123, 312, or 231 but not 212).When all fours limbs are within the arm, the animals were judged to enter the arm. The percentage of alternation was calculated according to the following equation:

$$\% \text{ alternation} = [(\text{number of alternations})/(\text{total arm entries}-2)] \times 100.$$

Maze arms were cleaned using 70% ethanol between tasks to remove residual odors.

4.4.2. Novel object recognition test (ORT)

The novel object recognition test was carried out as described previously [6-8]. The apparatus consisted of a square arena (50 cm x 50 cm x 40 cm high). The height of the objects was enough to prevent from mice climbing. The

ORT consisted of three different sessions: habituation, sample phase trial and test phase trial. About 24 h before the test, each mouse was individually habituated to the test box, with 10 min exploration in the absence of objects. In the sample phase trial, each mouse was placed into the observation box where two identical objects (objects O1 and O2) were placed in two adjacent corners and was allowed to explore for 5 min. The animals were considered to be exploring the object when the head of animal was facing the object or when the animal was touching or sniffing the object. The time spend exploring each object was recorded. In the test phase trials performed 30 min after the sample phase trials, one of the two objects was replaced by novel object. The total time spent exploring each familial object and novel object was analyzed. The box arena and objects were cleaned using 70% ethanol between trials to prevent a build-up of olfactory cues. Memory is assessed by measuring animal's ability to recognize an object previously presented by the time animals spent exploring a familiar object during a 5 min observation period. The discrimination index was calculated according to the following equation

$$DI = [(T_N - T_F)/(T_N + T_F)] \times 100$$

Here, T_N and T_F are the time spent to explore new and familiar objects during a 5-min observation period, respectively.

4.4.3. Morris water maze test

The Morris waster maze test was performed to elucidate the spatial reference memory of the animals as previously described [9] using a 1.1 m diameter circular pool. The mice received daily a block of four trials during a 5-days period of training sessions. In each trial, the mouse was placed into the pool at one of 4 start positions 90° apart around the edge of the pool by facing the wall of the tank and allowing the mouse to swim to the hidden transparent platform. This platform was submerged 1.5 cm below the surface of water in the center of one quadrant (Q1) and therefore invisible. The platform position remained stable during training session. If the mouse had not found the platform during a 60-s period, it was placed onto the platform by the experimenter. The mouse was allowed to remain on the platform for 10 s before being removed to an opaque high sided plastic chamber for 60 s. The next trial was then performed. After completion of the four trials, the mice were kept warm for an hour and then return to their home cage. For each trial, the latency to reach the platform (escape latency) and distance covered were recorded via video capture. The data for each day were averaged over the 4 trials before being used for result interpretation. In the retention phase, the platform was removed (in sixth day) and a single 60-s probe trial was run to evaluate how well the mice had learned and remembered the exact location of the platform. The time spent in the target quadrant (Q1) and the other quadrant (Q2-Q4) of the pool were recorded and compared among groups.

4.5. Determination of serum 17β-estradiol level

Twenty hours after completing the behavioral experiments, cardiac puncture was performed under Nembutal (60 mg/kg, i.p.) anesthesia to obtain approximately 1 ml blood sample from each mouse. The whole blood was centrifuged at 3000 rpm and 4°C for 15 min to isolate serum. The supernatants were frozen at -70°C for use. The serum level of 17β-estradiol was determined by electro-chemiluminescence immunoassay according to the manufacturer's instructions Name of the Company.

4.6. Dissection of uteri and brain tissues

The animals were sacrificed by decapitation. Their uteri, frontal cortex and hippocampus were dissected out and kept at -80°C until use. Weights of the uteri from each animal group were recorded.

4.7. Measurement of lipid peroxidation activity

Lipid peroxidation in the frontal cortex and hippocampus homogenate were measured as previously described [10-12]. Briefly, the hippocampus was homogenized in 10 vol. of ice-cold phosphate buffer (5mM, pH 7.4) using a

Potter-Elvehjem homogenizer with a Teflon pestle. The homogenates were mixed with the same volume of 10% (w/v) trichloroacetic acid and then centrifuged at 8000 x g and 4 ℃ for 10 min. The supernatant was incubated with 0.8% (w/v) 2-thiobarbituric acid at 100 °C for 15 min. After a cooling period, the content of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation, was spectrophotometrically measured at 532 nm using malondialdehyde (MDA) as a standard. The amount of TBARS was expressed as nmol of MDA/mg protein. The protein contents of hippocampus homogenates were determined by the Bradford method [13].

4.8. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

After completing the behavioral experiments, mice were decapitated under anesthesia. Hippocampus and frontal cortices were quickly dissected out and kept in -80°C until use. Mouse cyclic AMP response element binding protein (CREB), brain-derived neurotrophic factor (BDNF) and β-actin mRNAs were semi-quantified by RT-PCR. Total RNA was extracted from the tissues with Sepazol® (Nacalai Tesque, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized with oligo (dT) primers and M-MLV® reverse transcriptase (Invitrogen, USA). PCR amplification was carried out using gene-specific PCR primer sets as follow: β-actin:5′-AAC GGT CTC ACG TCA GTG TA-3′ (sense) and 5′-GTG ACA GCA TTG CTT CTG TG-3′ (antisense); BDNF: 5′-GAC AAG GCA ACT TGG CCT AC-3′ (sense) and 5′-CCT GTC ACA CAC GCT CAG CTC-3′ (antisense); CREB: 5′-TAC CCA GGG AGG AGC AAT AC-3′ (sense) and 5′-GAG GCA GCT TGA ACA ACA AC-3′(antisense). PCR products were separated by bis-acrylamide gel electrophoresis, the target cDNA were detected under ultraviolet light in the presence of ethidium bromide, photographed and semi-quantified by Syngene gel documentation (Ingenius L, Cambridge, UK) and the GeneTools match program.

4.9. Statistical analysis

Data are expressed as the mean ± S.E.M. and were examined by paired Student's t-test for two groups or one way ANOVA followed by the Tukey test for multiple comparisons among different groups. Differences of $p<0.05$ were considered significant. The analysis was conducted using SigmaStat® ver. 3.5 (SYSTAT Software Inc., Richmond, CA, USA)

5) Results

Effects of miroestrol and estrogen on OVX-induced cognitive deficits.

To determine whether miroestrol modulates the cognitive function in an estrogen deprivation model of mice, spatial and non-spatial working memory performances of OVX mice were elucidated in the Y-maze test and the novel object recognition test, respectively. OVX mice received once daily administration of vehicle corn oil, 17β-estradiol or miroestrol for 8 weeks before starting the behavioral experiments. The vehicle-treated OVX mice showed significantly less spontaneous alteration than the sham-operated group, indicating impairment of spatial working memory caused by OVX. 17β-estradiol- and miroestrol-treated OVX groups showed significantly improved spontaneous alternation performance in the test compared with the vehicle-treated control OVX mice (Fig.2A).

The novel object recognition test also revealed OVX-induced cognitive deficits in mice. In the sample phase trial, the total time spent exploring the identical objects presented did not differ among each animal group (data not shown). However, in the test trials, the sham-operated group spent significantly more time exploring the novel object than the familiar object, while the vehicle-treated control OVX mice failed to discriminate these two objects, indicating OVX-induced impairment of non-spatial working memory. On the other hand, the OVX animals that received hormone replacement therapy with 17β-estradiol (1 μg/kg) and supplementation of miroestrol (1 mg/kg) for 8 weeks exhibited significantly improved discrimination performance in the test phase trials (Fig. 2B).

Next we examined the effects of miroestrol on spatial cognitive performance relevant to reference memory of OVX animals using the water maze test. The escape latency of each animal group was decreased by the daily

training, indicating some degree of learning ability. Statistical analysis revealed that the vehicle-treated control OVX mice had significantly deteriorated abilities to learn the location of the hidden platform and retrieve the platform location in the training test and probe test, respectively, compared with the sham-operated animals, indicating the OVX-induced reference memory deficit (Fig. 2C, D). Repeated hormone replacement therapy with 17β-estradiol and supplementation of miroestrol significantly ameliorated the acquisition and retrieval performance of reference memory impaired by OVX (Fig. 2C, D).

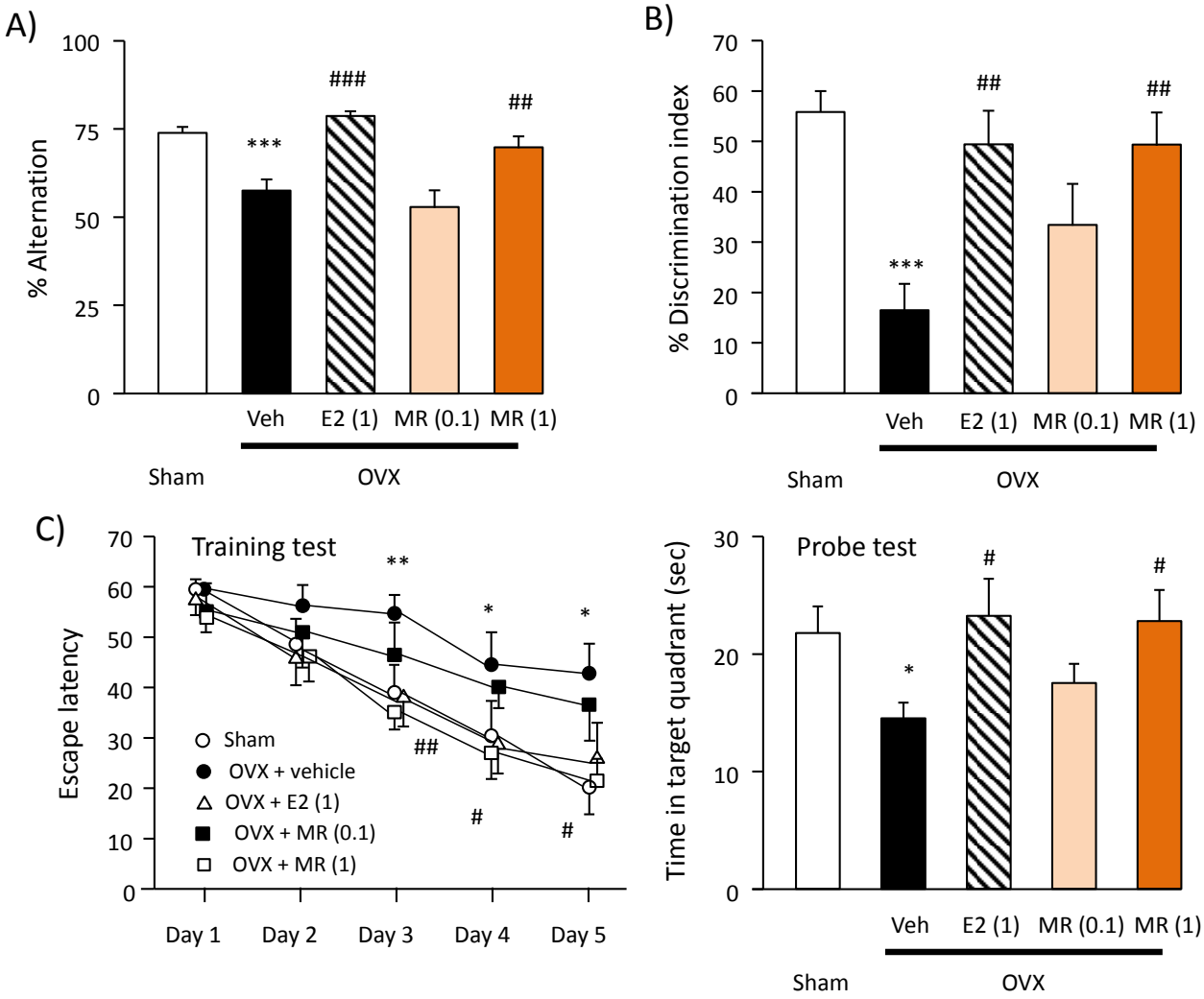

Fig. 2 Effects of miroestrol and 17β-estradiol (E2) on OVX-induced cognitive impairment in the Y-maze (panel A), the object recognition test (panel B) and the water maze test (panel C). Each column represents the mean ± S.E.M. (n=10-12). *P<0.05, **P<0.01, and ***P<0.001 vs. the sham-operated group. #P<0.05, ##P<0.01, and ###P<0.001 vs. the OVX group.

Changes in uterus weights and serum 17β-estradiol level after miroestrol administration

The OVX group showed a significant decrease in uterus weight and a serum 17β-estradiol level compared with the sham group (Table 1). Hormone replacement therapy of OVX mice with 17β-estradiol for 8 weeks significantly increased both the uterus weight and the serum 17β-estradiol level. Similarly, miroestrol treatment significantly and dose-dependently increased the uterus weight in OVX animals without altering the serum 17β-estradiol level.

Table 1　Effects of 17b-estradioal (E2) and miroestrol (MR) on the uterus weight and the serum estradiol level

Treatment	Uterus weight (g)	Serum E2 (pg/ml)
Sham	0.212 ± 0.016	25.3 ± 2.2
OVX + vehicle	0.075 ± 0.010***	12.8 ± 2.3***
OVX + E2 (1)	0.261 ± 0.030###	32.9 ± 5.0###
OVX + MR (0.1)	0.178 ± 0.010##	15.9 ± 1.2
OVX + MR (1)	0.268 ± 0.027###	16.8 ± 1.2

The number in the parenthesis is a dose of each drug (mg/kg, i.p.). Each datum represents the mean ± S.E.M. (n=4-6)

Effects of miroestrol and estrogen on lipid peroxidation in brain of OVX mice

To investigate the possible involvement of oxidative stress in the OVX-induced cognitive impairment and the effects of miroestrol, we determined the MDA level, a biomarker for lipid peroxidation, in the frontal cortex and hippocampus of experimental animals. OVX induced a significant increase in the MDA contents in these brain regions compared with the sham-operated animals. Repeated treatment with 17β-estradiol (1 μg/kg) or miroestrol (1 mg/kg) over 8 weeks significantly decreased the MDA levels in the frontal cortex and hippocampus of OVX mice (Fig. 3).

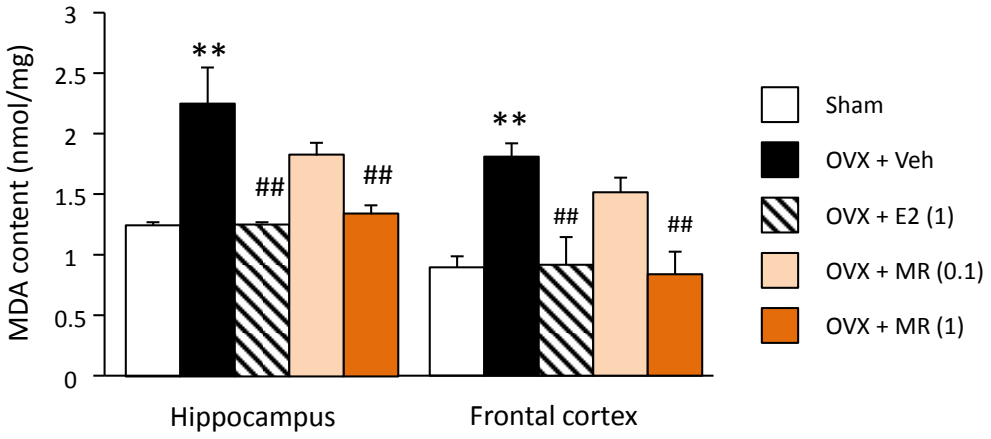

Fig. 3　Effects of miroestrol and 17β-estradiol (E2) on OVX-induced oxidative damage in the fortal cortex and hippocampus in mice. TBARS in the brain homogenate was determined using malondialdehyde (MDA) as a standard and was expressed as nmol of MDA/mg protein. Each data column represents the mean ± S.E.M from 5-6 mice. **P<0.01 and ***P<0.001 vs. the sham-operated group. #P<0.01 and ###P<0.001 vs. the OVX group.

Effects of miroestrol and estrogen on OVX-induced changes in the hippocampal and frontal cortex expression of genes encoding CREB and BDNF

Semiquantitative analysis of the CREB and BDNF mRNAs expression revealed that, in the OVX group, the expression levels of these genes in the hippocampus and cerebral cortex were significantly down-regulated compared with those in the sham group in a manner reversible by 17β-estradiol treatment (Fig. 4). Like 17β-estradiol treatment, miroestrol administration dose-dependently normalized the down-regulated expression of these genes in OVX animals.

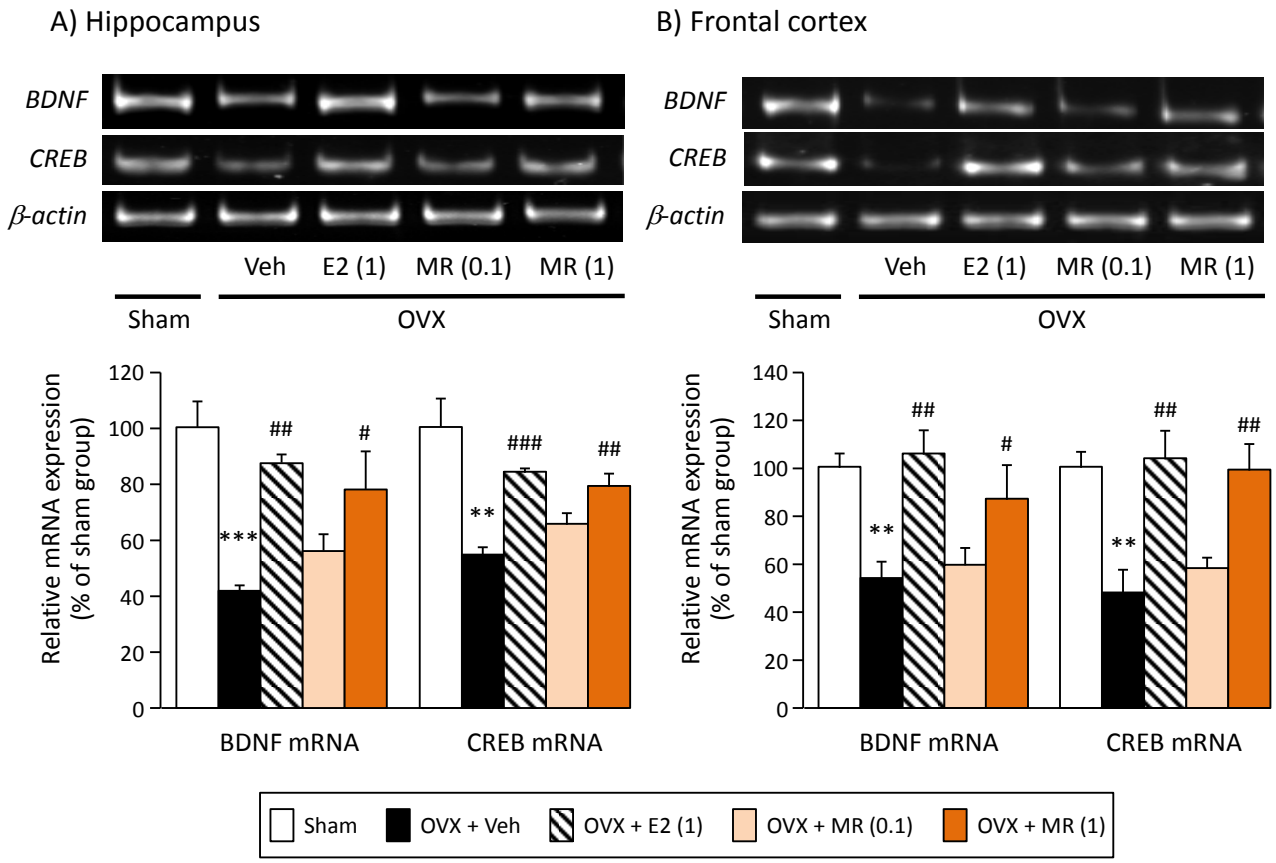

Fig. 4 Effects of miroestrol on the expression levels of BDNF and CREB mRNAs in the hippocampus and frontal cortex of sham and OVX mice. The expression of BDNF and CREB mRNAs were semi-quantitatively analyzed as described in the text. Each data column represents the mean ± S.E.M. (n=4-5). *P<0.05 and **P<0.01 vs. the sham-operated group. #P<0.05 and ##P<0.01 vs. the OVX group.

6) Discussion

The present study demonstrated that miroestrol ameliorated estrogen depletion-induced learning and memory deficits and brain membrane damage and suggested that these effects of miroestrol are attributable to suppression of oxidative stress and estrogen receptor-mediated facilitation of BDNF and CREB gene transcription in the brain. Our findings raised the possibility that miroestrol and its origin plant *Pueraria candollei* var. *mirifica* (KwaoKrueaKhao) are beneficial to cognitive deficits like AD in which menopause/ovariectomy is implicated as risk factors.

Evidence indicates that estrogen depletion by OVX/menopause is implicated in AD as one of the important pathogenic factors and impairs cognitive function in rodents [14, 15]. In addition, lines of studies have demonstrated that hormone replacement therapy is beneficial not only to various menopausal symptoms but also to risk of AD after climacterium [16]. In this study, we found that miroestrol, a phytoestrogen from the tuberous root of *Pueraria candollei* var. *mirifica* (KwaoKrueaKhao), as well as hormone replacement therapy with 17β-estradiol, significantly ameliorated OVX-induced deficits of non-spatial cognitive performance, which was elucidated in the object recognition test, and spatial cognitive performance, which was tested in the Y-maze test and the water test. Taking together, the present findings suggest that miroestrol administration is also effective in prevention of cognitive dysfunction which is likely to be caused after climacterium in humans.

It should be noted that the levels of TBARS, a marker of oxidative stress-induced membrane damage, in the hippocampus and frontal cortex were significantly elevated in the OVX animals compared those in the sham-

operated group and that that elevation was blocked by miroestrol in a dose-dependent fashion. These findings suggest that OVX-induced oxidative stress via estrogen deprivation is severe enough to cause membrane damage in the frontal cortex and hippocampus, leading to cognitive dysfunction. This idea is supported by the data that estrogen replacement therapy with 17β-estradiol almost completely attenuated the OVX-induced cognitive deficits and oxidative membrane damage in these brain regions. Moreover, considering that miroestrol administration exerted the effects similar to 17β-estradiol, it is likely that the protection by miroestrol of the cortical and hippocampal membrane function against OVX-induced oxidative stress also contributes to amelioration of both non-spatial and spatial cognitive performance of OVX animals, since object recognition performance and spatial cognitive performance mainly depend on the neuronal function in the entorhinal/frontal cortex [17, 18] and the hippocampus [17], respectively.

Miroestrol is a chromene derivative with a chemical structure similar to an estrogenic hormone, estradiol [19] and is reported to exhibit an estrogenic-like effect through binding to intracellular estrogen receptor α (ER-α) and ER-β, which function as ligand-activated transcription factors [20]. In the present study, we found that miroestrol dose-dependently increased the uterus weight without affecting the serum estradiol level in OVX animals, suggesting that estrogenic activity exhibited by miroestrol binding to ER in the brain is involved in the mechanism by which the cognitive function of OVX animals is improved. In fact, evidence indicates that the hippocampus and prefrontal cortex possess moderate to high levels of ERα and ERβ [21, 22] and that activation of these ERs in the brain by estrogen enhances BDNF gene transcription through an estrogen receptor responsive element on the promoter region and thereby modulates neurogenesis and synaptic plasticity in the hippocampus, a molecular biological mechanism implicated in learning and memory [23]. Therefore, considering the present data that miroestrol reversed OVX-induced down-regulation of the BDNF and CREB mRNAs' expression in the frontal cortex and hippocampus, it is likely that the ameliorative effect of miroestrol on cognitive deficits in OVX animals is at least partly due to facilitation of BDNF- and CREB-medicated neurogenesis and synaptic plasticity in the brain.

Hormone replacement therapy with 17β-estradiol has been reported to prevent or delay cognitive decline in postmenopausal women, as well as in an OVX mice model [24, 25], via multiple mechanisms including stimulation of neurogenesis in the hippocampus [26] and antioxidant properties [27]. However, this strategy is also known to increase risks of malignancy and breast cancer in humans as one of its adverse effects. [28-30]. Taking together with these facts, our findings suggest that miroestrol may provide an alternative approach for prevention/therapy of cognitive dysfunction caused by estrogen depletion.

In conclusion, miroestrol exhibits the neuroprotective effect in OVX animals, a mouse model of estrogen-deprivation. Long term supplementation of miroestrol improves OVX-induced cognitive deficit and brain oxidative damage in a manner similar to those of 17β-estradiol. Miroestrol and its origin plant *Pueraria candollei* var. *mirifica* (KwaoKrueaKhao) may become alternatives to prevention of cognitive deficits like AD in which menopause/ovariectomy is implicated as risk factors.

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