Estrogenic Effects of the Herbal Formula, Menoprogen, in Ovariectomized Rats

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Despite the health risks for postmenopausal women, the indications and ideal candidates for hormone replacement therapy remain unclear. The present study used ovariectomized rats to examine the safety and effects of the Chinese herbal formula Menoprogen (MPG), which is prescribed for menopausal syndrome. Daily oral MPG (1000 mg/kg body weight) for 2 weeks significantly recovered uterine and adrenal gland atrophy and restored serum estradiol, estrone and progesterone levels that were decreased in rats by bilateral ovariectomy. However, yeast two-hybrid and nuclear receptor cofactor assays showed that MPG did not bind estrogen receptors α (ERα) and β, and immunohistochemical staining revealed that unlike 17β-estradiol, MPG did not stimulate the protein expression of ERα, progesterone receptor, c-jun and c-fos in the uterus. No side effects of MPG were confirmed in vivo. These findings suggest that MPG would be useful for treating women with premenopausal and postmenopausal syndromes.

Key words Menoprogen; estrogen; menopausal syndrome; Chinese herbal formula

Menoprogen (MPG), a herbal formula developed in China to treat perimenopausal symptoms, comprises the herbs Lycii fructus, Rehmanniae radix, Mori fructus and Carthami flos, which are commonly used in traditional Chinese medicine. MPG clinically elevates blood estrogen levels and is regarded as useful for improving menopausal symptoms.1–3) The major constituents of MPG have been determined by HPLC.4) MPG increases the weight of the ovary, uterus and adrenal gland in the aging rat, as well as the levels of 17β-estradiol (E2) and progesterone (P4), but decreases those of follicle-stimulating hormone and luteinizing hormone.5–7)

Many postmenopausal women undergo hormone-replacement therapy (HRT) or estrogen-replacement therapy (ERT) to improve their quality of life. However, the incidence of uterine carcinoma is high in postmenopausal women, which has raised concerns among patients and clinicians about the safety of HRT or ERT. Although MPG seems to be a promising alternative to these therapies, the mechanism of MPG actions and risks of uterine carcinoma remain unclear. The present study investigates the effects and safety of MPG in bilaterally ovariectomized rats.

MATERIALS AND METHODS

Animals Eight-week-old female Wistar rats (190—210 g, Japan SLC Inc., Shizuoka, Japan) underwent bilateral ovariectomy (OVX) or sham operation (Sham), and then were acclimatized for 14 d in a light-, temperature- and humidity-controlled environment (lights on, 07:00—19:00; 22±2 °C and 50±5%, respectively) with access to a pelleted commercial diet (MM-3; Funabashi Farm, Chiba, Japan) and water ad libitum. The animals were handled in accordance with the Guide for Animal Care Committee of the University of Toyama.

Preparation of MPG and Reagents MPG is obtained by Nanjing Mayflower Pharmaceutical Technology Corporation Ltd. (Nanjing, China), and an aqueous extract of a mixture of the traditional Chinese herbs, Lycii fructus, Rehmanniae radix, Mori fructus and Carthami flos. Decoctions contain over 30% and 20% polysaccharides and flavonoids, respectively. We purchased E2 from Sigma-Aldrich Japan Co. (Tokyo, Japan), α-nitrophenyl β-D-galactopyranoside (ONPG) from Nacalai Tesque (Kyoto, Japan) and antibodies to ERα, ERβ, β-estradiol, MPG in water (500 mg or 1000 mg/kg body weight, p.o.) daily for 2 weeks, and 5 groups of 6 OVX rats were administered daily for 2 weeks with the following: water (10 ml/kg body weight, p.o.), MPG in water (500 mg or 1000 mg/kg body weight, p.o.) or E2 (10 µg/kg body weight, subcutaneously (s.c.)) dissolved in saline containing 1% Tween 20. Thereafter, the rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally (i.p.)) and blood samples that were collected from the inferior vena cava were centrifuged at 3000×g for 15 min and stored for serum analyses. The rats were perfused transcardially with saline and 4% paraformaldehyde, and then uterine and adrenal glands were cryoprotected in 4% paraformaldehyde containing 30% sucrose and fixed for immunohistochemistry. Vaginal smears were obtained from Sham rats between 9:00 and 10:00 h to determine the phase of the estrous cycle, and rats were collected at the time of di-estrous.

Blood Analysis Serum concentrations of E2, P4, estrone (E1) and testosterone were measured by chemiluminescence immunoassays (CLIA), alanine aminotransferase (ALT) was measured by UV, urinary nitrogen (UN) was measured colorimetrically and creatinine (CRE) was measured using an enzymatic assay provided by BML Co. (Tokyo, Japan).

Immunohistochemistry) Frozen sections of uteri (20 µm) cut using a microtome (Leica, Wetzlar, Germany) were dipped in 0.3% hydrogen peroxide/phosphate buffer (PB) for 15 min, rinsed three times in PB, and then incubated in blocking solution (3% goat serum and 0.3% Triton X-
1000 (PB) for 1 h at room temperature, followed by overnight at 4°C with monoclonal antibodies against vascular endothelial growth factor (VEGF, 1:1000, Santa Cruz Biotechnology, CA, U.S.A.), aquaporin 2 (AQP2, 1:500, Santa Cruz Biotechnology), estrogen receptor α (ERα, 1:1000, Dako Cytomation, Kyoto, Japan), progesterone receptor (PR, 1:1000, Dako Cytomation), c-fos (1:1000, Sigma, MO, U.S.A.) and c-jun (1:500, Santa Cruz Biotechnology). On the following day, the sections were rinsed three times in PB, and antigen was visualized after a 2 h incubation at room temperature with secondary antibody anti-rabbit immunoglobulin G (IgG) (1:300, Alexa Fluor 594, Molecular Probes, OR, U.S.A.) for VEGF and AQP2, goat anti-mouse IgG (1:300, Zymed, CA, U.S.A.) for ERα, PR, c-fos and c-jun. The sections were further rinsed three times in PB. Antigens, ERα, PR, c-fos and c-jun were labeled using the 3,3′-diaminobenzidine (DAB) reaction. The stained sections were mounted and coverslipped with Vectashield (Vector Laboratories, CA, U.S.A.) for VEGF and AQP2, and with Softmount (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for ERα, PR, c-fos and c-jun. Immunolabelling was detected in uteri using a fluorescence and visible light microscope (AX-80; Olympus, Tokyo, Japan). The area of uterine tissues was measured using a Meta Imaging Series 7.0 (MetaMorph/MetaVue/MetaFluoro; Molecular Devices, Toronto, Canada).

**Yeast Two-Hybrid Assay** Yeast cells expressing ERα and estrogen receptor β (ERβ) were shaken overnight at 30°C in synthetic defined medium without tryptophan and leucine and then incubated with E2 and MPG (final concentration; 10 and 100 μg/ml) for 4 h at 30°C. The growth of the yeast cells was monitored by measuring turbidity at 600 nm. The treated yeast cells were collected by centrifugation (12000×g, 5 min, 25°C) and resuspended in 200 ml of Z-buffer containing 1 mg/ml of zymolase at 37°C for 15 min. The reaction was started by adding 40 ml of 4 mg/ml substrate ONPG. When the color yellow appeared (incubation time: t), 100 ml of 1 m Na2CO3 was added to stop the reaction. The absorbance of the solution (150 ml) was measured at 420 and 550 nm.

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\text{OD}_{450} = \frac{A_{420} - 1.75 \times A_{550}}{(1/0.05 \times A_{400})}
\]

**Nuclear Receptor Cofactor Assays (RCAS)** Interactions between ERα/β and MPG were monitored using EnBio RCAS for ERα, ERβ kits (Fujikura Kasei Co., Ltd., Ibaraki, Japan) according to the manufacturer’s instructions. MPG was dissolved in dimethyl sulfoxide (DMSO) to final concentrations of 10, 100 and 1000 μg/ml. The relative activity of E2 (positive control) was calculated using the formula: \[\frac{B/B_{	ext{max}}=(C-B)/(A-B)}{B_{	ext{max}}},\] where A is the OD_{450} value of the positive control in an SRC(+) well minus the OD_{450} value of the positive control in an SRC(−) well; B is the OD_{450} value of the negative control in an SRC(+) well minus the OD_{450} value of the positive control in an SRC(−) well; and C is the OD_{450} value of the test sample in an SRC(+) well minus the OD_{450} value of the test sample in an SRC(−) well.

**Statistical Analysis** All values are represented as means±S.E.M. Significant differences between Sham-Water and OVX-Water, and OVX-Water and OVX-E2 were analyzed using Student’s t-test (Prism; GraphPad, CA, U.S.A.). The statistical significance between OVX-Water and OVX-MPG (500 mg and 1000 mg/kg body weight) was evaluated by one-way ANOVA followed by Dunnett’s test (Prism) and that among OVX-Water, MPG (500 mg and 1000 mg/kg body weight) and E2 was evaluated by one-way ANOVA followed by Dunnett’s test (Prism).

**RESULTS**

**Effects of MPG on Uterine and Adrenal Gland Weight in Ovariectomized Rats** Ovariectomy induced significant uterine and adrenal gland atrophy (OVX-Water) compared with Sham-Water (Fig. 1) and this was significantly recovered by daily administration of MPG (1000 mg/kg/body weight) or E2 for 2 weeks. The same MPG regimen administered to Sham rats did not affect uterine and adrenal gland weight. The MPG dose of 1000 mg/kg was selected because it elicited recovery in the OVX rats.

**Effects of MPG on Uterine Tissues** Figure 2 shows significant atrophy in cross-sections of uteri from the OVX-Water group compared with the Sham group. Both MPG and E2 recovered areas of atrophied uterine tissues compared with the OVX-Water group.

**Recovery of VEGF and AQP2 Protein Expression by MPG** We monitored protein expression using VEGF as a marker to investigate the metamorphosis of uterine tissue. Ovariectomy decreased VEGF protein expression in the uterus of the OVX-Water group compared with the Sham group (Fig. 3). Both MPG and E2 recovered VEGF protein expression in the uterus compared with the OVX-Water group. Because body fluids/water affect metamorphosis of the uterine cavity, we examined uterine water circulation using AQP2 as a marker of protein expression. Both MPG and E2 recovered uterine AQP2 protein expression compared with the OVX-Water group (Fig. 3).

**Concentrations of Serum Hormones** Ovariectomy sig-
Fig. 2. Effects of MPG on Uterine Tissue
Cross-sections of uterine tissue were measured. Sham-Water group is expressed as 100%. ***p<0.001 vs. Sham; ###p<0.001 vs. OVX-Water group. Scale bar, 1 mm.

Fig. 3. Recovery of Vascular Endothelial Growth Factor (VEGF) and Aquaporin 2 (AQP2) Protein Expression Induced by MPG
Uterine tissues stained with antibody to VEGF and AQP2. Scale bar, 200 μm.

Fig. 4. Concentrations of Serum Hormones
Serum E2 (A), E1 (B), P4 (C) and testosterone (D) measured by CLIA. **p<0.01, *p<0.05 vs. Sham; ##p<0.01, #p<0.05 vs. OVX-Water group.
significantly decreased serum E2, P4, E1 and testosterone levels in the OVX-Water group compared with the Sham group (Figs. 4A—D) and significantly recovered plasma E2, E1 and P4 levels in OVX-MPG and OVX-E2 rats (Figs. 4A—C). Serum testosterone levels were hardly recovered by MPG or E2 (Fig. 4D).

**Induction of β-Galactosidase Activity in Yeast Two-Hybrid Assays and Relative Activity in RCAS** Estradiol dose-dependently induced β-galactosidase activity associated with ERα and ERβ binding (Fig. 5A). Doses of 10 and 100 μg/ml of MPG did not apparently elicit β-galactosidase activity associated with ERα and ERβ binding. The relative activities of ERα and ERβ in RCAS were defined compared with 0 and 100 as negative and positive controls, respectively. The relative activity of MPG, 10, 100 and 1000 μg/ml was almost zero against ERα and ERβ binding (Fig. 5B).

**ERα and PR Protein Expression in the Uterus** ERα and PR proteins were expressed in the uteri of the Sham group (Fig. 6). Ovariectomy suppressed ERα and PR protein expression in the uterus of the OVX-Water group compared with the Sham group. Estradiol stimulated ERα and PR protein expression in the uterus of OVX rats, whereas MPG did not.

**Effect of MPG on Uterine c-Jun and c-Fos Protein Expression** Estrogens reportedly induce the overexpression of proto-oncogenes such as c-jun and c-fos in the OVX uterus.10—12) We confirmed c-jun and c-fos protein expression in uteri from the Sham group (Fig. 7). Ovariectomy weakened c-jun and c-fos protein expression in the uterus of the OVX-Water group compared with the Sham group. Estradiol stimulated c-fos and c-jun protein expression in the OVX rat uterus, whereas MPG did not.

**Confirmation of MPG Side Effects** We measured serum levels of ALT, a marker of liver function, as well as
those of UN and CRE, markers of kidney function, to verify the side effects of MPG. None of ovariectomy (Fig. 8), MPG or E2 altered serum ALT, UN and CRE levels compared with the Sham group.

DISCUSSION

The uterus is the main target organ of estrogen in women. MPG significantly recovered the weight and area of the uterus atrophied by OVX (Figs. 1, 2), indicating that MPG exerts estrogen-like effects. The recovery of uterine weight induced by exogenous E2 is based on endometrial proliferation and water absorption as indicated by VEGF and AQP2 in the uterine endothelial membrane, respectively. The growth and proliferation of vascular endothelial cells in the abundantly vascularized endometrium is promoted by VEGF, the synthesis and action of which is promoted by E2.13,14) The aquaporin subtype AQP2 is mainly located in luminal and glandular epithelium, and its expression decreases in the uterus of premenopausal women.15) Here, we demonstrated that MPG increased the relative weight of the uterus and slightly recovered metamorphosis in the uterus of OVX rats (Figs. 1—3), suggesting that MPG has estrogenic action. Because ovarian function declines in postmenopausal women, androgens that originate and are secreted from the adrenal gland are metabolized to E1 in peripheral organs, especially adipose tissue. Therefore, E1 rather than E2 is the main type of estrogen in postmenopausal women.16) MPG increased the weight of the adrenal gland (Fig. 1) and the E1 serum level (Fig. 4B).

MPG prepared by water extraction contains polysaccharides and flavonoids; MPG did not bind to ERα and ERβ in the yeast two-hybrid assay (Fig. 5A), and RCAS also resulted in scarce interaction (Fig. 5B). These findings indicated that the components of MPG induced estrogenic effects through targets and signaling pathways other than those of E2; for example, by promoting adrenal gland secretion or local biosynthesis at peripheral tissues.17)

The estrogen-induced immunohistochemical expression of ERα and PR has shown that these biomarkers are closely associated with endometrial carcinogenesis.18) The proto-oncogenes c-jun and c-fos are associated with rapid cellular events, such as apoptosis, and their own expression.19) The overexpression of both c-fos and c-jun in the OVX uterus is induced by estrogens, and is closely related to estrogen-associated carcinogenesis.20—22) None of ERα, PR, c-jun and c-fos proteins were detectable in the uterus of OVX rats administered with MPG (Figs. 6, 7), suggesting that the risk of endometrial carcinogenesis associated with MPG is very low. Furthermore, the absence of MPG-induced changes in biochemical parameters such as ALT, and kidney injury evaluated by UN and CRE in OVX rats suggest that MPG does not lead to liver and kidney damage.

The frequency of diseases associated with female hormones is increasing as society ages. MPG is a promising therapeutic agent for menopausal woman because of its low risk of causing hormone-dependent breast and uterine cancers. MPG should become an alternative to HRT/ERT for post-menopausal women.

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REFERENCES AND NOTES

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