The Effect of Herbal Medicine on Nerve Growth Factor in Estradiol Valerate-induced Polycystic Ovaries in Rats

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Abstract: A type of polycystic ovary resembling some aspects of human polycystic ovarian syndrome (PCOS) can be induced in the rat with a single injection of long-acting estradiol valerate. Among several theories behind the development of polycystic ovaries (PCO), the involvement of the sympathetic nervous system draws much attention, and herbal medicine is known to relieve the abnormal symptoms of PCO. Two herbal formulas, Changbudodam-Tang (cang fu dao tan tang) and Yongdamsagan-Tang (long dan xie gan tang), were used in the present study. The administration of herbal medicine was done every other day for 60 days. The morphological changes of ovaries from herbal medicine treatment were compared to those from an oil-treated control group and an estradiol valerate-injected group. This study
also examined the possible hypothesis of neurogenic participation in terms of nerve growth factor (NGF) in the pathology of ovarian dysfunction. The nerve growth factor was analyzed in the central nervous system and ovaries by immunohistochemistry. The main findings of the present study were: (1) PCO were fully developed in rats with a single intramuscular injection of estradiol valerate, (2) PCO resulted in the expression of NGF in the ovaries and the brain tissues, and (3) herbal medicine administration significantly decreased the elevated NGF staining in the ovaries without affecting the brain tissues significantly.

**Keywords**: Changbudodam-Tang (Cang-Fu-Dao-Tan-Tang); Yongdamsagan-Tang (Long-Dan-Xie-Gan-Tang); Polycystic Ovary; Nerve Growth Factor.

**Introduction**

Both hormonal and intraovarian signals act in synchrony to control follicular development, steroidogenesis and ovulation. This orderly ovarian function is further regulated by sympathetic innervation (Lara et al., 1990). When the peripheral sympathetic activity is disrupted in a rodent model of the human syndrome of polycystic ovary (PCO), the altered neurophysiological environment may lead to the progression of cystic ovarian disease (Lara et al., 1993).

The definite underlying mechanism of PCO formation remains unknown. The resultant complex disease of polycystic ovary syndrome (PCOS) in women involves anovulation, hyperandrogenism, obesity and insulin resistance (Dahlgren and Janson, 1994; Franks, 1995). The anovulation is associated with a disturbing feedback system by ovarian estrogens to the hypothalamus, resulting in a high elevated luteinizing hormone concentration that is detrimental to follicular growth (Rebar et al., 1976; Baird et al., 1977; McKenna, 1988). Another endocrine characteristic of PCOS is a decreased concentration of sex hormone binding globulin (SHBG). This low level of SHBG is connected to a relative increase in unbound concentrations of androstenedione and testosterone, which is clinically related with hyperandrogenism of hirsutism (Rebar et al., 1976; Baird et al., 1977; McKenna, 1988).

For an experimental induction of PCO, a long-acting estradiol valerate (EV) has been used (Brawer et al., 1978 and 1986). The morphological changes include atretic antral follicles, follicular cysts with a well-developed theca cell layer, a diminished granulosa cell compartment and luteinized cysts (Brawer et al., 1978 and 1986). Previous studies have shown that PCOS is associated with an abnormal activation of the sympathetic nervous system, resulting in the increased density of catecholaminergic nerves (Somenova, 1969), impaired metabolism of norepinephrine (Garcia-Rudaz et al., 1998) and increased activity of sympathetic nerves via the superior ovarian nerve (Lara et al., 1993). The development and function of the ovarian sympathetic innervation depend on the ability of the ovary to produce nerve growth factor (NGF), a target-derived neurotrophin required for the development of the peripheral sympathetic system (Levi-Montalcini, 1987). In the rat ovary, NGF is principally synthesized in cells of the follicular wall (Dissen et al., 1996) and activation of NGF may be a factor involved in enhancing norepinephrine outflow to the gland in the EV-induced PCO (Lara et al., 2000).
There are several herbal formulas known to relieve the abnormal symptoms of PCO. In fact, both Changbudodam-Tang (cang fu dao tan tang) and Yongdamsagan-Tang (long dan xie gan tang) were prescribed for specific symptoms associated with PCO (Luo, 1994). Based on the role of NGF as a neurotrophin for the sympathetic nervous system, we used the experimentally induced murine PCO model to study the effects of herbal medicine by analyzing NGF expression in the brain tissues and the ovaries.

Materials and Methods

Animals

Thirty-two virgin adult cycling Sprague-Dawley rats (Daehan Laboratory, Daejun, Korea) weighing 190–210 g and with regular four-day estrous cycles were used. The rats were housed in a room kept at 25 ± 1°C, four to a cage, with free access to food and water for about a week before and throughout the study period.

PCO Induction

Based on the modified protocol of Stener-Victorin et al. (2000), all rats received either a single i.m. injection of estradiol valerate (EV, Sigma, USA) for the induction of a PCO (EV control) or sesame oil (0.2 ml/rat, Sigma, USA) for the control (oil control). For the fully developed polycystic ovary, the dose of 4 mg of EV and timing of 60 days after injection were chosen.

Medicinal Extract Preparation

Herb experts purchased all herbs used in this study in a local market with confirmation of origin. Two herbal formulas, Changbudodam-Tang and Yongdamsagan-Tang (Table 1) were used in this study for the treatment of PCO. A total of 93.75 g and 52.5 g of each respective formula was boiled in 1000 ml of distilled water in a flask while circulating the cold water to prevent evaporation of essential substances. After boiling for three hours, the decoction was filtered, and then centrifuged at 5000 rpm for 20 minutes. The supernatant was vacuum-concentrated with a rotary evaporator and freeze-dried for 48 hours to produce a powder, ready for the experiments.

Study Protocol

Eight rats in the EV control group and eight rats each in the two herbal medicine-treated groups were injected i.m. with 4 mg EV in 0.2 ml oil/rat. Another eight rats in the oil control were injected with 0.2 ml oil/rat. The herbal medicine-treated EV groups were subjected to oral administration by a zonde needle (Natsume, Japan) every other day for 60 days, beginning 2 days after the EV injection. Doses of 50 and 40 mg/kg were used for C-Tang and Y-Tang, respectively.
Ovarian Morphology

At the end of the experiments, all 32 rats were perfused transcardially following chloral hydrate (500 mg/kg, i.p.) anesthesia with a fixative of 4% paraformaldehyde solution in 0.1 M sodium cacodylate buffer with 4% sucrose added, pH 7.4. The dissected ovaries were placed in the same fixative overnight at 4°C and then embedded in paraffin. Samples were sectioned at 4 µm and stained with hematoxylin and eosin. Follicles containing an oocyte with a nucleus were counted and analyzed by an experienced pathologist. The morphometric analysis was performed using a Zeiss digital image analysis system. If ovum degeneration or at least one pyknotic granulosa cell was seen, the follicle populations were classified as atretic; otherwise, they were classified as healthy. Morphological characteristics of follicular atresia were, for instance, scattered pyknotic nuclei in the granulosa cell layer, detachment of the granulosa cell layer from the basement membrane, fragmentation of the basal lamina, and the presence of cell debris in the antrum of the follicle.

Nerve Growth Factor Measurement by Immunohistochemistry

The ovary, pituitary and hippocampus were excised, transferred to a fixative of 4% paraformaldehyde solution at least for a day and immersed again in the same fixative for another day. The fixed tissue samples were paraffin formatted for the microtome slicing by 4 µm thickness, and the sliced tissue sections were mounted on the X-tra™ slides (Surgipath, Richmond, USA).

For the immunohistochemical reaction for NFG, the slide glasses were deparaffinated and hydrated by immersing sequentially to xylene and descending concentrations of ethanol solution (100%, 95%, 90% and 80%) for 5 minutes each and distilled water for 10 minutes. To enhance antigen retrieval, sections were pretreated in the microwave (Pelco™ laboratory microwave oven, Ted Pella Inc., CA, USA) in 0.01 M citrate buffer (pH 6.0) three times for 5 minutes at 360 watts, and cooled down in the jar at room temperature for 20 minutes. Then, the slides were rinsed with 50 mM Tris buffered saline (TBS, pH 7.5). After quenching
the endogenous peroxidase activity in 3% hydrogen peroxide for 10 minutes, blocking reagent was added for 10 minutes. The slides were then washed as before, and were subsequently subjected to the primary antibody reaction. Primary antibody rabbit anti-mouse NGF antibody (clone 2.5s, Serotec, Kidlington, Oxford, UK, dilution of 1:50) was applied in a moist chamber overnight at 4°C. For each case, a corresponding section was incubated in TBS without the primary antibody as a control for non-specific staining. After washing with TBS, a biotinylated link antibody was added for 10 minutes, followed by streptavidin peroxidase for an additional 10 minutes. After washing with TBS, the sections were stained with 3-amino-9-ethyl carbazole (AEC, Vector laboratories, Burlingame, CA, USA). The sections were then counterstained with Mayor’s hematoxylin and mounted.

Results

Ovarian Morphology

A relatively normal appearance of the ovaries was seen in the oil control group. It was associated with numerous primary, secondary and growing follicles in the cellular stroma. Antral and atretic follicles were observed infrequently (Fig. 1). The ovaries of EV control animals showed the cystic follicles that are consistent with the fully developed PCO. They consisted of multiple follicular cysts, atretic follicles, abundant cortical stroma and well-developed theca interna. Focal areas of stromal hyperthecosis were noted (Figs. 2 and 3). These morphological aspects are in accordance with a previous report by Brawer et al. (1986). In the C-Tang (Fig. 4) and Y-Tang (Fig. 5) groups, corpora lutea and corpora albicantia were significantly increased while the number of cystic follicles were obviously decreased compared with the EV control. More growing secondary follicle numbers were also noted compared with the EV control. The morphological changes from C-Tang and Y-Tang were not discernible.

Figure 1. Oil control group. Microscopic finding shows secondary follicles (asterisks) and some atretic follicles (open asterisks).
Figure 2. EV control group. Microscopic finding illustrates many cystic follicles (asterisks). Decrement of the number of primary and secondary follicles is noted.

Figure 3. EV control group. Microscopic finding demonstrates atretic follicles (open asterisks), follicular cysts (asterisks) with well-developed theca interna and abundant cortical stroma with hyperthecosis.

Figure 4. C-Tang group. Microscopic finding shows many corpora lutea (asterisks), and some growing secondary follicles (open asterisks).
Tissues from the ovary, pituitary and hippocampus were used for NGF measurements by immunohistochemistry. A few positive scattered stromal cells for NGF in oil control ovary are illustrated in Fig. 6. The hippocampus and pituitary had only a few positive cells for NGF (figures not shown). Many strong NGF positive thecal and stromal cells in the ovary (Fig. 7) and neuronal cells in the hippocampus were noted while diffusely weak ones were found in the pituitary gland. With the administration of herbal medicine, thecal NGF immunoreactivity was decreased in follicles, not in stromal cells (Fig. 8). The intensity of NGF staining in the pituitary and hippocampus was also decreased compared with that of EV control tissues though it was not significant. In general, there was no significant difference of NGF immunoreactivity in all the organs that were observed between the C-Tang and Y-Tang groups.

Table 2. NGF-immunoreactive Staining in the Various Experimental Groups

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Ovary</th>
<th>Pituitary</th>
<th>Hippocampus</th>
</tr>
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<tr>
<td></td>
<td>Theca Interna</td>
<td>Stromal Cells</td>
<td>Corpus Luteum</td>
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<tr>
<td>Oil control</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>EV control</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Herbal formula</td>
<td>−</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

Oil control (0.2 ml, n = 8); EV control (4 mg of estradiol valerate injection, n = 8); herbal formula (n = 16).
−, Negative.
+, Scattered or clusters of some positive staining cells.
++, Several dozens of positive staining cells.
+++, Positive staining at localized area.
Figure 6. Oil control group. A few scattered positive immunoreactivities for NGF (arrowheads) are noted in the ovarian stromal cells. ABC method, counterstained by hematoxylin.

Figure 7. EV control group. Strong positive immunoreactivity for NGF is shown in many of thecal and stromal cells of the ovary. ABC method, counterstained by hematoxylin.

Figure 8. C-Tang group. A few scattered positive immunoreactivities for NGF (arrowheads) are seen in luteinized cells of the ovary. ABC method, counterstained by hematoxylin.
Weights of Body and Ovaries

Ovarian weights (mg) in all groups were presented in Table 3. Ovarian weight in the EV control group was significantly lower compared to those in the oil-injected group and in the herbal medicine-treated group (p < 0.01). No fluctuations of rat body weight (g) were seen when measured once a week for 8 weeks (data not shown).

<table>
<thead>
<tr>
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<th>Weight (mg)*</th>
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<tbody>
<tr>
<td></td>
<td>Oil Control (n = 8)</td>
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<td></td>
<td>0.040 ± 0.001</td>
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*Weight of the ovary shown as mean ± SE in the different groups: oil control (0.2 ml); EV control (4 mg of estradiol valerate injected); EV + C-Tang treated (estradiol valerate plus Changbudodam-Tang administered); EV + Y-Tang treated (estradiol valerate plus Yongdamsagan-Tang administered).

†p < 0.01, EV control versus oil control.

Discussion

Three important observations were made in the present study: (1) PCO was fully developed in the rat with a single intramuscular injection of EV, (2) PCO resulted in the expression of NGF in the ovaries and the brain tissues, and (3) herbal medicine administration significantly decreased the elevated NGF staining in the ovaries with little effect on the brain tissues.

As the most common endocrine disorder in women at their fertile ages, the PCOS has the morphological characteristic as the presence of multiple medium-sized atretic and antral follicles in the ovary (Franks, 1995). At present, no solid animal model that mimics all of the abnormal pathological conditions underlying the human PCOS is present. Nevertheless, a single injection of EV to adult rats has been shown to reproduce several endocrinological and morphological ovarian abnormalities of PCOS (Lara et al., 2000). The histological examination of the ovaries in the present study revealed that EV dissolved in sesame oil was able to induce the well-defined PCO at day 60 with multiple follicular cysts, atretic follicles, abundant cortical stroma, hyperthecosis and reduced numbers of corpora lutea and albicantia. This is well in accordance with a recent paper (Stener-Victorin et al., 2000) which used electroacupuncture to soothe hyperactivity in the sympathetic nervous system. The dose of 4 mg is twice that described previously (Brawer et al., 1978 and 1986) to achieve the fully developed PCO in the rat. The differences of rat strain and preparatory procedure of injectable EV were taken into account. In addition, the ovarian weight in the EV-treated rats was significantly lower compared to that in the oil-injected control counterparts. It is very similar to the findings of Brawer et al. (1986) and Stener-Victorin et al. (2000). Decreased numbers of corpora lutea and albicantia seen in the present study might explain the reduction in weight of the ovaries. Both herbal formulas were quite effective since no substantial influence of EV in ovarian morphology was seen at day 60.
The present findings of high expression of NGF in the ovaries and brain tissue support recent reports that ovarian NGF concentrations in rats with experimentally induced PCO are elevated (Lara et al., 2000; Stener-Victorin et al., 2000). It further suggests that overproduction of NGF caused by EV might be related to hyperactivation of the ovarian sympathetic input. This enhanced activity of the neurotrophic-neurogenic control system contributes to the process by which EV induces ovarian cysts and disrupts ovulation in rats. Since the sympathetic nerves arriving at the ovary are via the superior ovarian nerves, our herbal formula seemed to disrupt the ovary-related nervous system at the early stage of ovarian change. This was proved true because increased ovarian NGF concentrations have been shown to precede the development of morphological changes in rats with PCO (Lara et al., 2000). Furthermore, cyclicity and ovulatory capacity in EV-treated rats were restored when superior ovarian nerves were transected (Barria et al., 1993). This was the main reason for beginning the herbal medicine treatment as early as 2 days after EV injection and to sacrifice at day 60 in the current study.

In conclusion, our study proved that a single intramuscular injection of EV was able to induce the PCO in the rat model. The medium for the induction of PCO was hyperactivity in the sympathetic nervous system that was shown as an expression of NGF in the ovaries and the central nervous system. Herbal formulas prescribed by our team were efficacious in relieving some of symptoms of PCO, and acupoint manipulation needs to be evaluated in the further studies.

Acknowledgments

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References


PCO TREATMENT WITH HERBAL MEDICINE


