Effects of Tokishakuyakusan on the ovary in hypophysectomized rats

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The prescription Tokishakuyakusan (TS; Dang-Gui-Shao-Yao-San in Chinese) is widely used in traditional Chinese medicine. Since delay of ovarian follicle maturation and fall of bone density are known in hypophysectomized (HPX) rats, we investigated the effects of TS on ovaries in HPX rats. Although TS did not recover the ovary weight decreased by hypophysectomy, it recovered the uterine weight. Two weeks after hypophysectomy, the expression of steroidogenic acute regulatory protein (StAR) mRNA was decreased, but recovered by administration of TS. On the other hand, the expression of pituitary adenylate-cyclase activating polypeptide receptor type 1 (PAC1) and progesterone receptor (PR) mRNA was increased, but reduced by administration of TS. However, the expression of estrogen receptor α (ERα) mRNA did not synchronize with PAC1 mRNA and PR mRNA expression. Moreover, TS was demonstrated to promote the follicle maturation by histological analysis, and to decrease significantly the urinary deoxyxypiridinoline (DPD) level. These findings suggest that TS promotes the maturation of follicles and suppresses the bone metabolism associated with pituitary hormones.

Key words Tokishakuyakusan, Kampo medicine, hypophysectomy, ovary, bone metabolism.

Abbreviations DPD, deoxyxypiridinoline; ERα, estrogen receptor α; HPX rats, hypophysectomized rats; LH, luteinizing hormone; PAC1, pituitary adenylate-cyclase activating polypeptide receptor type 1; PACAP, pituitary adenylate-cyclase activating polypeptide; PCR, polymerase chain reaction; PR, progesterone receptor; StAR, steroidogenic acute regulatory protein; TS, Tokishakuyakusan.

Introduction

Tokishakuyakusan (TS; Dang-Gui-Shao-Yao-San in Chinese), a famous traditional Chinese prescription, is used frequently and effectively in obstetrics and gynecology departments in Japan, China and Korea. TS has been reported to improve ovarian dysfunction, such as luteal insufficiency, 11 and amenorrhea, 12 and to have a luteotropic effect, such as release of estrogen and progesterone in ovary, and stimulation of LH accumulation. 3,4 Our previous study reported that the secretion of gonadotrophic hormone might be promoted by TS through the hypolathamic expression of pituitary adenylate-cyclase activating polypeptide (PACAP), PACAP type I receptor (PAC1) mRNA and PAC1 protein in hypophysectomized (HPX) and ovariecetomized rats. 7

Ovaries, whose hormonal functions are controlled by the pituitary, are a main organ of producing and releasing female hormones. When the pituitary is removed (hypophysectomy), the follicle growth and maturation in ovaries become abnormal. 8,10 The biosynthetic cascade of female steroid hormones starts with the conversion of cholesterol to pregnenolone. Steroidogenic acute regulatory protein (StAR) is a key transporter of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where cholesterol is converted to pregnenolone. 22 Then, mutation in the StAR gene impedes gonadal steroidogenesis. 13,14 Ovulation and the subsequent luteinization of the ruptured follicles are induced by the midcycle gonadotropin surge. PACAP has the potential of being a local regulator of ovarian physiology 15,21 and periovulatory progesterone production, in particular. 22,23 PACAP and PAC1 mRNA are expressed in the same preovulatory follicles, and PACAP acts as an auto- or para-regulator through PAC1 in granulosa cells and cumulus cells of large preovulatory follicles. 24 The intracellular progesterone receptor (PR), which belongs to a family of the ligand-induced, hormonally regulated nuclear transcription factor, is known to mediate many progesterone actions, although there are other progesterone-binding proteins that have been implicated in mediating progesterone actions. 25,26 PR mRNA and PR protein are localized to various types of ovarian cells. These cells transiently express the PR gene in response to the preovulatory gonadotropin surge as shown experimentally in rats. 27

The HPX rats have been used as a model to study the effects of pituitary hormone deficiency on bone. 28,30 Deoxyxypiridinoline (DPD) is one of the markers of bone resorption, and present only in bone collagen. 11,32

Since, the in vivo effects of TS on ovaries and bone metabolism originated from pituitary hormones have not yet been clarified. We report in this paper the changes in StAR, PAC1, PR, estrogen receptor α (ERα) mRNA, histological observation in the ovaries, and urinary DPD levels in HPX female rats after oral administration of TS.

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**Materials and Methods**

**Animals.** Ten-week-old female Wistar rats (Japan SLC Inc., Shizuoka, Japan) were HPX or sham-operated (Sham) at 8 weeks of age. The rats were maintained in a light, temperature and humidity controlled environment (lights on, 07:00-19:00 h; 22±2°C and 50±5%, respectively) with access to food and water *ad libitum*. The animals were handled in accordance with the Guide for Animal Experiments of University of Toyama. Sham rats (control group, n=4) were given water (10 ml/kg, p.o.) once each day for 7 days, and HPX rats were divided into 3 groups of 3 animals each and administrated drugs once per day for 7 days as follows; (1) water (10 ml/kg, p.o.); (2) TS dissolved in water (1000 mg/kg, p.o.) and (3) a mixture of progesterone (300 µg/kg, s.c.) and 17β-estradiol (3 µg/kg, s.c.) dissolved in 0.9% sodium chloride containing 1% Tween 20. An experimental schedule is shown in Fig. 1. On day 7, urine samples were collected from metabolic cages to measure a bone resorption maker DPD.35

**Reagents and Drugs.** TS was composed of six medicinal plants (Angelicae Radix, 9g; Paconiae Radix, 18g; Atractylodis Rhizoma, 12g; Alismatis Rhizoma, 12g; Hoelen, 12g; Cnidii Rhizoma, 9g) and these crude drugs were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan). The mixture of TS (72g) was extracted with 1000 ml of distilled-water at 100°C for 1 h. The decotion was filtered, concentrated under reduced pressure, and lyophilized. The powdered extract was dissolved in water immediately before oral administration. Progesterone (P4) and 17β-estradiol (E2) (Sigma-Aldrich Japan Co., Tokyo, Japan), and ISOGEN (Nippon Gene, Toyama, Japan) were purchased from the respective companies indicated in parentheses, and PCR primers were obtained from Nippon EGT Co., Ltd. (Toyama, Japan).

**Reverse transcription-polymerase chain reaction (RT-PCR).** After 8 days, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and decapitated, and then ovaries were excised, immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from ovaries using ISOGEN, and dissolved in diethylpyrocarbonate-water. The RNA concentration was measured by spectrophotometry at 260 nm. The first standard cDNA was synthesized using 200 units of SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA, USA) from 2.0 µg of total RNA and 5.0 µM oligo (dT) primer in a 20 µl mixture. PCRs proceeded in 10 µl volumes containing 1.0 µl of the first standard cDNA, 0.5 µM of sense and antisense primers (Table 1), 250 µM dNTPs and 2 units of Taq DNA polymerase (Promega, Madison, WI, USA) in a reaction buffer containing 2.5 mM MgCl₂. Reactions proceeded in a thermal cycler (Astec, Fukuoka, Japan). Thermocycling was performed using the following protocol: 72°C for 5 min, x-cycles (94°C for 1 min, y-°C for 2 min, and 72°C for 2 min), and then, 72°C for 10 min. The extension cycles and annealing temperature were shown in Table 1. Accession number in GenBank is as follows: StAR, U17280; PAC1, NM_007407; PR, M15716; ERα, Y00102; β-actin, V01217. The PCR products were resolved by electrophoresis on 6% polyacrylamide gel, stained with ethidium bromide. The bands were visualized under UV light and quantified with a densitometer using ATTO Densitograph 4.0 software (ATTO, Tokyo, Japan).34,35

**Immunohistochemistry.** In another set of experiments, rats were perfused transcardially with 0.9% saline and 4% paraformaldehyde, and ovaries were cryoprotected in 4% paraformaldehyde containing 30% sucrose, then frozen sections (10 µm) were cut using a microtome (Leica, Wetzlar, Germany). The sections were dipped in 0.3% hydrogen peroxide/phosphate buffer (PB) for 15 min, rinsed three times

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**Fig. 1** An experimental schedule from birth to dissection.

**Table 1.** Primers employed and expected size of the PCR amplified cDNA

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Sense primer (5'-3')</th>
<th>Anti-sense primer (5'-3')</th>
<th>Product size (bp)</th>
<th>Extension cycles; x-cycles</th>
<th>Annealing temp. (°C); y-°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>StAR</td>
<td>GCAGCAAGGCAACCTGGTG</td>
<td>TGATTGCTTCCGCAGCC</td>
<td>406</td>
<td>23</td>
<td>59</td>
</tr>
<tr>
<td>PAC1</td>
<td>TTTTACGCATCATCATCCTCTT</td>
<td>CTCCTTGACCTCTTTCTTCCTTT</td>
<td>374</td>
<td>40</td>
<td>59</td>
</tr>
<tr>
<td>PR</td>
<td>CCCACAGAAGTTGCACGACTCTC</td>
<td>TACCTCGACATCTTCCCGGG</td>
<td>326</td>
<td>32</td>
<td>61</td>
</tr>
<tr>
<td>ERα</td>
<td>AATTCTGACAATCGAGCCGAG</td>
<td>GTGCTCTCAGATCTCCATCCCTTC</td>
<td>345</td>
<td>35</td>
<td>58</td>
</tr>
<tr>
<td>β-actin</td>
<td>AACGCTCTGACGACTGTA</td>
<td>GTGACAGCATGGCTCTGGT</td>
<td>222</td>
<td>20</td>
<td>57</td>
</tr>
</tbody>
</table>
in PB, incubated in blocking solution (3% goat serum and 0.3% Triton X-100/PB) for 1 h at room temperature, and then kept overnight at 4°C with a monoclonal antibody against PR (1:1000, Dako Cytomation, Glostrup, Denmark). On the following day, the sections were rinsed three times in PB, and antigen was visualized after 2 h incubation at room temperature using secondary goat anti-mouse IgG (Alexa Fluor 488, Molecular Probes, OR, USA). The sections were further rinsed three times in PB. The stained sections were mounted and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA). The immunolabelling was detected in the ovary using a fluorescence microscope (AX-70; Olympus, Tokyo, Japan).

Statistics. All values are represented as means ± S.E.M. Significant differences between water-treated Sham and HPX groups were analyzed by Student’s t-test. Statistical significance between water-treated and TS- or P4+E2-treated HPX groups were also evaluated by Student’s t-test (Prism; GraphPad, San Diego, CA, USA).

Results

Effects of TS on ovary and uterus weights of HPX rats. Two weeks after hypophysectomy, the ratio (%) of the weight of the ovary to the body weight was decreased in the water-treated HPX group, compared with that of the Sham group. Administration of TS or P4+E2 did not change this ratio (Fig. 2A). On the other hand, the ratio (%) of the weight of the uterus to the body weight was decreased in the water-treated HPX group, compared with that of the Sham group. Administration of TS significantly increased this ratio, and administration of P4+E2 also significantly increased this ratio, compared with the water-treated HPX group (Fig. 2B).

Effects of TS on StAR mRNA expression in the ovary of HPX rats. The expression of StAR mRNA was slightly reduced in the water-treated group two weeks after hypophysectomy, compared with that of the Sham group (Fig. 3). TS significantly recovered StAR mRNA expression when compared with the water-treated HPX group. However, administration of P4+E2 did not affect StAR mRNA expression, compared with the water-treated HPX group (Fig. 3).

Effect of TS on PAC1 mRNA expression in the ovary of HPX rats. Two weeks after hypophysectomy, PAC1 mRNA expression was significantly increased in the water-treated HPX group, compared with that in the Sham group (Fig. 4). Administration of TS significantly reduced PAC1 mRNA expression, compared with the water-treated HPX group. However, administration of P4+E2 did not appreciably change PAC1 mRNA expression, compared with the water-treated HPX group (Fig. 4).

Effects of TS on PR and ERα mRNA expression in the ovary of HPX rats. Two weeks after hypophysectomy, PR mRNA expression was significantly increased in the water-treated HPX group, compared with that of the Sham group (Fig. 5A). Administration of TS significantly reduced PR mRNA expression, compared with the water-treated HPX group. However, administration of P4+E2 did not change PR mRNA expression, compared with the water-treated HPX group (Fig. 5A). In addition, two weeks after hypophysectomy, ERα mRNA expression was increased in the water-treated HPX group compared with that of the Sham group (Fig. 5B). The administration of both TS and P4+E2 did not change ERα mRNA expression, compared with the water-treated HPX group.

Effect of TS on PR protein expression in the ovary of HPX rats. PR protein was widely expressed in the ovary. PR protein expression in the ovary was almost at the same levels among Sham-operated, water-treated, TS-treated, and P4+E2-treated HPX groups (Fig. 6). On the other hand, ovaries of the water-treated HPX group showed obviously small granular cells, compared with those of the Sham (Fig. 6A, B). Administration of TS induced growing of follicles,

Fig. 2 Effects of TS on the weight of ovary and uterus in HPX rats.
Sham, sham-operated rats; TS, Tokishakuyayusan; P4, progesterone; E2, 17β-estradiol. Statistical significance was examined by Student's t-test: * p < 0.05; ** p < 0.01.
compared with water-treated HPX group (Fig. 6B, C). Especially, ovaries of the TS-treated HPX group held follicles approaching maturity (fam) (Fig. 6C, arrows). The ovaries of P4+E2-treated HPX group did not hold such follicles in comparison with those of the TS-treated HPX group (Fig. 6C, D), but included growing follicles, compared with the water-treated HPX group (Fig. 6B, D).

**Effect of TS on DPD in HPX rat urine.** Two weeks after hypophysectomy, urinary DPD was obviously increased in the water-treated HPX group, compared with the Sham group (Fig 7). Administration of TS significantly decreased urinary DPD level, compared with the water-treated HPX group. However, administration of P4+E2 did not affect the urinary DPD level at all (Fig. 7).

**Discussion**

We investigated the effects of TS on the ovary using HPX female rats. At first, we examined the effects of TS on ovary weight of HPX rats. The pituitary controls the function of ovary producing and releasing female hormones. Advanced follicle growth and maturation in ovaries were reported to become abnormal by removing the pituitary, resulting in a uterine atrophy. The uterine atrophy is recovered by administration of exogenous female hormones. Following hypophysectomy, the weight of ovaries in the
water-treated HPX group was significantly decreased in two weeks. Administration of TS did not recover the weight of ovaries in comparison with that of the water-treated HPX group (Fig. 2A). The uterine weight was significantly decreased in the water-treated HPX group, but recovered by administration of TS in comparison with that in the water-treated HPX group (Fig. 2B). These findings indicated that TS had a female hormone-like effect or assisted to produce and release ovarian hormones, without increase of the weight of ovaries.

Since TS was reported to have a luteotropic effect releasing estrogen and progesterone in ovary and stimulating cAMP accumulation, we investigated effects of TS on StAR mRNA expression in the ovaries of HPX rats. StAR is responsible for transporting cholesterol from the outer to the inner mitochondrial membrane, possibly as the first rate-limiting step in steroidogenesis. Mutation of StAR gene was reported to cause severe impairment of gonadal steroidogenesis. Two weeks after hypophysectomy, the water-treated HPX group showed significantly decreased expression of StAR mRNA, compared with a Sham group, and administration of TS significantly recovered the expression of StAR mRNA in comparison with that of the water-treated HPX group (Fig. 3). These findings suggested that TS stimulated StAR and promoted to release female hormones. Rao et al. reported that continued administration of estradiol and follicle-stimulating hormone caused a decrease in the proliferative activity of both granulosa and theca cells. In our present study, administration of P4+E2 showed no significant change in the expression of StAR mRNA in comparison with that of the water-treated HPX group (Fig. 3).

PACAP has effects on the female reproductive system, and in particular, for periiovulatory progesterone production. PACAP binds to at least three types of G
protein-coupled receptors; type I (PAC₁), type II, and vasoactive intestinal peptide (VIP) receptor. The PAC₁ is expressed in the central nervous system and various peripheral tissues such as ovary.16,21). Two weeks after hypophysectomy, PACAP mRNA levels were reported to decrease significantly in the rat hypothalamus.36 In our present study, following hypophysectomy, the water-treated HPX group showed significantly increased expression of PAC₁ mRNA in ovaries, compared to the Sham group (Fig. 4). This finding suggested that hypophysectomy led to a decrease the PACAP mRNA levels in hypothalamus, and to activate upwards expressing PAC₁ mRNA in ovaries. Administration of TS, but not of P4+E2, significantly reduced the expression of PAC₁ mRNA in ovaries in comparison with that of the water-treated HPX group (Fig. 4). These results suggested that TS stimulated the release of PACAP from hypothalamus.

The intracellular PR, which is a key regulatory molecule in the ovary, and acts on the downstream of LH during ovulation, is known to mediate many, if not all, progesterone actions.37-40 PR mRNA and PR protein have been localized to various ovarian cell types. However, one consistent cellular localization for PR mRNA and PR protein is luteinizing granulosa cells, not in luteal cells, and spontaneously immortalized granulosa cells.41-43 Interestingly, Park et al. cloned a 5'-flanking region of the rat PACAP gene and identified a consensus PR element.44 Furthermore, PR activation was shown to mediate LH-induced PAC₁ gene expression in rat granulosa cells.45 In our study, the expression patterns of mRNA were similar between PAC₁ (Fig. 4) and PR (Fig. 5A) by administration of TS, but not of ERα (Fig. 5B). Administration of TS did not change the expression of ERα, compared with the water-treated HPX group (Fig. 5B), because TS did not appreciably bind to ER.55 However, following administration of P4+E2, the expression of ERα mRNA was slightly decreased, compared with that of the Sham group. As for ERα in the P4+E2-treated HPX group, the expression level was decreased possibly by competition in binding with ERα between P4 and E2 (Fig. 5B). Then administration of TS produced follicles approaching maturity (fam) as shown in Fig. 6. Following hypophysectomy, which results in the absence of LH, TS seemed to complement the pituitary functions and/or support the process of ovulation.

On the other hand, hypophysectomy induced the discontinuance of bone growth.29-31 Skeletal tissue contains a network of nerve fibers expressing several neuropeptides, including PACAP. This peptide was demonstrated to regulate osteoclast formation and osteoclast activity, and mouse osteoclasts expressed VIP-1 and PACAP, but not VIP-2 receptor mRNA.46 PACAP inhibited bone resorption by isolated rabbit osteoclasts.47,48 DPD is one of the markers of bone resorption.52 When bone resorption is promoted, the urinary DPD level increases to lead osteoporosis. In our previous study, one week after hypophysectomy, the water-treated HPX group showed an increase of the urinary DPD level (451.7±25.30 nM/mM creatinine), and administration of TS did not decrease the DPD level in comparison with that in the water-treated HPX group (accepted). In the present study, two weeks after hypophysectomy, the water-treated HPX group showed an increase of the urinary DPD level (767.3±54.15 nM/mM creatinine), and administration of TS decreased the DPD level in comparison with that of the water-treated HPX group (Fig. 7). Differences in previous and present studies were observed for levels of pituitary hormones, especially hypothalamic PACAP,38 possibly due to the difference in period after hypophysectomy. These results suggested that TS inhibited osteoclast by PACAP/ PAC₁ on bone. However, we can not rule out discrepancies involved in the inhibition of osteoclast regulation by PACAP/PAC₁.

In summary, we demonstrated the effects of TS on ovaries and hormone deficiency on bone in HPX rats. TS may promote the maturation of follicles and the inhibition of the bone metabolism originated from pituitary hormones.

Acknowledgments

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References

12) Clark, B.J., Wells, J., King, S.R., Stocco, D.M.: The purification,


当帰芍薬散は古来より婦人科領域で使われている代表的漢方剤の1つである。また下垂体摘出ラット卵巣では卵泡の発育遅延、骨密度の低下を引き起こすことが知られている。そこで我々は、ラットの下垂体摘出後、当帰芍薬散の経口投与を1週間行い、投与後の卵巣を解析した。下垂体摘出による卵巣重量低下を当帰芍薬散は回復させなかったが、子宮重量低下に対する回復はみられた。また、下垂体摘出により卵巣内StAR蛋白質のmRNA発現量は減少したが、当帰芍薬散を投与することにより発現量の回復がみられた。下垂体アデニル酸シクラーゼ活性化学種変替受容体（PAC）プロゲステロン受容体（PR）のmRNA発現量は下垂体摘出により上昇したが、当帰芍薬散はこれを抑制した。しかし、エストロゲン受容体α（ERα）のmRNA発現量の変化は見られなかった。さらに組織学的観察より、当帰芍薬散は卵胞発達を促進する傾向が見られた。また、当帰芍薬散は骨代謝のマーカーである尿中DPDレベルの上昇を抑制した。以上の結果から、当帰芍薬散は卵巣における卵胞の発達を促進し、下垂体ホルモン由来の骨代謝を抑制することが示唆された。

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