SHORT COMMUNICATION

Preventive effects of a herbal medicine on bone loss in rats treated with a GnRH agonist

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Abstract

The study was designed to evaluate the effects of a traditional Chinese herbal medicine Hochu-ekki-to (Bu-zong-yi-qi-tang), which was composed of 10 herbal medicines and had been used for the treatment of oligospermia and as a postoperative medication in Japan, on bone loss in rats treated with a gonadotropin-releasing hormone (GnRH) agonist. Female rats at 40 weeks of age were divided into 4 groups of 8 rats each. In the three experimental groups, each animal received subcutaneous injections of the long-acting GnRH agonist, buserelin acetate, once every four weeks throughout the experiment.

Beginning at 48 weeks of age, the experimental groups were given diets containing conjugated estrogens or Hochu-ekki-to for 8 weeks. The administration of the GnRH agonist reduced the bone mineral density in the whole femur to 91.0% of that in the control group. However, administration of conjugated estrogens and Hochu-ekki-to increased the serum concentrations of estradiol 16.8- and 5.3-fold respectively compared with concentrations in the GnRH agonist-treated group, resulting in the augmentation of the bone mineral density to 110.3% and 106.2% respectively. These findings indicate that Hochu-ekki-to enhances the reduced bone mineral density and causes a slight elevation of the serum estradiol levels in the chemically castrated rats.

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Introduction

The chronic administration of a gonadotropin-releasing hormone (GnRH) agonist offers a means of treating patients with symptomatic endometriosis, uterine adenomyosis and leiomyoma, because the administration of such potent agonists leads to an acute, reversible hypo-estrogenism via a desensitization of the pituitary gland to hormonal stimulation, possibly by the down-regulation of GnRH receptors in women (1). However, GnRH agonist treatment induces adverse effects, particularly increased bone remodeling and bone loss due to a temporary clinical menopausal pattern. A traditional Chinese herbal medicine, Hochu-ekki-to (HET; Bu-zong-yi-qi-tang) has been used for the treatment of oligospermia (2) and as a postoperative medication in Japan. We have experienced a clinical case in which the progress of the patient from 66 to 76 years of age could be monitored (data not shown). The diagnosis at the first medical examination of the patient was postmenopausal osteopenia and senile colpitis – i.e. the bone mass was 71.1% of the age-matched average value. The bone mass increased to 90.7% of the age-matched average value 2 years after the beginning

of HET treatment, with body weight gain (+5 kg) for 5 years. In the present study, we investigated the effects of conjugated estrogens as an add-back replacement drug, or HET on femoral bone mineral density (BMD) in female rats chronically treated with the long-acting GnRH agonist, buserelin acetate.

Materials and methods

The long-acting GnRH agonist, buserelin acetate ([D-Ser(tBu)⁶Pro⁹]GnRH; Suprecur MP 1.8; a gift from Hoechst Marion Roussel, Ltd, Bridgewater, NJ, USA; 100 mg/kg body weight) was used. Conjugated estrogens, i.e. mainly estrone sodium sulfate and equilin sodium sulfate extracted from pregnant mare urine (Premarin, Asahi Chemical Industry Co., Tokyo, Japan) were given as a diet containing Premarin (0.313 mg in 1 kg diet). HET (a gift from Tsumura Co., Tokyo, Japan, lot no. 270041010) is composed of ten herbal drugs: a mixture consisting of 4.0 g *Astragali radix* (Ougi), 4.0 g *Atractylodis lanceae rhizoma* (Soujyutsu), 4.0 g *Ginseng radix* (Ninjin), 3.0 g *Angelicae radix* (Touki), 2.0 g *Bupleuri radix* (Saiko), 2.0 g *Zyzyphi fructus* (Taisou), 2.0 g *Aurantii nobilis pericarpium* (Chinpi), 1.5 g

Glycyrrhizae radix (Kanzou), 1.0 g *Cimicifugae rhizoma* (Shouma) and 0.5 g *Zingiberis rhizoma* (Shoukyou) was prepared, from which 5.0 g HET were extracted with hot water, filtered, lyophilized, and stored at 4 °C. In the present study, HET was given as a diet containing HET (12.5 g extract in 1 kg diet).

Aged female Sprague-Dawley rats (Sankyo Laboratory Service Co., Tokyo, Japan) were used in the present study. Throughout the experiment, all rats were kept under controlled lighting and temperature, with tap water available *ad libitum*, and were weighed every 7 days. Beginning at 40 weeks of age, the animals were divided into four groups of eight rats each. In the three experimental groups, each animal received subcutaneous injections of the long-acting buserelin acetate once every 4 weeks throughout the experiment. The animals in the control group and the first experimental group were fed a commercial diet (CE-2, CLEA Japan, Co., Tokyo, Japan) containing 1.18% calcium and 1.03% phosphorus, and were given drinking water available *ad libitum*. Beginning at 48 weeks of age, the animals in the second and third experimental groups were fed the same commercial diet containing either conjugated estrogens or HET respectively. All animals were bled, at the age of 56 weeks, by cardiac puncture under deep urethane anesthesia (1.5 g urethane/kg body weight; Merck, Darmstadt, Germany), and the anterior pituitary, ovaries, uterus, liver, spleen and bilateral femurs were removed. Each organ was weighed, and each femur was fixed in 99.5% ethanol and stored. All experimental procedures conformed to the regulations described in the US NIH Guide for the Care and Use of Laboratory Animals.

The serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were determined with radioimmunoassay kits (a gift from the National Institute of Arthritis, Metabolism and Digestive Disease Rat Pituitary Hormone Distribution Program and Dr AF Parlow, MD, USA). The serum levels of estradiol were also determined with a radioimmunoassay kit (Diagnostic Products Co., Los Angeles, CA, USA). Serum calcium concentration and alkaline phosphatase activity were determined with commercial kits (Calcium C-test and Alkaline phosphatase B-test, Wako Pure Chemical Industries, Osaka, Japan).

Each fixed femur was dissected free from adhering soft tissues, and microradiographed (Softex, Softex Co., Tokyo, Japan) (at 90 kV, 1 mA for 60–90 s) together with a standardized step-wedge made of synthetic hydroxyapatite (HA; Mitsubishi Kasei Co., Ltd, Tokyo, Japan). Since there is a linear relationship between the logarithms of HA density ($\mu\text{g}/\text{mm}^2$) of the step-wedge and gray levels (256 steps) of the microradiographic image of the step-wedge (3), the BMD ($\mu\text{g HA}/\text{mm}^2$) was determined by analyzing the gray levels in the microradiograph with an image analyzer (Winroof, Mitani Corp., Fukui, Japan). Then, the BMD of the whole femur was determined by analyzing the total content of HA in the femur ($\mu\text{g HA}/\text{femur}$) and the femoral volume, and was expressed as $\mu\text{g HA}/\text{mm}^3$.

The statistical significance of differences between groups was evaluated by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Scheffé's multi-comparison test. A value of $P < 0.05$ was considered significant.

Results

Although the subcutaneous injections of the GnRH agonist, buserelin acetate, did not affect the body growth in rats, the diet containing conjugated estrogens significantly decreased the body growth to 83.2% of that in the GnRH agonist-treated group ($P < 0.01$) (data not shown).

Chronic treatment using the GnRH agonist significantly reduced the wet weights of the ovaries ($P < 0.01$), uterus ($P < 0.05$) and liver ($P < 0.01$), although the treatment did not affect the weights of the anterior pituitary and spleen. Treatment with conjugated estrogens increased the weights of the pituitary ($P < 0.05$), uterus ($P < 0.05$) and liver ($P < 0.01$) compared with those in the GnRH agonist-treated group. However, administration of HET did not affect the body growth and the organ weights compared with those in the GnRH agonist-treated group (data not shown).

Treatment with buserelin acetate did not affect serum FSH levels, but it reduced serum levels of LH and estradiol although not significantly (Table 1). The administration of conjugated estrogens and HET significantly elevated serum estradiol levels ($P < 0.05$ and

Table 1 Serum concentrations of LH, FSH, estradiol and calcium, and alkaline phosphatase (ALP) activity in rats treated with GnRH agonist (buserelin acetate) alone or with either conjugated estrogens or HET. Results are means $\pm$ S.E.M.

	GnRH agonist	Estrogen	HET	Normal control
LH (ng/ml)	0.60 $\pm$ 0.14	0.40 $\pm$ 0.12	1.23 $\pm$ 0.40	2.01 $\pm$ 0.84
FSH (ng/ml)	5.29 $\pm$ 0.41	4.73 $\pm$ 0.43	6.17 $\pm$ 0.66	7.60 $\pm$ 0.95
Estradiol (pg/ml)	1.97 $\pm$ 0.57	33.12 $\pm$ 14.22*	10.36 $\pm$ 1.09**	65.89 $\pm$ 60.84
Calcium (mg/dl)	9.82 $\pm$ 0.21	11.01 $\pm$ 0.18	9.79 $\pm$ 0.20	10.73 $\pm$ 0.27
ALP (IU/l)	278 $\pm$ 34	226 $\pm$ 15	208 $\pm$ 26	310 $\pm$ 44

* $P < 0.05$, ** $P < 0.01$ compared with GnRH agonist-treated group.

Figure 1 Effects of chronic treatment with a GnRH agonist with or without conjugated estrogens or HET on BMD ($\mu\text{g HA/mm}^3$) in rat whole femur. Data are the means $\pm$ S.E.M. of 8 samples. * $P < 0.05$, ** $P < 0.01$ compared with the GnRH agonist group.

$P < 0.01$ respectively) compared with the GnRH agonist-treated group. There were no differences among groups in serum calcium concentration and serum alkaline phosphatase activity.

The BMD in the whole femur was significantly reduced by treatment with buserelin acetate ($P < 0.01$) (Fig. 1). The simultaneous administration of conjugated estrogens or HET significantly prevented the loss of BMD induced by buserelin acetate treatment ($P < 0.01$ and $P < 0.05$ respectively) (Fig. 1).

Discussion

Some Chinese herbal medicines have been used for the treatment of postmenopausal osteoporosis and osteopenia induced by ovariectomy or GnRH agonist treatment. The efficacy of Hachimi-jiou-gan (Ba-wei-di-huang-wan) for the treatment of bone loss was reported in postmenopausal women by Koyama (4). Hidaka *et al.* demonstrated the effectiveness of Hachimi-jiou-gan and Unkei-tou (Wen-jing-tang) in ovariectomized rats (5). On the other hand, HET has been used as a postoperative medication in Japan. For example, Kaneko *et al.* reported that HET suppressed IgE production in mice (6). Harada *et al.* (7) and Haranaka (8) reported the antitumor effects of HET. In the present study, we

investigated the preventive effects of estrogens or HET on the loss of BMD in female rats chronically treated with a potent GnRH agonist. As Gaumett *et al.* reported (9), estrogenic treatment induced a significant decrease in the body weights of chemically castrated rats. Estrogens are suggested to be related to body growth. DeMoss and Wright have demonstrated that there is a clear relationship between skeletal mass and body mass in mature animals (10). Although chronic treatment with the GnRH agonist in the present study did not affect anterior pituitary weight, serum LH, but not FSH, levels were lowered by the treatment, resulting as expected in atrophy of the ovaries and uterus and a decrease in serum estradiol levels. An increase in serum bone alkaline phosphatase activity was observed in 28 patients treated with a GnRH agonist for 6 months (11), and in postmenopausal women (12), and resulted in an increase of bone resorption in ovariectomized rats (9). Estrogenic treatment is known to reduce the elevated bone turnover in menopausal women (12, 13). The BMD value was decreased to 91.0% of that of the normal control animals by the 16-week treatment with the GnRH agonist. However, the simultaneous 8-week administration of conjugated estrogens or HET elevated the serum estradiol levels, resulting in enhancement of the BMD values compared with those in the rats

treated with the GnRH agonist alone. Ota *et al.* demonstrated that herbal Shakuyaku (*Paeoniae radix*), Keihi (*Cinnamomi cortex*) and Botanpi (*Moutan cortex*) stimulated the aromatase activity in human granulosa cells and increased estradiol secretion *in vitro* (14). Tamaya *et al.* reported that some components of herbal medicines influenced the steroid effects via glucocorticoid and mineralocorticoid receptors and to a lesser extent via estrogen receptors – i.e. as phytoestrogens, or serum sex hormone-binding globulin and corticosteroid-binding globulin (15). Ingredients of *Astragali radix*, *Ginseng radix* and *Glycyrrhizae radix* in HET have been suspected to have a weak estrogenic action as phytoestrogens, and chronic administration of *Glycyrrhizae radix* has been known to lead to hypopotassemia. Our findings indicate that HET could be useful when combined with careful monitoring of the biochemical markers of osteoblastic activity or bone resorption and the BMD of the patients with bone mineral disorders.

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