Journal of Food and Drug Analysis, Vol. 13, No. 3, 2005, Pages 232-238　　藥物食品分析　第十三卷　第三期

Effects of Chia-wei-hsiao-yao-san on Bone Loss in Ovariectomized Rats

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(Received: January 28, 2005; Accepted: May 18, 2005)

ABSTRACT

Preventive effects of two traditional Chinese medicines, Chia-wei-hsiao-yao-san and Kuei-lu-erh-hsien-chiao, on the bone loss induced by ovariectomy in Long-Evans rats were investigated. The rats were divided randomly into five groups. The first group was given a sham operation with administration of distilled water, whereas the second, third and fourth groups were ovariectomized with administration of distilled water, Kuei-lu-erh-hsien-chiao and Chia-wei-hsiao-yao-san, respectively. The rats of the fifth group were embedded by intraperitoneal with a silicon tube containing 17 β-estradiol (10% in cholesterol) at a dose of 10 μg/kg. Eight weeks later, the mean differences of spinal bone mineral density (BMD) in groups 1, 4 and 5 were significantly higher than that in group 2. The mean differences of plasma alkaline phosphatase (ALP) in groups 1 and 5 were significantly lower than those in group 2, but there were no significant differences between groups 1 and 5. In histologic analysis, the cross sections from lower third of the tibial shaft in group 3 showed more mucopolysaccharide accumulation than those in groups 4 and 5. The urinary *N*-telopeptide of type I collagen (NTx) levels did not differ significantly among groups 1, 2, 3, 4 and 5. Nevertheless, the mean differences of urinary deoxypyridinoline (Dpd) levels in group 5 were significantly higher than those in group 2. 17 β-estradiol and Chia-wei-hsiao-yao-san showed more profound effect than Kuei-lu-erh-hsien-chiao in the prevention of bone loss in the ovariectomized (OVX) rats. Although 17 β-estradiol exerted more profound effect, Chia-wei-hsiao-yao-san showed good compliance and safety for relief of climacteric symptoms for post-menopausal women in a previous study. Therefore, Chia-wei-hsiao-yao-san might be an alternative for preventing bone loss.

Key words: 17 β-estradiol, Chia-wei-hsiao-yao-san, Kuei-lu-erh-hsien-chiao, ovariectomized, osteoporosis

INTRODUCTION

Estrogen has long been touted as a beneficial factor in preventing bone loss in premenopausal women. Women who were menopausal or received the oophoreectomy lose this protection because of dramatic decreases in estrogen levels due to natural atrophy of the ovaries[1,2] or surgical deprivation of the ovaries. Bone mass decreases significantly during the postmenopausal[3] and surgical menopausal states. The ovariectomized rat model is used to mimic the trabecular bone loss observed in the post-menopausal and surgical menopausal states[4].

Estrogen replacement therapy in postmenopausal and surgical menopausal women restores the protective actions against bone loss[5,6]. However, this treatment has several side effects, such as increased risk of breast cancer and resumption of menses[7,8]. Chia-wei-hsiao-yao-san was used as a safe and efficacious therapy that might be an alternative for relief of climacteric symptoms in post-menopausal women who refuse or have contraindications for hormone replacement therapy[9]. Chia-wei-hsiao-yao-san and Kuei-lu-erh-hsien-chiao have been reevaluated by clinicians[10,11]. According to the traditional Chinese medicines, bone loss is categorized as bone wilting, vacuity taxation or lumbar pain and the kidney governs the bone and engenders bone and marrow. These illustrate the close relationship between the kidney and the bone. Kuei-lu-erh-hsien-chiao includes Testudinis Plastrum, Cervi Cornu, Lycii Fructus and Ginseng Radix. Testudinis Plastrum and Cervi Cornu are collagen-rich materials, and Testudinis

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Plastrum is believed to enhance physical activity and to promote the function of kidney[12]. The formula of Chia-wei-hsiao-yao-san contains Angelicae Radix, Atractylodis Rhizoma, Paeoniae Radix, Bupleuri Radix, Hoelen, Glycyrrhizae Radix, Moutan Cortex, Gardeniac Fructus, Zingiberis Sieatum Rhizoma and Menthae Herba. Chia-wei-hsiao-yao-san is used to treat gynecological diseases and postmenopausal symptoms[9], such as severe flushes, insomnia, muscle aches and pains, formication, fatigue, palpitations and mood swings. It also exerts beneficial effect on the premenstrual tension syndrome, and is a novel formula for hypofunction of the reproductive system[13,14]. The effectiveness of Chia-wei-hsiao-yao-san on repairing the ovary function apparently correlates to the efficacy in curing osteoporosis.

Measurement of Bone Mineral Density (BMD) for the diagnosis of bone loss by computed X-ray densitometry (CXD) method has been recognized[15], but the decrease of BMD is not obvious in a short time interval[16,17]. Alkaline phosphatase (ALP) is a bone formation marker. In addition, other indicators of bone resorption, the deoxypyridinoline (Dpd) and *N*-telopeptide of Type I collagen (NTx) in human urine have been used to evaluate the effect of antiresorptive therapy on bone loss in postmenopausal women[17-19]. Bone formation markers and bone resorption markers are biomarkers of bone turnover rate.

In this study, we examined the effectiveness of Chia-wei-hsiao-yao-san and Kuei-lu-erh-hsien-chiao on preventing the progress of bone loss induced by ovariectomy in the rats.

Table 1. Herbal constituents and contents of Kuei-lu-erh-hsien-chiao and Chia-wei-hsiao-yao-san

Herbs	Contents (%, w/w)	
	Chia-wei-hsiao -yao-san	Kuei-lu-erh -hsien-chiao
Angelicae Radix	12.1	
Atractylodis Rhizoma	12.1	
Paeoniae Radix	12.1	
Bupleuri Radix	12.1	
Hoelen	12.1	
Glycyrrhizae Radix	6.1	
Moutan Cortex	7.6	
Gardeniac Fructus	7.6	
Zingiberis Sieatum Rhizoma	12.1	
Menthae Herba	6.1	
Testudinis Plastrum		29.4
Cervi Cornu		58.8
Lycii Fructus		6.5
Ginseng Radix		5.3

MATERIALS AND METHODS

I. *Traditional Chinese Medicines and Chemicals*

Herbal constituents and contents of Kuei-lu-erh-hsien-chiao and Chia-wei-hsiao-yao-san refer to the Yi-Fang-Ji-Jie[20]. All materials of Kuei-lu-erh-hsien-chiao and Chia-wei-hsiao-yao-san were extracted with water and filtered to remove the insoluble debris. Then the extracts of Kuei-lu-erh-hsien-chiao and Chia-wei-hsiao-yao-san were concentrated 5.67 and 4.46 times to get the final products, respectively. Their herbal constituents and contents are shown in Table 1. 17 β-estradiol was purchased from Sigma Chemicals (St. Louis, MO, USA). All other reagents were purchased from chemical companies.

II. *Ovariectomy and Administration of Traditional Chinese Medicines*

Five- to six-week-old female Long-Evans rats with body weight from 100-180 g were purchased from the National Laboratory Animal Breeding and Research Center (Taiwan). All rats were under regulated 12 hr/12 hr light-dark illumination cycles at constant temperature (24 ± 0.5°C) and humidity (45-50%). The test subjects had free access to standard chow. Body weight was measured at a specified time during the course of the experiment. Seven weeks later, the first mature rats model at 3 months old[3] were used. They were divided randomly into five groups (Table 2). The first group was given a sham operation, while the second, third, fourth and fifth groups were ovariectomized under nembutal (pentobarbital sodium; 50 mg/kg: Abbott Laboratories, IL) anesthesia. A small midline dorsal skin incision (1-2 cm) was made just caudal to the 13th rib. Bilateral ovariectomy was performed according to the procedures described by Waynforth and Flecknell[21]. Simultaneously, the rats of the fifth group were embedded with a silicon tube containing 17 β-estradiol (10% in cholesterol) in intraperitoneal at a dose of 10 μg/kg. The silicon tube was refilled once a month for eight weeks. The fourth group received Chia-wei-hsiao-yao-san (2.5 g/kg/day) via gastric gavage; the third group received Kuei-lu-erh-hsien-chiao (2.5 g/kg/day), and the first and second groups received distilled water during the experimental period. The traditional Chinese medicines were given daily for eight weeks, and each dose followed the previous one after 24 hr. The total volume of solution gavaged for each dose was controlled within 1 to 1.5 mL.

Table 2. Classification and treatment of the experimental rats

Groups	n	Operation	Treatment
1. (sham+D.W.)	6	Sham	Distilled water
2. (OVX+D.W.)	8	Ovariectomized	Distilled water
3. (OVX+ Kuei-lu-erh-hsien-chiao)	8	Ovariectomized	Kuei-lu-erh-hsien-chiao
4. (OVX+ Chia-wei-hsiao-yao-san)	9	Ovariectomized	Chia-wei-hsiao-yao-san
5. (OVX+ 17β-estradiol)	7	Ovariectomized	17 β-estradiol

III. *Measurement of Bone Mineral Density (BMD)*

BMD was quantitatively determined by the CXD method[15] (HOLOGIC QDR™ 1500, Hologic INC, Waltham, MA, USA) before operation and after termination. The rats were anesthetized prior to measurement. The densitometry pattern of the lumbar spines on an X-ray picture was read by a personal computer using a software program for rat bone density. Bone mineral results were reported for these regions by a BMD index.

IV. *Histologic Analysis*

Rats with pentobarbital were perfused with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 1.4) through the left ventricle for 15 min. The tibiae were immediately extracted and immersed in the same fixative for 3 days at 4°C. The specimens were decalcified with 10% EDTA solution for 3 days at 4°C. The samples were embedded in paraffin for histological examination. The proximal tibial heads were transected, and the cross sections from lower third of the tibial shaft were made. All the sections were dehydrated by a stepwise application of 70 and 99.9% (v/v) ethanol solutions and stained with hematoxylin and eosin[22].

V. *Biochemical Analysis*

Before the operation and eight weeks after the operation, blood samples from the retro-orbital plexus were taken from all rats. Heparin plasma was then separated by centrifugation at 2,000 rpm for 10 min at 4°C and analyzed for ALP. Urine samples were collected for 24 hr in metabolic cages at the same time. Urine samples were tested for creatinine (CRTN), Dpd and NTx levels. The assay values of urine were corrected for kidney function by urinary CRTN levels. CRTN levels and ALP activity were analyzed using Toshiba biochemical analyzer (TBA-80FR, Tokyo, Japan). CRTN and ALP were tested by Jaffe-rate method (seiken reagent kit, Tokyo, Japan) and Bessey-Lowry-Brock method (seiken reagent kit, Tokyo, Japan), respectively.

Dpd values were analyzed using the METRA DPD EIA Kit (Quidel Co., CA, USA) by an automated chemiluminescence system (ACS: 180®, Bayer, NY, USA). Dpd assay is a competitive enzyme immunoassay in a microtiter strip-well format utilizing a monoclonal anti-Dpd antibody coated on the strip to capture Dpd. Dpd in the sample competes with conjugated Dpd-alkaline phosphatase for the antibody, and the reaction is detected with a pNPP substrate. Dpd values must be corrected for variation in urine concentration by dividing the Dpd value (nmol/L) by the CRTN value (mmol/L). The final Dpd result is expressed as nmol/mmol CRTN.

NTx assay was performed using the Vitros NTx reagent pack (Ortho-Clinical Diagnostics, Amersham, UK) and Vitros immunodiagnostic products NTx calibrators on the Vitros Eci immunodiagnostic system. A competitive immunoassay technique was used. NTx values are corrected for urinary dilution by urinary CRTN analysis and expressed in nanomoles bone collagen equivalents per liter (nM BCE) per millimole CRTN per liter (mM creatinine).

VI. *Statistics*

Data were expressed as mean ± standard error. The mean differences were calculated from the values subtracted by the baseline (pre-operation) from the values after 8 weeks of treatment after operation. The mean difference level in group 2 was compared to those in groups 1, 3, 4, and 5 using Wilcoxon's Sum of Ranks test. When the probability was less than 0.05, the difference was considered statistically significant.

RESULTS

I. *Body Weight*

As shown in Figure 1, 8 weeks after the ovariectomy, the body weights in group 2 were significantly higher than those in groups 1, 4 and 5. There were no significant differences in the body weights between groups 2 and 3. The body weights of groups 4 and 5 were almost the same as those of group 1.

II. *Bone Mineral Density (BMD)*

As shown in Figure 2A, the mean differences of BMD levels in group 2 were significantly lower than those in group 1 ($p < 0.01$). The mean differences of BMD in groups 3, 4 and 5 were higher than those in group 2. BMD significantly differed between groups 2 and 4 ($p < 0.05$) and between groups 2 and 5 ($p < 0.01$). However, there was no significant difference between groups 2 and 3 ($p > 0.05$).

III. *Plasma ALP Activity*

The mean differences of ALP in groups 2, 3 and 4 were higher than those in groups 1 and 5. There were significant differences between groups 1 and 2. The mean difference of ALP in group 5 was significantly lower than that in group 2 (Figure 2B).

IV. *Urine Analysis*

The mean differences of the Dpd levels in group 5 were higher than those in groups 1, 2, 3 and 4, and there were significant differences between groups 2 and 5 (Figure 2C). However, there were no significant differences in urinary NTx levels among groups 1, 2, 3, 4 and 5 (Figure 2D).

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V. *Histologic Analysis*

On transections of the proximal tibial heads, the epiphyseal plates in groups 1 were more compact than those in groups 4 and 5 (Figure 3A-C), but there were no obvious differences among them. The cross sections from lower third of the tibial shaft in group 3 showed more mucopolysaccharide accumulations than those in groups 4 and 5 (Figure 3D-F).

DISCUSSION

Hidaka *et al.*[23] strongly asserted that traditional Chinese (Kampo) medicines, Unkei-to, Hachimi-jio-gan, and Juzen-taiho-to were as effective as 17 β-estradiol in preventing the development of bone loss induced by OVX in rats for a period of 49 days. Therefore, we evaluated the effectiveness of Chia-wei-hsiao-yao-san and Kuei-lu-erh-hsien-chiao on bone loss induced by OVX in rats throughout 8 weeks.

Due to higher food consumption, fat and protein gain, and food efficiency in the OVX rats[23,24], ovariectomy caused an increase of their body weights (Figure 1). A gain in weight was possibly a partial protection against the development of osteoporosis in the long bone[25]. Treatment of 17 β-estradiol or Chia-wei-hsiao-yao-san almost abolished the OVX-induced obesity, which may be corelated with the diuretic action of Paeoniae Radix and Hoelen. However, Kuei-lu-erh-hsien-chiao cannot prevent the OVX-induced obesity in this study.

Although our results indicated that the spinal BMD of the OVX rats showed a small increase as compared to previous studies[26,27], the mean differences of BMD levels in group 2 were significantly lower than those in group 1 ($p < 0.01$). In humans, girls receiving oophoreectomy during teen age continue to grow. Even so, the mean differences of BMD in groups 1, 3, 4 and 5 were higher than those in group 2, and there were significant differences between groups 1, 4, 5 and 2, respectively. The differences between groups 1, 5 and 2 were more significant than those between groups 4 and 2. Although the mean differ-

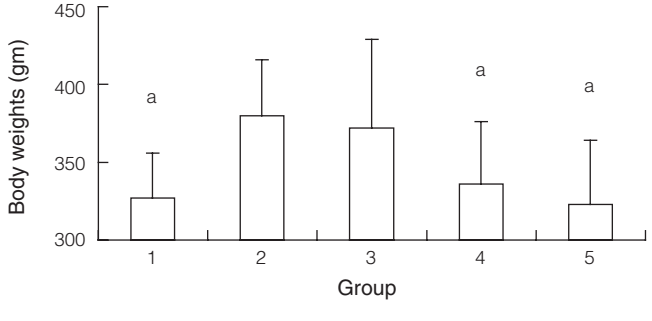

Figure 1. The body weights of various groups 8 weeks after operation.
[a]The mean differences differed from those in group 2 were significant ($p < 0.05$).

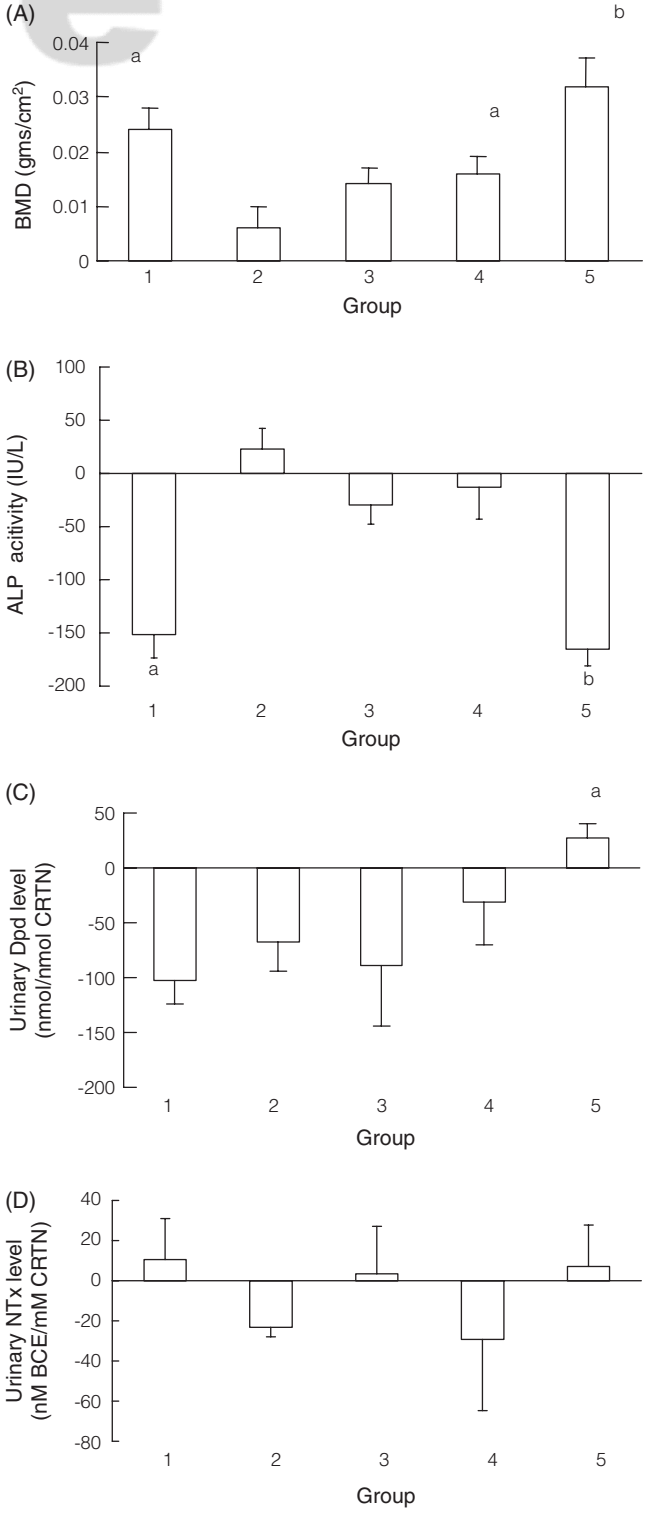

Figure 2. The mean differences of (A) BMD (gms/cm^2), (B) plasma ALP (IU/L), (C) urinary Dpd (nmol/mmol CRTN) and (D) urinary NTX (nM BCE/mM CRTN) from the values that substracted the baseline (pre-operation) from the analyzed values after 8 weeks of treatment after the operation in various groups
[a]The mean differences differed from those in group 2 were significant ($p < 0.05$).
[b]The mean differences differed from those in group 2 were significant ($p < 0.01$).
[c]The standard error of the mean differences.

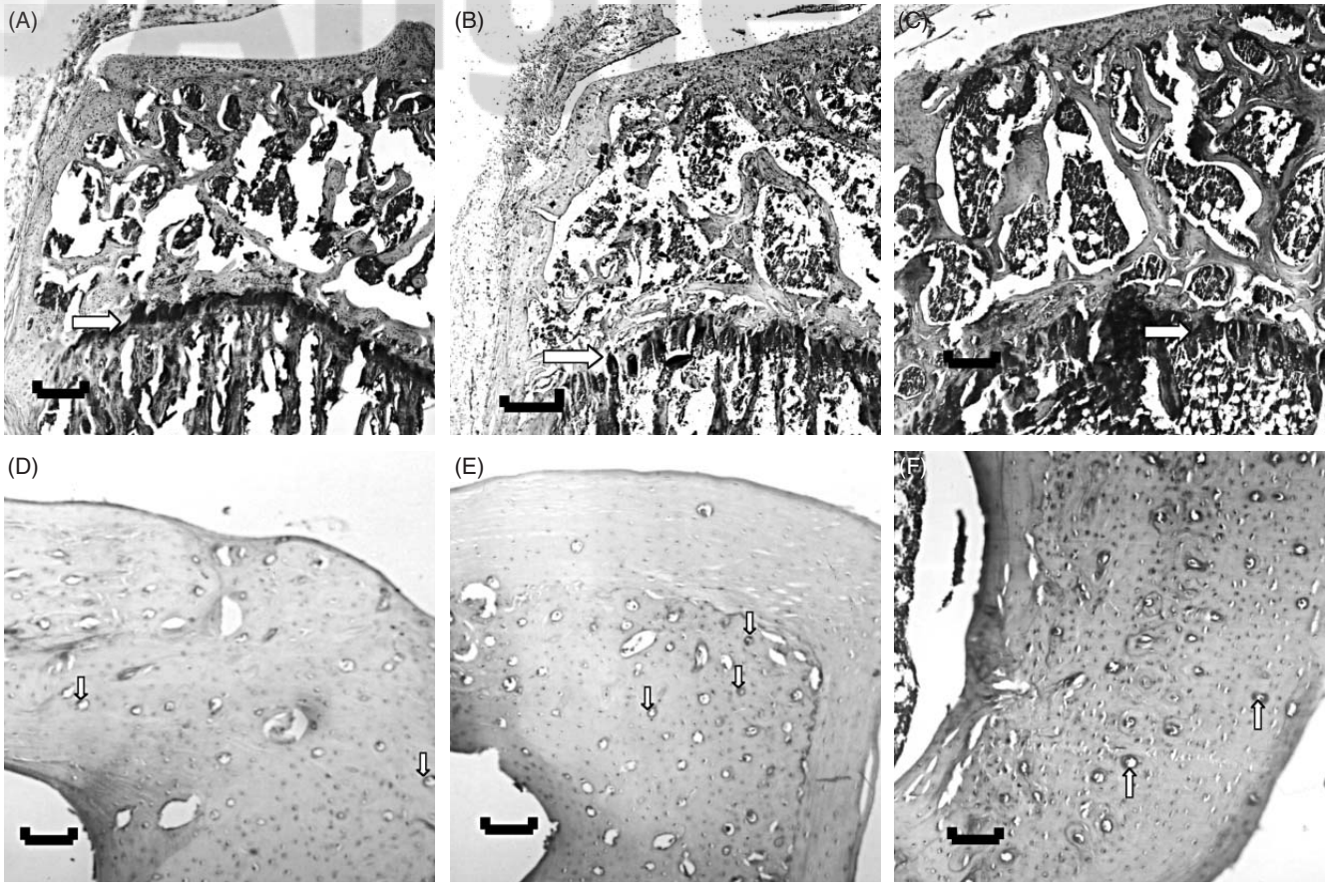

Figure 3. Histologic analysis of the epiphyseal plates (arrow) in the transections of the proximal tibial heads from groups 1 (A), group 5 (B) and group 4 (C). Histologic analysis of mucopolysaccharide accumulations (arrow) in the cross sections of lower third of the tibial shaft from group 5 (D), group 4 (E) and group 3 (F). represent 100 μm.

ences of BMD in group 3 were higher than those in group 2, there were no significant differences between the two groups. If the duration of the experiment is longer, there might be significant difference between these two groups. Losses of tibial cortical bone in human are seen in immobilization[28] and senile osteoporosis[29]. Young *et al.*[30] demonstrated that restraint in the semirecumbent position produced regional losses of bone mostly in the anterior-proximal tibiae. Tibial bone showed lesions of whole osteons after 4 weeks of restraint. The cement line of osteons was partially replaced by what appears to be a substantial accumulation of mucopolysaccharide. In our results, the epiphyseal plates in groups 1 was more compact than those in groups 5 and 4, and that in group 5 was more compact than those in group 4. Nevertheless, there were no obvious differences among them. The cross sections from lower third of the tibial shaft in group 3 showed more mucopolysaccharide accumulations than those in groups 4 and 5. Furthermore, the mucopolysaccharide accumulations in group 4 were more than those in group 5. Therefore, Kuei-lu-erh-hsien-chiao is not as effective as 17 β-estradiol or Chia-wei-hsiao-yao-san in preventing bone loss. Moreover, 17 β-estradiol showed more profound effect than Chia-wei-hsiao-yao-san.

Gala *et al.*[31] reported that OVX rats showed a significant decrease ($p < 0.05$) in BMD and a significant increase in ALP with regard to their respective sham group. Furthermore, Nawawi *et al.*[32] demonstrated that there were significantly higher serum TALP (total alkaline phosphatase), BSALP (bone specific alkaline phosphatase) and urinary Dpd levels in non-HRT (hormone replacement therapy) treated patients compared to the controls. There were no significant differences in the levels of above three bone markers between the HRT treated patients and control subjects. In our results, the plasma ALP levels significantly decreased in groups 5 and 1. Although the plasma ALP levels also decreased in groups 3 and 4, but there were no significant differences between groups 3, 4 and 2, respectively. It was referred that the doses of Kuei-lu-erh-hsien-chiao and Chia-wei-hsiao-yao-san might be lower than the effective dose. Although the BSALP activity was more specific than the TALP activity as bone marker, the BSALP activity was not detectable in rats.

Both bone resorption and bone formation are promoted by the ovariectomy, and the prominent increase of the bone resorption is known as high-turnover osteoporsis[33]. Urinary NTx and Dpd are bone resorption markers, whereas serum ALP and osteocalcin are bone formation

markers. In our results, there was a significantly negative correlation between plasma ALP activity and BMD in the OVX rats (data not shown), but there was no significant correlation between urinary Dpd or urinary NTx levels and BMD (data not shown). In contrast to previous reports[31,34,35], Frolik *et al.*[36] showed that ethynyl estradiol lowered serum ostecalcin level within 1 week of treatment by 18%, with a more pronounced reduction of 34% at 3 weeks in OVX rats. Urinary Dpd level was reduced with time. Swaminathan[37] demonstrated that urinary Dpd levels showed diurnal variation, the individual variation was large, and BSALP was much more stable and less variant within each test subjects. We cannot infer to the BMD levels by the urinary Dpd or NTx determination, but they can provide us with an early index of the degree of bone resorption. As the result, some protective strategies can be performed in time.

We conclude that Kuei-lu-erh-hsien-chiao (2.5 g/kg/day) cannot prevent bone loss induced by ovariectomy in rats, but 17 β-estradiol (10 μg/kg) and Chia-wei-hsiao-yao-san (2.5 g/kg/day) have beneficial effects in preventing bone loss in the OVX rats. Although 17 β-estradiol showed more profound effect than Chia-wei-hsiao-yao-san, the beneficial effects of Chia-wei-hsiao-yao-san were not mediated by hormone replacement. Chia-wei-hsiao-yao-san showed good compliance and safety for relief of climacteric symptoms in postmenopausal women[9]. Therefore, Chia-wei-hsiao-yao-san might be an alternative choice for preventing bone loss.

ACKNOWLEDGEMENTS

The study was supported by the research grant from the National Science Council, Executive Yuan, Taiwan, ROC (NSC89-2314-B-037-090). We would also like to thank the Chuang Song Zong Pharmaceutical Company, Kaohsiung, Taiwan, for donating the traditional Chinese medicines, Kuei-lu-erh-hsien-chiao and Chia-wei-hsiao-yao-san.

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