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Effects of Herbal Preparations Containing Isoflavones on Bone Metabolism in Postmenopausal Women

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ABSTRACT

The effects of the mixed herbal preparation "Tzuo-Kuai-Wuan" (TKW) on bone metabolism were compared with a single herb containing equivalent amounts of isoflavones, daidzein and genistein, and a combined estrogen-progesterone therapy (EPT). One hundred women completed the trial, 33 in group A, consuming a single herb, 30 in group B, consuming TKW, and 37 in group C, receiving EPT. In both herbal groups, the serum levels of daidzein significantly increased after treatment for 6 months (224% in group A and 234% in group B, both $p < 0.01$), though the serum levels of genistein did not change significantly. In group C, serum levels of either isoflavone changed only slightly. After 6 months of treatment, the bone formation marker (bone specific alkaline phosphatase [BSAP]) significantly increased in group B, but not in group A (25.4% vs. 15.9%, $p < 0.01$ and $p = 0.09$, respectively). The elevation of BSAP was related to increased serum daidzein and genistein (ß = 0.498 and 0.248, $p < 0.01$ and $p = 0.03$, respectively). The bone resorption marker (urinary deoxypyridinolines/creatinine [Dpd/Cr]) was nearly the same at beginning of the study and after treatment in both herbal groups. In contrast, BSAP and Dpd/Cr significantly decreased after 3 and 6 months of treatment in group C (BSAP: 10.4% and 30.3%; Dpd/Cr: 21.7% and 43.8%, all $p < 0.01$ in paired *t*-tests). Thus, we conclude that TKW stimulates bone formation, in contrast to EPT which primarily inhibits resorption. This effect appears to be related to the amount of isoflavones. Further, the stronger effect in women consuming TKW than consuming a single herb that contains equivalent amounts of isoflavones suggests higher synergistic or additive effect of other components than isoflavones in TKW.

Key words: bone metabolism, daidzein, genistein, herb, isoflavones, osteoporosis

INTRODUCTION

Isoflavones are believed to be helpful in preventing cardiovascular diseases and beneficial to the skeletal system[(1)]. More specifically, major isoflavones, i.e., genistein and daidzein, have been shown to prevent osteoporosis in ovariectomized rats[(2-4)] possibly by means of induction of apoptosis of osteoclasts through Ca^{2+} channel signaling, modulation of cytokine production, increase in calcitonin and action on osteoblasts or calcium content, and alkaline phosphatase activity in bone tissue[(5-8)]. The mechanism of action of genistein and daidzein still requires clarification, but it may be very different from that of estradiol.

The prevalence of osteoporotic hip fracture and climacteric symptoms is higher in the West than in Asia. Traditional Chinese medicine and soy food containing isoflavones are commonly used in Taiwan to treat climacteric symptoms and to prevent osteoporosis. Few human studies have shown that isoflavones in soy protein favorably influence bone health[(9-11)]. However, there are very few reports concerning the effects of herbal compounds that may contain some unknown active substances[(12,13)]. This study focused on herbal preparation, "Tzuo-Kuai-Wuan" (TKW), which contains isoflavones that we believe are the major active components. We hypothesized that the effects of TKW on bone metabolism should be similar to those of a single herb containing equivalent amounts of isoflavones. Randomized trial was designed to treat postmenopausal women with TKW, a single herb containing equivalent amounts of isoflavones, or a combined estrogen-progesterone therapy (EPT). The objective of this investigation was to compare the effects of these three different prescriptions on bone metabolism and to see whether TKW demonstrated any synergistic effect.

MATERIALS AND METHODS

I. *Inclusion and Exclusion Criteria*

Healthy Taiwanese women between 45 and 60 of age

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with spontaneous menopause for at least one year and not taking any hormone therapy for at least one month were enrolled. All test subjects were willing to comply with the protocol and signed informed consent. The study was approved by the Institutional Review Board of Mackay Memorial Hospital.

Inclusion criteria included a serum estradiol < 20 pg/mL and FSH > 50 mIU/L. Routine mammography, Pap smears, and transvaginal sonography were done to rule out breast or gynecological abnormality and to measure endometrial thickness. The bone mineral density (BMD) of the lumbar spine was measured by double-energy X-ray absorptiometry (DXA, Norland XR-36 Bone Densitometer). Patients with a history of hysterectomy or ovariectomy, gynecological or breast abnormality, systemic disease, or treatment affecting bone or lipid metabolism were excluded. Patients with osteoporosis, defined as a BMD T-score ≤ -2.5; poor liver function, with either an AST or ALT greater than twice of the upper limit of normal; or poor renal function, with creatinine > 1.3 mg/dL; were also excluded.

II. *Randomization and Treatment*

Test subjects were randomized to group A, consuming a single herb, group B, consuming TKW, or group C, receiving standard EPT. All herbal preparations certified with GMP were purchased from a Chinese herbal drug manufacturer.

TKW consisted of 7.1% each of *Cornus officinalis* Sieb. et Zucc., *Lycium barbarum* L., *Cervus nippon* Temminick., *Chinemys reevesii* (Gray), *Psoralea corylifolia* L., *Schisandra chinensis* (Turczz.) Baill., *Angelica sinensis* (Oliv.) Diels, *Eucommia ulmoides* (Oliv.), 9.5% of *Cyathula officinalis* Kuan, and 12% each of *Rehmannia glutinosa* Libosch., *Dioscorea opposita* Thunb. and *Cuscuta chinensis* Lam. The single herb was processed Dihuang (*Rehmannia glutinosa* Libosch). The isoflavone contents in each herbal preparation were quantified by the method previously described by Hsu *et al.*[14], and had been calculated to equivalent amounts. Although daidzein and genistein have not been previously found in *Rehmannia glutinosa*, we confirmed the presence of above two compounds in the plant by LC/MS.

Subjects in group A consumed 2.5 g of processed Dihuang twice daily; those in group B, consumed 5 g TKW twice daily; and those in group C took one daily dose of 0.625 mg conjugated estrogen and 2.5 mg medroxyprogesterone acetate. All women were also given 1000 mg/day of calcium carbonate. Patients were asked to refrain from consuming foods high in phytoestrogens during the study period.

III. *Followup*

Test subjects were evaluated by monthly questionnaire and physical examination to evaluate compliance and side effects. Serum levels of genistein and daidzein were analyzed at 0, 3, and 6 months after enrollment, along with biochemical markers of bone formation (bone specific alkaline phosphatase [BSAP]) and resorption (urinary deoxypyridinolines/creatinine [Dpd/Cr]). All patients were followed monthly for 6 months.

IV. *Specimen Collection*

Blood and urine were collected at 0, 3, and 6 months after enrollment, with each collection being done between 7:30 and 10:30 AM following a minimum 8-hr fast. Serum was immediately separated and the specimens frozen at -70°C until assayed. Urinary creatinine concentrations were measured by a standard automated method. All serum and urine assays were simultaneously performed in triplicate with the same batch kits to minimize inter-assay variation.

V. *Analysis of Bone Metabolism Markers*

Bone specific (skeletal) alkaline phosphatase (BSAP) was assayed in serum using an ELISA system (Metra^{TM} BAP, Quidel Corp., San Diego, CA, USA). This assay had only 16% cross-reactivity with the circulating liver isoenzyme. The sensitivity of the assay was 0.7 U/L, and the intra- and inter-assay CVs were less than 6% and 8%, respectively[15].

Urinary deoxypyridinoline (Dpd), a cross linked bone type 1 collagen released during resorption and excreted unmetabolized in urine, was quantitatively measured with an ELISA kit (Metra^{TM} DPD, Quidel Corp., San Diego, CA, USA). The intra- and inter-assay CVs were less than 8% and 5%, respectively. The sensitivity was 1.1 nM[16].

VI. *Serum Isoflavones Concentrations*

Isoflavones were detected and quantified according to the methods of Hsu *et al.*[14]. Briefly, serum samples were deproteinated with 1% TCA. After centrifugation, the clear supernatant was subjected to acid hydrolysis with 2 M HCl at 100°C for 2 hr. The hydrolysate was neutralized and passed through a reverse-phase C18 column (Waters, Milford, MA, USA). Isoflavonoid compounds were eluted with 2 mL of methanol and the eluate dried under nitrogen flow. The dried material was redissolved in 100 μL of methanol and subjected to high performance liquid chromatography (HPLC) analysis.

VII. *Statistics*

Data were analyzed with independent and paired *t*-tests and linear regression using SPSS 10.0 for Windows.

RESULTS AND DISCUSSION

One hundred and sixty-six postmenopausal women who met the inclusion criteria were initially enrolled, but 31 were excluded primarily due to low BMD T-scores.

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Therefore, 135 women were randomized into group A, B, or C.

One hundred women completed the trial, 33 in group A, 30 in group B, and 37 in group C. Twenty-nine women resigned from the study due to change of mind (7 in A, 6 in B, and 1 in C), lack of improvement in severe climacteric syndromes with treatment (2 in A and 3 in B), and poor compliance with medication, defined as monthly consumption of less than 3/4 of the recommended doses (3 in A, 5 in B, and 2 in C). One patient in group B was incidentally found to have colon cancer and dropped out. Five patients in group C discontinued the study because of severe vaginal bleeding and breast tenderness.

The baseline demographics of the women who completed the trial are listed in Table 1. There were no significant differences among the three groups.

Various side effects were noted in the women who completed the trial, most commonly gastrointestinal symptoms (13 in A, 10 in B, and 3 in C) including diarrhea, followed by abdominal fullness and dry mouth. Two women in each herbal group and 5 women in group C had mild vaginal bleeding. Breast tenderness occurred in 1 woman in group A, 2 in group B and 4 in group C.

The serum levels of daidzein and genistein at beginning of the study were nearly the same in all groups. After 6 months of treatment, serum daidzein significantly increased in both herbal groups (224% in group A, 234% in group B, both p <0.01) with no significant difference between the magnitude of increase (p = 0.792). Neither group had significant changes in genistein after treatment (Table 2).

The serum levels of BSAP at beginning of the study were nearly the same in all groups. At 6 months of treatment, BSAP was significantly increased in group B women from 24.8 ± 8.7 to 31.1 ± 10.4 U/L (25.4%, p < 0.01), while the increase in group A from 25.1 ± 8.5 to 29.1 ± 10.6 U/L was not statistically significant (15.9%, p = 0.09). Comparing the mean increase of BSAP in group A and B, there was a significant difference between them (p = 0.02). The BSAP in group C was significantly decreased after 3 and 6 months of treatment with EPT (from 23.0 ± 9.5 to 20.6 ± 8.9 and 18.0 ± 8.2 U/L, both p < 0.01 in paired t-tests) (Figure1).

The Dpd/Cr was nearly the same at beginning of the study in all groups. Although it did not significantly change in either herbal group (from 6.92 ± 2.36 at baseline to 6.80 ± 2.45 at 3 months and 6.80 ± 1.99 at 6 months in group A and from 6.96 ± 2.75 at baseline to 7.49 ± 2.82 at 3 month and 7.33 ± 2.54 at 6 months in group B, none p < 0.05 in all paired t-tests), the Dpd/Cr decreased significantly in group C after treatment (from 7.67 ± 2.94 at baseline to 5.35 ± 2.16 at 3 months and to 4.31 ± 1.82 at 6 months, p < 0.01 for both) (Figure2).

Women treated with TKW had a significantly higher increase in BSAP than women who received the single herb (25.4 % vs. 15.9%, p = 0.02). This negated our null hypothesis that herbal preparations containing equivalent amounts of isoflavones will have equal effect on bone metabolism. Instead, TKW had a stronger effect on bone formation than the single herb.

Isoflavones have been thought to act on bone by mimicking the effects of estrogen, that is, through inhibition of osteoclasts. However, a biphasic effect of isoflavones

Table 1. Baseline demographics of the study population

	Group A (N = 33)	Group B (N = 30)	Group C (N = 37)	p
Age (yr)	52.3 ± 4.6	53.2 ± 4.6	54.3 ± 4.8	NS[d]
BMI (kg/m^2)	25.8 ± 2.9	24.9 ± 2.3	26.0 ± 3.1	NS
LMP[a] (yr)	3.8 ± 5.8	3.9 ± 6.6	4.6 ± 6.4	NS
Estradiol (pg/mL)	<20	<20	<20	NS
FSH[b] (mIU/L)	95.6 ± 39.3	97.9 ± 43.1	88.5 ± 48.2	NS
BMD[c]	–0.4 ± 1.3	–0.6 ± 1.3	–0.7 ± 1.2	NS

[a]Last menstrual period.
[b]Follicle-stimulating hormone.
[c]Bone mineral density, represented by the T-score [SD], the number of standard deviations below the mean BMD value of young, normal Taiwanese females.
[d]Not significant.

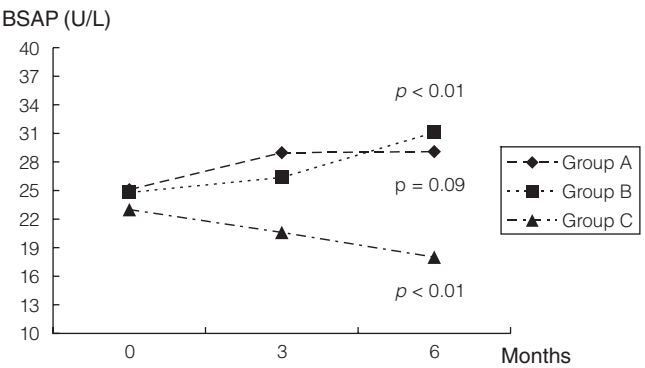

Figure 1. Bone formation marker (serum BSAP) in patients consuming a single herb (Group A), "Tzuo-Kuai-Wuan" (Group B), or EPT (Group C).

Table 2. Serum daidzein and genistein levels at 0, 3, and 6 months after enrollment

		At baseline	After 3 months	After 6 months	p[a]
Serum daidzein level (uM)	Group A (N = 33)	0.94 ± 0.93	1.19 ± 0.74	2.11 ± 1.37[a]	< 0.01
	Group B (N = 30)	0.85 ± 0.83	1.16 ± 0.88	1.99 ± 1.50[a]	< 0.01
	Group C (N = 37)	1.20 ± 0.75	1.16 ± 0.65	1.18 ± 1.30	NS[b]
Serum genistein level (uM)	Group A (N = 33)	1.11 ± 0.95	1.15 ± 0.93	1.36 ± 1.05	NS
	Group B (N = 30)	1.22 ± 0.86	1.53 ± 0.97	1.33 ± 0.71	NS
	Group C (N = 37)	1.02 ± 0.83	1.00 ± 0.96	090 ± 0.63	NS

[a]Significance in paired t-test in each group compared with baseline.
[b]Not significant.

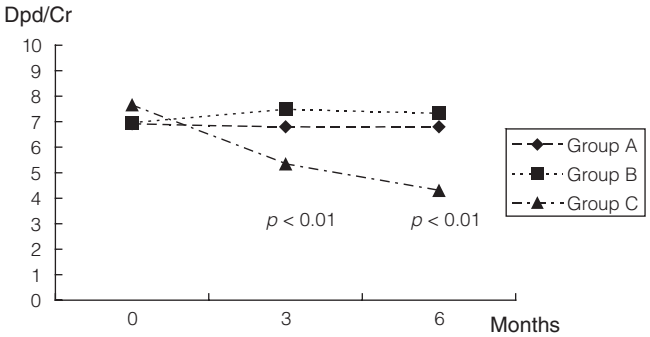

Figure 2. Bone resorption marker (urinary Dpd/Cr) in patients consuming a single herb (Group A), "Tzuo-Kuai-Wuan" (Group B), or EPT (Group C).

on expression of the estrogen receptor in MCF-7 cell lines has been reported[17]. At concentrations ranging from 10^{-2} to 10 uM, isoflavones may also stimulate estrogen receptor ß in osteoblasts. These are compatible with the serum concentrations of isoflavones in women consuming herbs in our study. At high concentrations, generally > 10 µM, isoflavones may also act through other mechanisms, such as inhibition of tyrosine kinases, inhibition of topoisomerase II, and induction of apoptosis in osteoclasts to inhibit bone resorption[18].

In this study, both herbal preparations significantly increased serum levels of daidzein but not genistein. This suggested differences in bioavailability of isoflavones in herbs, a topic that has yet to be explored fully. Many factors might be involved, including the intestinal microflora, drug interaction, diet, bowel disease, and individual variation. Having analyzed the relationship between serum levels of isoflavones and bone turnover markers, we found that the increased serum daidzein level in herbal groups was more related to difference in BSAP and urinary Dpd/Cr than to genistein (Pearson correlation: ß = 0.498 vs. 0.248 in BSAP, and ß = 0.302 vs. 0.059 in Dpd/Cr, respectively, both $p < 0.01$ for daidzein, p = 0.03 and 0.51 for genistein in BSAP and Dpd/Cr, respectively). This was similar to the results of a previous study by Picherit *et al.*[19]. Differences in both bioavailability and potency might explain the difference between the two isoflavones. Xu *et al.*[20] also concluded that daidzein was more bioavailable than genistein in adult women. In contrast, genistein has been shown to be more potent than daidzein in stimulating alkaline phosphatase activity in an endometrial adenocarcinoma cell line[21]. However, the relative potency and mechanisms of these two isoflavones in preventing osteoporosis in humans remain unknown. Regardless of the exact mechanisms and relative benefits, it appears from our study that the isoflavones in medicinal herbs, especially daidzein, actively promote bone formation rather than inhibiting bone resorption, an observation supported by the studies of Oh *et al.*[13] and Atkinson *et al.* [11].

Moreover, the increased serum levels of daidzein and genistein in each herbal group after 6 months did not differ significantly (the mean of increase in serum daidzein were 1.14 vs. 1.17 µM in group A and B, respectively, p = 0.96; the mean of increase in serum genistein were 0.25 vs. 0.11 uM in group A and B, respectively, p = 0.24). Therefore, the increased BSAP activity could not be explained simply by the increased serum levels of the two major isoflavones. Synergism of the compounds might be present, or there could be some substances other than the two major isoflavones in TKW that had an additive effect.

The Dpd/Cr and BSAP decreased significantly in women receiving EPT (30.3% and 10.4% at 3 months, then 43.8% and 21.7% at 6 months, all $p < 0.01$). In contrast, BSAP significantly increased in women taking TKW appeared after 6 months of treatment but not after 3 months of treatment (25.4% at 6 months, $p < 0.01$, but only 6.5% at 3 month, p = 0.54). The effect of estradiol in inhibiting bone resorption was fast and strong, while herbal regimens containing isoflavones stimulated bone formation slowly and weakly.

In terms of the effect of calcium supplements on markers of bone turnover, Hla *et al.*[22] found that women taking ≥250 mg of calcium per day had significantly reduced levels of osteocalcin and urinary type I collagen cross-linked *N*-telopeptides. Karkkainen *et al.*[23] investigated the acute effects of calcium loads of 0, 250, and 1000 mg in the morning and found no significant changes in the markers of bone formation and resorption they measured, regardless of the calcium dose. Given these discrepancies, the actual effect of calcium supplements was unclear. However, we controlled this problem by giving all our patients the same dose of calcium.

In this pilot study, we have demonstrated that isoflavone-containing herbal regimen TKW appears to stimulate bone formation and thus prevent osteoporosis. However, the complexity of the interventions and short duration of the study limited our ability to demonstrate other measurable clinical effects, such as changes in BMD or the incidence of osteoporotic fractures. More detailed prospective studies are required to measure the actual clinical outcomes of interest. The stronger effect on bone formation of TKW than the single herb also provided a strong impetus to investigate other agents in the compound that may have additive or synergistic effects on bone formation.

CONCLUSIONS

Our study demonstrated that the herbal regimen containing isoflavones may prevent osteoporosis by stimulating bone formation, an effect quite different from that of estradiol which inhibits bone resorption. The stronger effect of TKW than the single herb, processed Dihuang, containing equivalent amounts of isoflavones suggested a synergistic or an additive effect of components in TKW. Our results also provided a strong impetus to investigate possible effects on bone metabolism of substances other than isoflavones in herbs.

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