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Effects of *R. Glutinosa* and *E. Senticosus* on Postmenopausal Osteoporosis

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In this study, we investigated the therapeutic effects of a novel formulation of low-dose calcium and vitamin D_3, blended with *Rehmannia glutinosa Libosch and Eleutherococcus senticosus Max* (RE+), in postmenopausal women. The controls were given either a placebo or high dose calcium and vitamin D_3 (Ca+D). Bone mineral density (BMD) in the L2-3 lumber spines and femur regions was assessed, and serum osteocalcin, bone-specific alkaline phosphatase (BALP), and cross-linked N-telopeptide of type I collagen (NTx) were used as markers of osteoblast and osteoclast activity. Furthermore, all variables were measured before and after 6 and 12 months of treatment. The osteocalcin level was higher in the RE+ group, and BALP was almost the same in all groups. Serum NTx was significantly decreased in the RE+ group after 12 months ($p<0.05$). The NTx in the Ca+D and placebo groups showed no significant change. The decrease of femur BMD was further demonstrated in the placebo group, but significantly increased in the RE+ group after 6 and 12 months of treatment ($p<0.05$). There were significant differences in the percent changes of femur BMD between the placebo and RE+ groups ($p<0.01$) and Ca+D and RE+ groups ($p<0.05$). The decrease of spine BMD in the placebo group was inhibited both in the Ca+D and RE+ groups, however, there was significant difference only between the placebo and RE+ groups ($p<0.05$). These findings suggest that continuous oral therapy of the RE+ formulation reduces rapidly decreasing bone mineral density in postmenopausal women more effectively than high doses of calcium and vitamin D_3 alone by inhibiting osteoclastic activity. Therefore, it seems that the RE+ has its own antiosteoporotic effects. We suggest larger clinical studies to determine the most efficacious dosage and benefits of this novel treatment.

Key Words: Osteoporosis, Postmenopausal women, Herbs, Bone mineral density, Biomarkers

INTRODUCTION

Osteoporosis is a progressive, systemic, skeletal disease characterized by low bone mass. Bone mass declines with age in both genders, but more rapidly in women and accelerated after menopause. This loss is mainly a result of estrogen deprivation secondary to an increase in bone remodeling (Mazess, 1982). Therapeutic modalities for preventing and treating osteoporosis, therefore, should increase bone mass, or, prevent further bone loss at the very least. Current therapies for treating osteoporosis include estrogen and hormone replacement therapies (ERT and HRT), bisphosphonates, calcitonin, and raloxifen. Because there are possible contraindications with ERT and HRT, such as breast cancer, endometrial adenocarcinoma, and other undesirable adverse effects, compliance with hormonal therapy is poor, leading to a loss of treatment efficacy (Groeneveld et al, 1994; De lignieres, 1996). Continued uterine bleeding with the use of HRT has also alarmed women to look for alternatives to traditional therapy (Huntley & Ernst, 2003). Various herbs, such as licorice, soybeans, black cohosh, red clover, kava, dong quai, ginseng, *Rehmannia glutinosa Libosch (RGX), Eleutherococcus senticosus Max (ES), Cimicifuga recemosa*, and *Pueraria labata* have commonly been used as alternative therapies. Some of these herbs are recommended for estrogenic activity and are used for postmenopausal symptoms and osteoporosis (Alkel et al, 2000; Amato et al, 2002; Mühlbaur et al, 2003; Xu et al, 2003).

RGX has widely been used as an herbal medicine in Eastern Asia for more than 2000 years. Depending on various processing methods, RGX has been categorized into three types; named in Korean as Saeng-Ji-Whang (fresh root), Gun-Ji-Whang (dried root), and Sook-Ji-Whang (steamed root). Dried or steamed roots have been used for treating many diseases because they have haemostatic, cardiotonic, diuretic, antibacterial, anti-inflammatory (Ni et al, 1992), anti-allergic (Kim et al, 1998), and anti-tumor properties (Kim et al, 1999). Many constituents have been isolated from both the *Rehmanniae Radix* (the root of R. glutinosa Libosch) and the fresh plant. The major con-

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ABBREVIATIONS: *RGX, Rehmannia glutinosa Libosch; ES, Eleutherococcus senticosus Max;* BMD, bone mineral density; BALP bone-specific alkaline phosphatase; NTx, cross-linked N-telopeptide of type I collagen; 5-HMF, 5-(Hydroxymethyl)-2-furaldehyde; Eleutheroside E, (−)-Syringaresinol-4-4'-O-β-D-Diglucoside.

stituents of the herb are β-sitosterol and mannitol. Other constituents include a small amount of stigmasterol and trace amounts of campesterol, catalpol, rhemannin, and vitamin A (Ni et al, 1992). Interlukin-1 (IL-1) is known to be a potent bone resorptive cytokine, and the tumor necrosis factor (TNF-α) appears to synergize with IL-1, therefore, they together can regulate bone metabolism; it is thought that RGX decreases osteoclastic activity by inhibiting IL-1 and TNF-α (Stashenko et al, 1987; Kim et al, 1999). Although the active substances have not yet been identified, findings have demonstrated that RGX attenuates the progress of bone loss in rats with ovariectomy-induced osteoporosis (Oh et al, 2003).

The extract of the root bark of ES has been used as a substitute for ginseng roots in the control of blood circulation, and it has extensively been used in Russia, China, Korea, and Japan as an adaptogen which increases nonspecific body resistance to stress and fatigue (Fujikawa et al, 1996; Davydov & Krikorian, 2000). Its shoot and root have been used as a remedy for many ailments, including hypertension, gastric ulcer, neuralgia, learning behavior difficulties, and cancer. More than 10 compounds have been isolated from ES, and heir pharmacological activities are thought to be mainly due to lignans and iridoid glycosides (Deyama et al, 2001). It is also known to have antioxidant properties and to modulate the effect or release of glucocorticoid by acting on glucocorticoid receptors (Gaffney et al, 2001). The extracts of ES have been shown to have inhibitory actions on mast cell-dependent anaphylaxis and anti-metastatic effects through the activation of macrophages and natural killer cells (Yi et al, 2002). Moreover, Kimura and Sumiyoshi (2004) reported that the water extract of ES significantly prolongs the swimming time in rats and inhibits the reduction of NK activity and the elevation of corticosterone induced by forced swimming; the extract of ES has also been effective in preventing bone resorption of steroid-induced osteoporosis in a rat model (Kropotov et al, 2002).

In the present study, we report on a preliminary clinical trial of a novel formulation for the treatment of osteoporosis. We compared the therapeutic outcomes of administration of this formulation, which contained low dose calcium and vitamin D_3 (Cholecalciferol) blended with RGX and ES, with the standard calcium and vitamin D_3 supplementation. Both herbs are commonly available as alternative medicine, although literature on these herbs in relation to osteoporosis is rather limited.

METHODS

Materials

The source of RGX was the root of *Rehmannia glutinosa Libosch* which was steamed with alcohol three times, and the source of ES was the stem bark of *Eleutherococcus senticosus Max.* Eighty percent of RGX and 20% of ES (wt/wt) were mixed and heated in distilled water for 8h at 97~98°C. After filtration with 150 mesh, the solvent was removed under reduced pressure to give a residue (ca. 40~45 brix). This extract was mixed with 50% carrier (maltodextrin) and spray-dried (OPB®). *Rehmanniae Radix* (Scrophulariaceae) was cultivated and harvested in the fall and imported from China (Lianonig Province). After being imported by the Poongsan Pharmaceutical Company, alcohol was added and processed in steam. *Eleutherococcus senticosus* (Araliaceae), widely distributed by China (East-North province), was obtained in the fall and also imported by the Poongsan Pharmaceutical Company. The marker substance, 5-HMF {5-(Hydroxymethyl)-2-furaldehyde}, was used for RGX, and Eleutheroside E {(−)-Syringaresinol-4-4'-O-β-D-Diglucoside} was used for ES. The contents of marker substances in the final extract were 5 mg/g of 5-HMF and 0.26 mg/g of Eleutheroside E. The 5-HMF was analyzed by isocratic RP-HPLC using Shim-pack ODS (250×4.6 mm, 5 um) column (Shimadzu, Japan), with 5% acetonitrile at a flow rate of 1.2 mL/min. The peak retention time was 11.0 min. The Eleutheroside E was analyzed by isocratic RP-HPLC using Very C-18 (150×4.6 mm, 5 um) column (Supelco, USA) with 15% acetonitrile at a flow rate of 1.0 mL/min. The peak retention time was 7.0 min, determined at 210 nm UV.

Study population

Subjects were recruited at Dankook University Medical Center through bulletin board and poster advertisements. Potential subjects were screened initially by interview to ensure that they were postmenopausal women who were free of chronic diseases, such as known cardiovascular disease, diabetes mellitus, or malignant tumors and previous pathological fractures, and who had a body mass index (BMI; in kg/m^2) between 20 and 30. Sixty-seven women were initially recruited, and 41 women were finally enrolled. We randomly assigned the participants to one of three groups. The women enrolled in the study were between 51 and 71 years old, had been postmenopausal for at least three years, and had osteopenia or osteoporosis having lumbar spine BMDs greater than 1.0 SD, below the young adult mean (T-score ≤ -1.0) (WHO, 1994). Subjects who had conditions affecting bone metabolism, such as kidney, liver, parathyroid, and thyroid disease were excluded from the study. Also excluded were those who had been receiving glucocorticoids, estrogens, thyroid hormone, fluoride, bisphosphonate, calcitonin, thiazide diuretics, and vitamin D- or calcium-containing drugs within the previous year, and those who were experiencing severe postmenopausal symptoms such as hot flashes or lumbar pain. All participants were prohibited from taking medications affecting bone metabolism and hormones, alcohol, excessive exercise, and from smoking during the duration of the study. The study was approved by the Human Research Review Committee of the Dankook University School of Medicine.

Study design and supplements

The 41 participants were randomly assigned to one of three groups: RE+, Ca+D, and placebo, resulting in 14, 14, and 13 volunteers, respectively. The BMD and biochemical markers were assessed at the beginning (T0), and at 6 (T6) and 12 months (T12) after administration of the supplements. The Ca+D participants were treated with vitamin D_3, 125 IU, and calcium (Oyster shell powder), and 1287.36 mg, in each serving of one tablet, and the placebo group was treated with tablets composed of only carboxymethylcellulose. The RE+ group was treated with the novel formulation of 250 mg of calcium (calcium citrate), 100 IU of vitamin D_3, blended with 250 mg of the herbs, *RGX* and *ES*, in each tablet. All doses were given twice a day

with ample amounts of water. The medications were provided for one month at a time, and we suggested that the participants keep to a normal diet. All subjects were given a monthly followup to ensure that they had regularly taken the medication and, accordingly, we gave them refills for the following month.

Bone density measurement

Bone mineral density (BMD) was measured by DEXA (Lunar DPX-MD+, GE Medical Systems, Madison, WI). It was estimated at the L2-3 spine and at the right hip, including the femoral neck, trochanter, Ward's triangle, and shaft. The standard for BMD was calculated by the software program and is presented as g/cm^3; all participants had a baseline BMD of less than 1.004 g/cm^3.

Biochemical measurement

Serum samples were used to estimate biochemical markers. All three markers were measured at the baseline and 6 and 12 months after treatment by using the competitive enzyme-linked immunosorbant assay (ELISA). Baseline serum calcium, phosphate, creatinine, and total alkaline phosphatase levels were also determined by routine methods employing an autoanalyzer (Shimadzu CL-7200, Shimadzu Co., Japan).

We used the intact Osteocalcin ELISA kit, having native human origin osteocalcin (BioSource International Inc., Camarillo, CA). The test had a sensitivity of 0.4 ng/mL with an assay range of 2~90 ng/mL. Bone-specific alkaline phosphatase (BALP) was analyzed with the METRA^{TM} BAPEIA kit (Quidel corporation, San Diego, CA), which quantifies BALP with monoclonal anti-BALP antibody. The test had a sensitivity of 0.7 U/L, with an assay range of 2-140 U/L.

NTx was measured by ELISA with the Osteomark^{TM} serum kit (Ostex International Inc., Seattle, WA). Assay values were standardized to an equivalent amount of bone collagen, expressed as nmol bone collagen equivalent (BCE) of NTx, with an assay range of 3.2~40 nM BCE. Fasting blood samples were collected between 8:00 and 10:00 a.m., and kept in at −70°C until analysis.

Statistical analysis

Data were presented as mean±SD. Mean differences in each group were evaluated by a student's t-test for paired samples. Percentage changes in BMD and biochemical markers were calculated [(post-treatment-baseline values)/baseline values×100] for each group. To determine whether the changes over the course of treatment were significantly different from the baseline in each group, paired t-tests were performed. The repetitive-measured analysis of variance (ANOVA) was used to detect differences in the changes recorded in each group for each variable. Differences were considered to be significant at $p<0.05$.

RESULTS

Clinical characteristics

The major clinical and biochemical characteristics of the subjects at baseline are shown in Table 1. There were no significance differences in variables among the three groups. During the course of the study, five participants withdrew from the experiment; two subjects in the placebo group had a BMD below the normal range, one was taking a hormone prescribed by a physician, another one showed insufficient compliance in the Ca+D group, and one in the RE+ group moved out of the area. In the initial days of therapy, two participants in the RE+ group and three in the Ca+D group experienced gastrointestinal side effects, including constipation, bloating, or diarrhea; these symptoms subsided after a few weeks, and the participants were able to continue the treatment. The adverse reactions were thought to be related to calcium and vitamin D_3 therapy, and were more severe in the Ca+D group who received the highest doses of calcium and vitamin D_3.

Bone mineral density

The initial mean values for bone mineral density at all sites were similar between the three groups. The changes of BMD in the L2-3 spine and the femur during the study are shown in Table 2, and the ratio changes, compared with the respective baselines, are shown in Figure 2. The BMD of the lumbar spine gradually declined in the placebo group, but it increased in the RE+ and Ca+D groups. The change in the RE+ at T6 was significant with T0 by the paired t-test ($p<0.05$) and compared with the placebo group by ANOVA ($p<0.05$). The BMD of the femur declined in the placebo group; the BMD increased in the RE+ and Ca+D groups, however, a significant treatment effect was found in T6 and T12, only in the RE+ group with T0 by the paired t-test ($p<0.05$) and compared with the placebo group by ANOVA ($p<0.05$). There were significant differences in the RE+ group, compared by repetitive-measured ANOVA with the placebo and Ca+D groups ($p<0.01$; $p<0.05$).

Biochemical markers

The principal biochemical markers of calcium metabolism measured during the study are shown in Table 3. The results in the three groups were similar at baseline. The

Table 1. Summary of volunteers' baseline values (Mean±SD)

Variables	Placebo	Ca+D	RE+
Number of participants (n)	11	12	13
Age (years)	57.3±6.1	60.4±5.0	61.4±4.6
Body mass index (kg/m^2)	25.5±1.6	23.5±3.7	24.6±2.7
Serum calcium level (mg/dl)	8.91±0.4	9.03±0.4	8.84±0.7
Serum phosphate level (mg/dl)	3.78±0.3	3.81±0.4	3.59±0.4
Serum creatinine level (mg/dl)	0.86±0.1	0.83±0.1	0.83±0.1
Serum total alkaline phosphatase level (IU/L)	76.5±15.4	70.7±11.7	78.4±22.9

Ca+D: formulation with calcium and vitamin D_3. RE+: formulation of calcium and vitamin D_3, blended with *Rehmannia glutinosa Libosch* and *Eleutherococcus senticosus Max.*

Table 2. Changes of BMD before and after treatments

Groups	0 (T0)	6 months (T6)	12 months (T12)
A: Changes in L2-3			
Placebo	0	−0.759±3.480	−0.986± 2.671
Ca+D	0	0.906±1.781	1.072±4.448
RE+	0	2.098±3.289[a,b]	1.616±3.961
B: Changes in total Femur[#]			
Placebo	0	−0.243±0.935	−0.322±1.574
Ca+D	0	0.942±1.688	1.239±3.985
RE+	0	2.044±2.398[a,b]	3.533±4.865[a,b]

Ca+D: formulation with calcium and vitamin D_3. RE+: formulation of calcium and vitamin D_3, blended with *Rehmannia glutinosa Libosch* and *Eleutherococcus senticosus Max.* Data ar mean of BMD change±SD, [a]$p<0.05$ compared with T0 with paired t-test, [b]$p<0.05$ compared with placebo group with ANOVA. There were significant differences in the changes between placebo and RE+ groups by repetitive-measured ANOVA ($p<0.05$). [#]Total femur: total right femur, including neck, wards, trochanter and shaft.

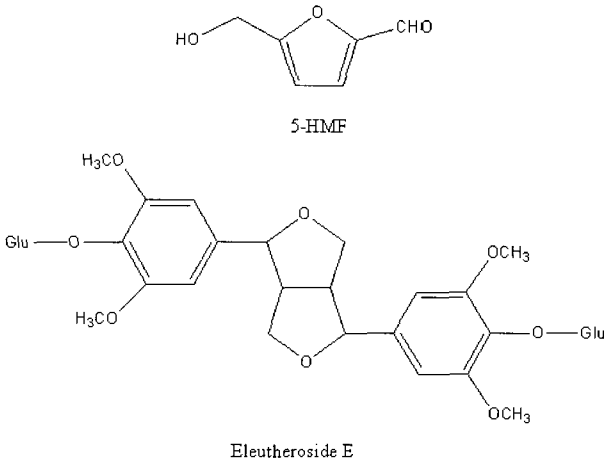

Fig. 1. The structures of marker substances. 5-HMF {5-(Hydroxymethyl)-2-furaldehyde} for *Rehmannia glutinosa Libosch* and Eleutheroside E {(−)-Syringaresinol-4-4'-O-β-D-Diglucoside} for *Eleutherococcus senticosus Max.*

Table 3. Changes of bone biomarkers

Types	0 month (T0)	6 months (T6)	12 months (T12)
A: Serum BALP (IU/L)			
Placebo	30.5±4.3	29.3±6.8	31.0±6.1
Ca+D	28.5±5.8	31.6±11.1	31.6±6.9
RE+	29.6±6.0	31.3±12.0	31.5±11.4
B: Serum osteocalcin (ng/ml)			
Placebo	4.15±1.15	4.62±2.63	4.73±1.29
Ca+D	4.56±3.56	4.85±2.16	5.35±1.19
RE+	4.58±2.89	5.13±3.94	6.00±2.69
C: Serum NTx (nM)			
Placebo	19.69±6.51	19.08±4.24	21.09±4.75
Ca+D	21.15±5.23	20.86±7.85	20.24±6.17
RE+	19.60±6.18	15.43±7.56	14.24±4.93*

Ca+D: formulation with calcium and vitamin D_3. RE+: formulation of calcium and vitamin D3, blended with *Rehmannia glutinosa Libosch* and *Eleutherococcus senticosus Max.* BALP: bone-specific alkaline phosphatase, NTx: cross-linked N-telopeptide of type I collagen. *$p<0.05$, paired t-test, compared with T0.

concentrations of serum BALP were almost constant in all three groups, but the concentration of serum osteocalcin increased in the RE+ group at 6 and 12 months, whereas it was almost constant in the placebo and Ca+D groups (Table 3). The percentage change of osteocalcin was slightly higher, but not significantly, in the RE+ group than in the placebo and Ca+D groups at six and 12 months, compared to the respective baseline values. Although the mean serum NTx concentration declined slightly, but insignificantly at 6 months, the decreased value in the RE+ group was significantly sustained for 12 months ($p<0.05$).

DISCUSSION

It has been well established that the rate of bone loss increases during menopause subsequently resulting in osteopenia and osteoporosis. The inadequate intake of calcium and vitamin D_3 can lead to reduced calcium absorption, increased serum parathyroid hormone concentrations, and ultimate bone loss. Thus, calcium and vitamin supplementation appear to be important for the prevention and cure of postmenopausal osteoporosis. Despite differences in the dose and length of supplementation, even the calcium therapy alone is worthwhile for decreasing bone loss. Beneficial effects have been seen with a calcium dose, ranging from 500 mg/day to 1,600 mg/day, and it has been reported that bone mass has increased throughout the skeleton with D3 and calcium supplementation (Reid et al, 1993; Dawson-Hughes et al, 1997; Riggs et al, 1998; Grados et al, 2003). On the other hand, Cooper et al (2003) found no extra benefit from the addition of vitamin D3 and calcium. Despite such disparity, there is a general consen-

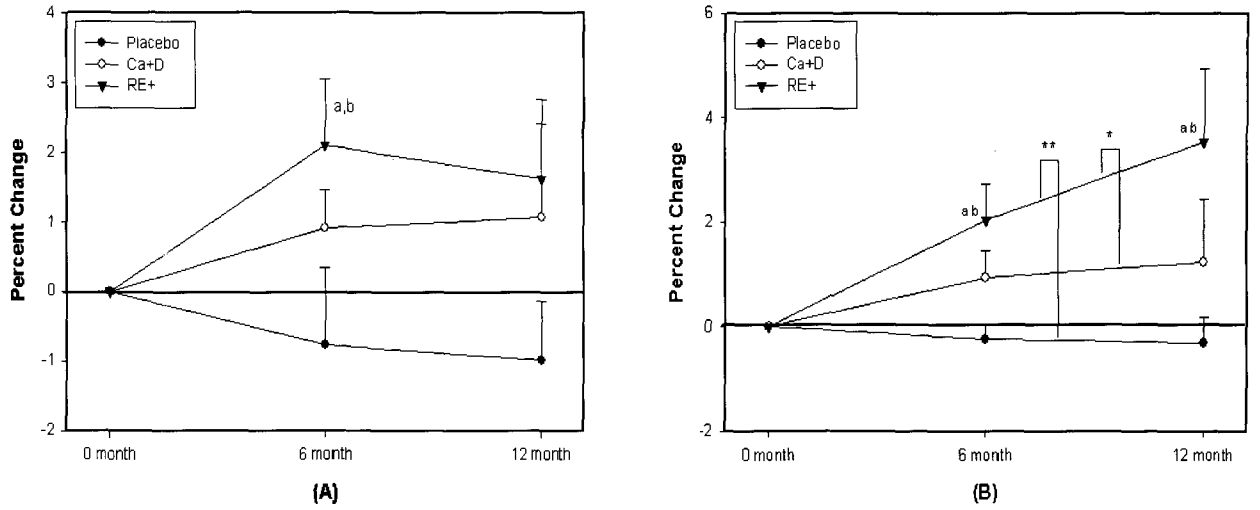

Fig. 2. Percentage change in BMD of (A) lumbar and (B) total femur in postmenopausal women during one year period by administration of Ca+D: formulation with calcium and vitamin D_3, RE+: formulation of calcium and vitamin D_3, blended with *Rehmannia glutinosa Libosch* and *Eleutherococcus senticosus Max.* Values are means±SE. $^{a}p<0.05$ compared with T0 with paired t-test, $^{b}p<0.05$ compared with placebo group with ANOVA. There were significant differences in the changes of femur BMD between two groups by repetitive-measured ANOVA (*$p<0.05$, **$p<0.01$).

sus on the beneficial effects of calcium and vitamin D_3 supplementation. Although it does not completely prevent postmenopausal bone loss, it is regarded as an adjunctive therapy which should be used for a long term (Dawson-Hughes et al, 2000; Boonen et al, 2004).

In this randomized trial, we established that there are highly beneficial effects of a new herbal formulation, including doses of calcium and vitamin D_3, by measuring both the spinal BMD (S-BMD), femoral BMD (F-BMD) and several biochemical markers throughout treatment. The S-BMD (L2-3 spine) was significantly increased after 6 month in the RE+ group compared with 0 month and placebo group (Fig. 2A). However, it should further elucidated the decrease of lumbar BMD at T12 than T6 in the RE+ group. The F-BMD (total right femur including neck, Wards, trochanter and shaft) was significantly increased after 6 and 12 months in the RE+ group compared with 0 month, placebo and Ca+D groups, whereas it was only slightly increased in the Ca+D group (Fig. 2B). When the Ca+D group was compared with the placebo group, our results were not consistent with the findings of Dawson-Hughes et al (1997); their study showed a significant benefit in BMD of the hip, spine, and total body. However, our findings are consistent with those of Chapuy et al (2002) and Chevelley (1994) who showed higher increments in F-BMD than in S-BMD.

Biochemical markers of bone formation and resorption are useful in assessing the bone turnover rate (Garcia-Perez et al, 2003). The resorption markers are better than formation markers for assessing hormone replacement therapy or antiresorptive therapy (Rosen et al, 1997; Yilmaz et al, 1999; Riera-Espinoza et al, 2004). Bone-specific alkaline phosphatase (BALP) and osteocalcin are formation markers useful in estimating osteoblast activity, and cross-linked N-telopeptide of type I collagen (NTx) is an ideal resorption marker to estimate osteoclast activity (Clemens et al, 1997; Ge et al, 1999).

We found a significant decrease of NTx in the RE+ group ($p<0.05$). This also correlated to the F-BMD which was significantly increased during the study period in the RE+ group ($p<0.05$). There was a positive, but insignificant, decrease in serum NT_x in the Ca+D group, whereas it was slightly increased in the placebo group (Table 3). This finding of a negative correlation of serum NTX with F-BMD is similar to the findings of Garcia-Perez et al (2003) on a group of women in the initial years of natural menopause. Moreover, both osteocalcin and BALP were increased in the RE+ and Ca+D groups more than in the placebo group (Table 3). Osteocalcin was increased more in the RE+ than in the Ca+D group, whereas BALP was almost the same as that in the RE+ and Ca+D groups. Since both resorption and formation markers diminish after antiresorptive treatment due to the inhibition of remodeling cycle as a whole, the increased levels of formation markers in the present study indicate unique bone-sparing effects, especially with these herbal extracts. The increased level of formation markers is possibly due to increased activity of osteoblasts, probably resulting from a transient artifact of bone remodeling or from physiological changes during menopause. Even though underlying mechanism involved in the increased osteoblastic activity after the administration of these herbs is not well known, Oh et al (2003) reported that RGX enhances the proliferation of osteoblasts. Similar trends of increasing osteocalcin (Chiechi et al, 2002) but decreasing NTx (Schreiber et al, 2001) have been observed with the supplementation of soy foods in postmenopausal women. Our present findings also showed an increase of bone mass, possibly correcting osteoporosis in postmenopausal women, by slightly increased osteoblastic activity along with significantly decreased osteoclastic activity, suggesting that these effects were induced by the novel formulation *RGX* and *ES* rather than by calcium and vitamin D_3 alone, since this formulation showed an effect on BMD and NTx much greater than the formulation containing a higher dose of calcium and vitamin

D_3 alone. Considering the above findings, calcium and vitamin D were included in the RE+ group for ethical reason, and it is certain that the antiosteoporotic effects of RE+ were induced by the herbs *RGX* and *ES*.

In conclusion, in the present study, the continuous oral therapy of the herbs, *RGX* and *ES*, was proved to be highly effective in controlling rapid reduction of bone turnover in postmenopausal women, and it showed fewer adverse effects than the administration of high dose calcium and vitamin D_3 supplementation alone. Because the number of participants in this study was limited, long-term evaluation with larger numbers of participants is highly recommended, in order to more precisely determine the effects of this formulation in preventing and treating osteoporosis

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