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Full Communication

Soy Isoflavones Mitigate Long-Term Femoral and Lumbar Vertebral Bone Loss in Middle-Aged Ovariectomized Mice

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ABSTRACT We evaluated the protective effects of soy isoflavones (SIF) against osteoporosis in middle-aged ovariectomized (OVX) mice. SIF (30 mg/kg or 60 mg/kg) or 17β-estradiol (E_2) was administered to OVX mice for 4 months after bilateral ovariectomy. We observed the biochemical markers of bone turnover, *e.g.*, alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP), in serum. We also observed the bone mineral density (BMD) in femurs and lumbar vertebrae. In addition, we examined trabecular bone and interstitial cells in the femur using hematoxylin and eosin staining. The decrease in ALP levels and the increase in TRAP levels normally resulting from ovariectomy were suppressed by administration of 60 mg/kg SIF or E_2. Administration of 60 mg/kg SIF or E_2 also maintained the BMD, trabecular bone, and interstitial cells in OVX mice compared to those in pre-OVX mice. These results suggest that 60 mg/kg SIF effectively mitigates ovariectomy-induced osteoporosis in middle-aged mice.

***KEY WORDS:** • alkaline phosphatase • bone mineral density • mice • ovariectomy • soy isoflavones • tartrate-resistant acid phosphatase*

INTRODUCTION

OSTEOPOROSIS IS A CHRONIC and progressive disease that is characterized by a decrease in bone mass and concomitant bone fragility. This is widely recognized as a major public health issue, as it causes problems such as fracture with trauma.[1] In particular, the extent of bone resorption exceeds its formation, resulting in osteoporosis and increased fracture risk in many postmenopausal women.[2,3]

Research on osteoporosis during the past 3 decades has shown a strong correlation between bone mineral density (BMD) and the elastic modulus or strength of trabecular bone. Fracture risk is determined according to BMD reduction.[4] However, even at a similar BMD, the fracture risk is known to strongly depend on age.[5] A number of alternative medicines have been used as traditional therapies for various diseases including osteoporosis.[6–11]

Soybeans are a rich source of the isoflavones genistein and daidzein.[12] Isoflavones have recently received considerable attention for their potential use in the prevention of postmenopausal bone loss because these compounds are found in natural foods and are structurally similar to mammalian estrogens.[13–15] Many reports have indicated that isoflavones show anti-osteoporotic activity *in vitro*[16,17] and *in vivo*.[7,8,12,14,18–22] Age-associated osteoporosis, which accompanies decreases in bone mass, is widely recognized as a major public health problem. The most dramatic expression of osteoporosis is the increased incidence of fractures of the proximal femur, the number of which increases with age.[23–26] It was reported that the effect of statins against osteoporosis in adult rats differed from that in aged mice.[27] In addition, few studies have assessed the anti-osteoporotic effects of soy isoflavones (SIF) in middle-aged ovariectomized (OVX) mice. Therefore, in this study, we investigated the

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protective effects of SIF against ovariectomy-induced osteoporosis in middle-aged mice.

MATERIALS AND METHODS

Experimental animals

The progeny of female ICR mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were used at 12 months of age. The animals were housed in an environmentally controlled facility, at a temperature of 22°C and relative humidity of 55% with a 12-hour light/12-hour dark cycle, and were provided free access to feed and water. The procedures for animal handling and care adhered to the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.[28] All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Ovariectomy

For ovariectomy surgery, the animals were placed under general anesthesia with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. Bilateral ovariectomy was performed from a dorsal approach with a small midline dorsal skin incision. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by the observation of the marked atrophy of uterine horns.

Administration of SIF

The OVX mice were randomly divided into four groups ($n = 10$ per group): vehicle (water)-treated group, 30 mg/kg SIF-treated group, 60 mg/kg SIF-treated group, and 1 mg/kg 17β-estradiol (E_2)-treated group. SIF was administered by oral gavage, and E_2 was injected intraperitoneally once daily 4 months after ovariectomy. An E_2 tablet was dissolved and adjusted with physiological saline to an appropriate concentration. SIF was provided by Dr. Chung's Food Co. Ltd. (Cheongju, Republic of Korea). After ovariectomy, the mice were fed orally with an experimental diet every day for 4 months, as described previously.[20]

Alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activities in serum

ALP activity was determined according to the method of Andersch and Szecypinski[29] using *p*-nitrophenyl phosphate as the substrate, and TRAP activity was determined according to the method of Tenniswood *et al.*[30] using *p*-nitrophenyl phosphate as the substrate. In brief, ALP and TRAP activities were determined using 10 m*M* *p*-nitrophenyl phosphate as the substrate in 0.1 *M* diethanolamine buffer (pH 9.8) and in 50 m*M* acetate buffer (pH 5.5) containing 20 m*M* sodium tartrate, respectively, for 30 minutes at 25°C. A unit of activity was defined as the release of 1 μmol of *p*-nitrophenol/minute. The samples were incubated at 37°C for 30 minutes. The light absorbance was measured at 405 nm for ALT activity, and the serum TRAP 5b activity was determined using the MouseBoneTRAP Assay (Immunodiagnostic Systems Ltd., Boldon, UK) according to the manufacturer's protocol.

Measurement of BMD in femurs and lumbar vertebrae

For the measurement of BMD, the animals were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. BMD was measured just before and 4 months after ovariectomy. The BMDs of the left femurs and lumbar vertebrae were measured by dual-energy X-ray absorptiometry (GE Healthcare Lunar, Madison, WI) by using the small animal scan mode.[31]

Histopathological analysis in the femur

The left femurs were removed and fixed in 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) for 24 hours. The femur tissues were decalcified by infiltration with Calci-Clear Rapid (National Diagnostics, Atlanta, GA) for 2 days and were then dehydrated in ethanol, defatted in xylene, and embedded with paraffin. The frontal sections were cut at a 6-μm thickness with a microtome (Leica, Wetzlar, Germany). Hematoxylin and eosin staining was used to evaluate the bone regeneration in femur tissues.

Quantification of data and statistical analysis

Data are expressed mean $\pm$ SEM values. Differences among the means were statistically analyzed by two-tailed Student's *t* test in order to elucidate the changes of serum ALP and TRAP levels of the vehicle-, SIF, and E_2-treated groups. In addition, differences among the means were statistically analyzed by analysis of variance, followed by Tukey's multiple range test in order to elucidate the effects of ovariectomy and SIF or E_2 treatment against ovariectomy-induced osteoporosis. Results were considered to be significant if $P < .05$.

RESULTS

ALP and TRAP activity in serum

The ALP in the serum was increased with the treatment of SIF and E_2 for 4 months after ovariectomy (Fig. 1A). In the 30 mg/kg SIF-treated group, the ALP level was increased, but not significantly, compared to that in the vehicle-treated group. However, in the 60 mg/kg SIF-treated group, the ALP level was increased to 124% compared to that in the vehicle-treated group. In the E_2-treated group, the ALP protein level was also significantly increased compared to that in the vehicle-treated group (Fig. 1A).

In contrast to the ALP levels, the TRAP levels in the serum were decreased with the treatment of SIF and E_2 for 4 months after ovariectomy (Fig. 1B). The TRAP levels were significantly decreased in the 60 mg/kg SIF-treated group, as well as in the E_2-treated group (Fig. 1B).

BMD in femurs and lumbar vertebrae

In the vehicle-treated group, the BMDs in both femurs and lumbar vertebrae were significantly decreased 4 months after ovariectomy: The BMD was decreased to 77.7% in the femurs (Fig. 2A) and to 71% in the lumbar vertebrae (Fig. 2B) of the pre-OVX group. In the 30 mg/kg SIF-treated group, the BMD in the femurs and lumbar vertebrae was also significantly decreased after ovariectomy compared to that in the pre-OVX group (Fig. 2). In the 60 mg/kg SIF-treated group, the BMD was 89.7% in the femurs and 91.5% in the lumbar vertebra of the pre-OVX group. In these groups, the reduction of BMD was not significant. In the E_2-treated group, the BMD was slightly decreased in both the femurs and lumbar vertebrae after ovariectomy compared to that in the pre-OVX group (Fig. 2).

Morphometric findings in trabecular bone in femur

In the vehicle-treated group, trabecular bone in the femur was poorly observed. In addition, interstitial cells were not abundant (Fig. 3A and B). In the 30 mg/kg SIF-treated group, trabecular bone was slightly better than that in the vehicle-treated group. In addition, interstitial cells were also slightly increased in this group (Fig. 3C and 3D). In the 60 mg/kg SIF-treated group, trabecular bone seemed to be intact, and interstitial cells were abundant compared to those in the vehicle-treated group (Fig. 3E and 3F). In the E_2-treated group, trabecular bone and interstitial cells were similar to those in the 60 mg/kg SIF-treated group (Fig. 3G and 3H).

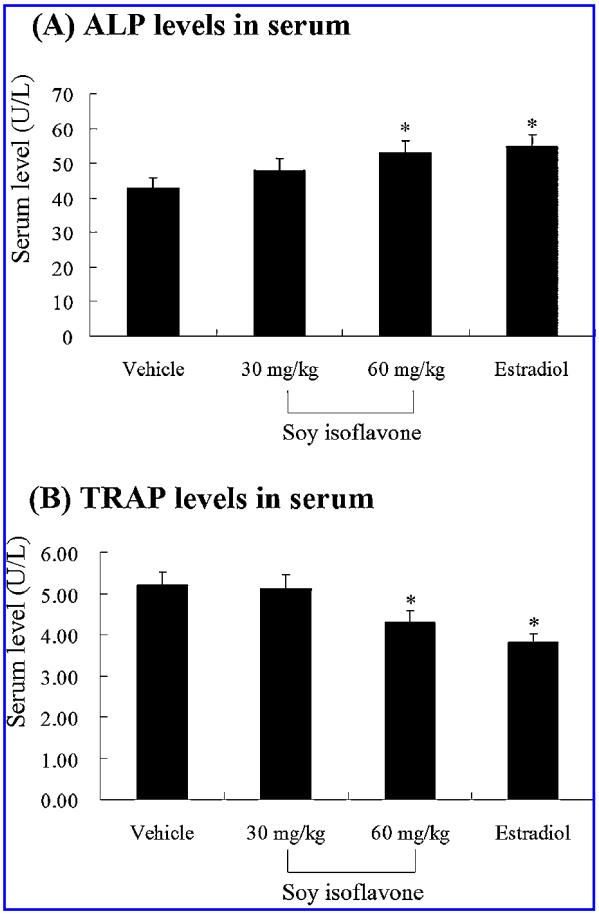

FIG. 1. Serum levels of bone turnover markers (ALP and TRAP) in vehicle-, 30 mg/kg SIF-, 60 mg/kg SIF-, and E_2-treated mice after ovariectomy. ALP levels are significantly increased in the 60 mg/kg SIF- and E_2-treated groups, whereas the TRAP levels in these groups are significantly decreased. *$P < .05$, significantly different from the vehicle-treated group. Data are mean ± SEM values ($n = 10$ per group).

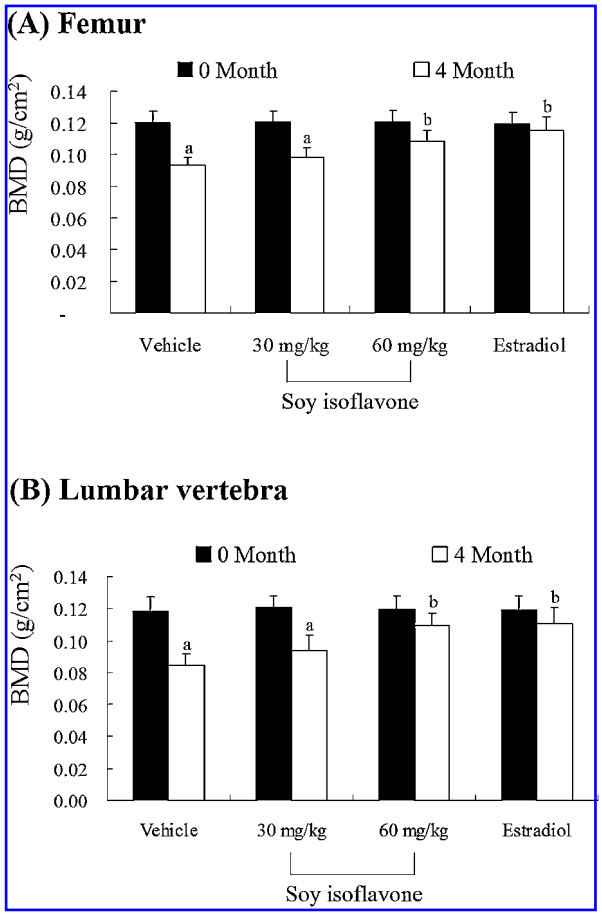

FIG. 2. BMD (in g/cm^2) in vehicle-, 30 mg/kg SIF-, 60 mg/kg SIF-, and E_2-treated mice after ovariectomy. The BMD is significantly decreased in the vehicle-treated group. In the 60 mg/kg SIF- and E_2-treated groups, the BMD is nearly maintained at the levels of the pre-OVX values. $^aP < .05$, significantly different from 0 month group, $^bP < .05$, significantly different from the vehicle-treated group. Data are mean ± SEM values ($n = 10$ per group).

DISCUSSION

Bone remodeling is mediated by the balanced activities of osteoclasts, which resorb existing bone, and of osteoblasts, which form new bone. In the present study, we observed the protective effects of SIF in a middle-aged mouse model of ovariectomy-induced osteoporosis because the incidence of osteoporosis is increasing worldwide as populations age

and because women are four times more likely than men to develop osteoporosis.[32] Data from several observational studies have suggested that populations with a high mean intake of soy, such as Japan, have a lower incidence of osteoporotic fractures than Western populations.[33,34] Isoflavone intakes of Japanese, Chinese, and Korean women consuming traditional diets are estimated at 15–50 mg/day[35–37] or in the vicinity of 1 mg/kg of body weight/day[38]; however, the doses reported to be biologically active in humans are lower than the doses generally reported to be active in rodents (10–100 mg/kg of body weight/day).[38] In this regard, we chose 30 and 60 mg/kg SIF to elucidate the effects against osteoporosis.

In the present study, SIF treatment in OVX mice maintained ALP and TRAP levels. This result suggests that SIF may increase the osteoblast activity and decrease the osteoclast activity, since ALP and TRAP are frequently used as bone formation and disruption markers, respectively, in the monitoring of drug actions.[39–41] In addition, we assessed the BMD in femurs and lumbar vertebrae and investigated histopathology in the femur in order to confirm protective effects of SIF against osteoporosis. SIF significantly improved the BMD score and maintained the structures of trabecular bone and interstitial cells in the femur compared to those in the vehicle-treated group. These results are supported by previous studies that found feeding soy protein to OVX rats prevented bone loss. In addition, soy protein has been found to attenuate menopausal bone loss, and it has been suggested that isoflavones in soy might be responsible for their protective effects against bone loss.[19–21,42] However, there are contradictory findings among clinical studies about the efficacy of SIF on bone metabolism in postmenopausal women. Inter-individual differences in intestinal bacteria metabolism of isoflavones to produce equol might partly explain these discrepancies.[43] In this study, however, we did not observe any inter-individual differences because we used the inbred strain of mice.

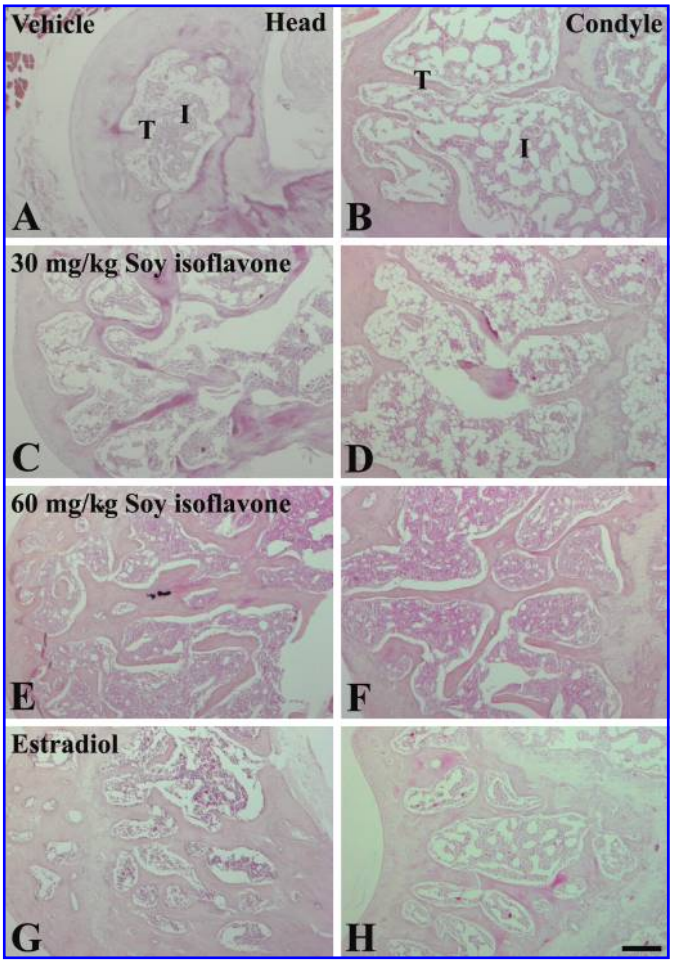

FIG. 3. Hematoxylin and eosin staining in the head (**A**, **C**, **E**, and **G**) and condyle (**B**, **D**, **F**, and **H**) of the femur in vehicle-, 30 mg/kg SIF-, 60 mg/kg SIF-, and E_2-treated mice after ovariectomy. Trabecular bone (T) and interstitial cells (I) appear intact in the 60 mg/kg SIF- and E_2-treated groups compared to those in the vehicle-treated group. Bar = 200 μm.

Genistein directly stimulates osteoblastic cell proliferation, protects osteoblastic cells from oxidative stress, and increases the apoptosis of osteoclast progenitor cells.[16,17] In addition, OVX rats treated with a genistein-rich SIF preparation or purified genistein retain more bone than vehicle-treated control animals.[44–46] Epidemiological studies have suggested that the low incidence of osteoporosis and heart disease in postmenopausal Asian women compared to American women is attributable to their higher intake of soybean-based foods.[47]

In conclusion, oral administration of 60 mg/kg SIF potently mitigates ovariectomy-induced osteoporosis in middle-aged mice.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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