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Herbal Extract Prevents Bone Loss in Ovariectomized Rats

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This research aims to test a new drug candidate based on a traditional medicinal herb, F1, an herbal extract obtained from *Astragalus membranaceus* and its main ingredient, 1-monolinolein that may have fewer side effects and less uterine hypertrophy. *In vitro* experiments, human osteoblast-like cell lines, MG-63 and Saos-2, were analyzed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and an alkaline phosphatase (ALP) assays. Mouse osteoclasts were induced through a calcium-deficient diet and inhibition effects were measured. *In vivo* experiments were done using ovariectomized (OVX) rats for 9 weeks. At necropsy, uterus weights were measured, trabecular bone area (TBA) of tibia and lumbar vertebra were measured bone histomorphology. In results, cell proliferation and ALP activity in Saos-2 by ether F1 or 1-monolinolein did not increased significantly compared to the control. The F1 inhibited osteoclast development ($IC_{25} = 3.37 \times 10^{-5}$ mg/mL) less than 17β-estradiol. The OVX rats administered F1 (2 mg/kg/day and 10 mg/kg/day) showed an increase in TBA of the tibia significantly (136.3±4.2% and 138.5±10.3% of control). In conclusions, the herbal extract, F1 inhibited tibia and lumbar bone loss and did not cause uterine hypertrophy. However, 1-monolinolein, the main ingredient of the herbal extract, did not inhibit bone loss.

Key words: Osteoporosis, Herbal extract, Osteoblast-like cells, Osteoclast, Ovariectomized rat, Trabecular bone area

INTRODUCTION

Postmenopausal women, because of estrogen loss, are at risk for type I osteoporosis (Gillespy and Gillespy, 1991). After menopause, the normal balance between bone formation and resorption is disrupted; osteoclasts (giant multi-nucleate cells) become more active, decreasing bone mass and increasing the chances of fracture. Thus, the development of an osteoclast inhibitor is very important for osteoporosis prevention. On the other hand, osteoblasts, bone-forming cells derived from mesenchymal cells in bone marrow, line up on the bone surface, especially in regions of new bone formation, lay down bone matrix in orderly lamellae and induce its mineralization. Thus, the increased activity of osteoblasts is helpful to treat and prevent osteoporosis. Osteoporosis medications in wide use include estrogen products, calcium, phosphate, fluorine-based drugs, ipriflavone, vitamin D_3, selective estrogen receptor modulators (SERMs), and aminobisphosphonate (Gillespy and Gillespy, 1991; Bryant and Dere, 1998; Wark, 1996; Agnusdei *et al.*, 1989).

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Estrogen-based drugs have been linked with adverse effects including uterine bleeding and cancer, when used over an extended period of time (Bergkvist *et al.*, 1989; Whitehead and Fraser, 1987). Therefore, we have attempted to develop a medication based on traditional medicinal herbs to replace estrogen-based drugs.

Astragali Radix is the dried root of *Astragalus membranaceous* Bunge (a perennial plant in the pulse family, Leguminosae), and contains glycosides and flavonoides, particularly isoflavonoides as its principle constituents. As a tonic used to strengthen muscles and bones, it is one of the most widely used medicinal herbs in Asian traditional medicine (Shin, 1988).

The ideal drug for osteoporosis prevention and treatment should increase the activity of osteoblasts and suppress the activity of osteoclasts. To determine whether *Astragalus membranaceous* has such properties or not, we conducted an *in vitro* experiment using substances from *Astragalus*

membranaceous isolated by fractionation, and investigated the effects of such fractions on osteoblast proliferation and osteoclast inhibition. In an *in vivo* experiment, isolates identified as metabolically active were administered to ovariectomized rats. Trabecular bone areas were measured to determine the isolates effects on bone growth and resorption.

MATERIALS AND METHODS

Herbal extraction

As reported in our previous studies (Kim *et al.,* 2003) the herbal extract was obtained from dried and powdered roots of *Astragalus membranaceous*. The extract was made by steeping the dried powdered roots in 80% EtOH for 1 week at room temperature; this step was repeated four times. Later n-hexane fractionation was conducted on the ethanol extract. The *n*-hexane faction was applied to a silica gel 60 column (Merck Art. 9385, 7734: 14×1, 128 cm) and eluted with $CHCl_3$: MeOH (= 9:1~1:1) and 100% EtOH sequentially with the flow rate of 5.7 mL/min. Then TLC analysis was performed on pre-coated Kiesel gel 60 F_{254} (0.25 mm, Merck Art. 5715, 5729, and 5789) and the eluate was divided into 13 fractions.

Osteoblast-like cell proliferation

MG-63, a human osteoblast-like cell line (Korea Cell Line Bank, Seoul, Korea), was grown in Dulbecco's modified eagle medium (DMEM) containing 10% FBS, penicillin (100 units/mL) and streptomycin (100 mg/mL) (Gibco BRL, Grand Island, N.Y., U.S.A.) (Koh *et al.,* 1997; Androanarivo *et al.,* 1992). The cells were placed in a 96-well plate at the rate of 20,000 cells/well. Then, 1% DMSO and $1,25(OH)_2D_3$ (Biomol Research Labs Inc., Plymouth Meeting, PA, U.S.A.), NaF (Sigma Chem. Co., St. Louis, MO, U.S.A.), 17β-estradiol (E2) (Aldrich, St. Louis, MO, U.S.A.), 1-monolinolein (Sigma Chem. Co., St. Louis, MO, U.S.A.) and F1 (10^{-3}~10^{-10} mg/mL) were applied to respective wells. After incubating the cells for 3 days, 0.5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (MTT: Sigma Chem. Co., St. Louis, MO, U.S.A.) was added to the culture and then incubated for another 4 hours. Formed formazan crystals were dissolved in DMSO solution, and $OD_{550\,nm}$ was measured with an ELISA reader (Ceres UV 900C, Bio-Tech Instrument, U.S.A.), and $OD_{550\,nm}$ of each reagent-treated well to control well was calculated as a percentage (Carmichael *et al.,* 1987).

Saos-2, a human osteoblast-like cell line (Korea Cell Line Bank, Seoul, Korea), was grown in RPMI 1640 medium containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). As with the MTT method described above, cell proliferation was calculated as percentage, and an ALP activity assay was also conducted. ALP activity was measured in a colorimetric assay using a biochemical analyzer (Airon 200, Crony Instruments, Rome, Italy) and an ALP assay kit (Trace Am. Inc., Miami, FL, U.S.A.) (Sabokar *et al.,* 1994).

Osteoclast cell culture and proliferation test

Osteoclasts may be directly isolated from bones (Takada *et al.,* 1992) or formed by growing bone marrow cells and ST-2 cells together in culture (Shioi *et al.,* 1994; Ragab *et al.,* 1998). We isolated osteoclasts from calcium-deficient mice. From the tibias and femurs of 10-week-old ICR mice (Daehan BioLink Co., Umsung, Chungbuk, Korea) that had been placed on a four-week calcium-deficient diet (ICN Biomedicals, Inc., Aurora, OH, U.S.A.), osteoclasts were isolated with α-MEM containing penicillin (100 units/mL) and streptomycin (100 μg/mL), suspended again in α-MEM containing 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL), and then inoculated in 24-well plates (3.5×10^6 cells/well). Then, various concentrations of test compounds (E2, genistein, 1-monolinolein or F1) were applied to the inoculated plates and incubated for 2 days. Osteoclast proliferation was measured by tartrate-resistant acid phosphatase (TRAP) staining (Sigma Chem. Co., St. Louis, MO, U.S.A.) and identifying TRAP-positive cells with 3 or more of nuclei (TRAP-Positive MNC) (ASBMR, 2000). E2 and genistein (Sigma Chem. Co., St. Louis, MO, U.S.A.) served as controls in osteoclast inhibition by F1 or 1-monolinolein experiments.

Animal studies

All animals were cared for at the Animal Resource Center, Korea Institute of Oriental Medicine, according to the USDA Animal Welfare Act and the NIH *Guide for the Care and Use of Laboratory Animals*. Ten-week-old (200~300 g) Sprague-Dawley rats (KRICT, Daejon, Korea) were allowed free access to solid food and water. Then, either a sham operation or ovariectomy (OVX) was conducted (Nordin *et al.,* 1980). One mg/kg/day of E2 (n = 18), and 1 (F11) (n = 11), 2 (F12) (n = 11) and 10 (F110) (n = 9) mg/kg/day of F1, and 2 (M2) (n = 9) and 10 (M10) (n = 11) mg/kg/day of 1-monolinolein were suspended in 10% Tween 80 (Sigma Chem. Co., St. Louis, MO, U.S.A.) solution and injected intraperitoneally into the animals once daily for 9 weeks beginning 1 week post operation; sham (n = 15) and control groups (n = 24) received 10% Tween 80 solution only. Blood samples were collected from the tail veins in each group before the operation, before administration of drug, and 3 weeks and 6 weeks after administration of experiment materials. After 9 weeks of administration, blood samples were collected from the inferior vena cava, and euthanasia and necropsy were conducted to separate the uterus, tibias, and 6th lumbar vertebrae. Complete blood cells (CBC) were counted from

the blood samples using a Coulter counter (Coulter JT, Coulter Electronics Inc., Hialeah, FL, U.S.A.); plasma was isolated and kept at -70°C for subsequent analysis. Concentrations of ALP, calcium, inorganic phosphate, creatinine, total cholesterol, HDL-cholesterol, LDL-cholesterol, and urea were determined using a biochemical analyzer (Airon 200, Crony Instruments, Rome, Italy) and reagents (Trace Am. Inc., Miami, FL, U.S.A.). The weights of uterus were measured and histological analysis was conducted on tibia and lumbar vertebrae (Kim *et al.,* 1998).

Histomorphological analysis

Collected tibias and lumbar vertebrae were fixed in 10% neutral formalin solution followed by decalcification with formic acid. Then, the decalcified bones were bisected anterior-posteriorly at the intercondylar space of tibia head and midline of body to spine of lumbar vertebra (L4-5) followed by dehydration sequentially with 70~100% ethanol; they were cleared with xylene and embedded in paraffin. The paraffin embedded tissues were cut into 5-micron sections with a microtome. Hematoxylin and eosin staining was performed and observed by light microscope. Histomorphometrys of the trabecular bone of tibia and lumbar vertebra were done using an image analysis system (Wild Leitz Co., Wetzler, Germany). The represented area of morphometry was absolute trabecular bone area(TBA) that appeared in the standardized central rectangular area (2×10^6 μm^2) of metaphysis below the epiphyseal plate in tibia and lumbar vertebra (Kim *et al.*, 1998; Parfitt *et al.*, 1987). Trabecular bone areas in standardized rectangular area were measured from two step sections of tibia and lumbar vertebra in each animal by two persons. Trabecular bone areas were described as the trabecular bone area (B.Ar : mm^2) as a proportion (B.Ar/TT.Ar = bone volume fraction = BV/TV; %) of total tissue area (TT.Ar : mm^2) (Ederveen *et al.*, 2001; Lark *et al.*, 2001).[6,16]

Statistics

Statistical tests used were the Bonferroni multiple comparision and ANOVA (Systat®, Systat Inc., Evanston, Il, U.S.A.); a value of $P<0.05$ indicated significance (Rosner, 1990).

RESULTS

Herbal extraction

The herbal extract of *Astragalus membranaceous* was fractionated sequentially and *n*-hexane fraction was the most active in osteoblast-like cell proliferation at our earlier studies (Kim *et al.,* 2000). F1 corresponds to 11th fraction of the column chromatography of *n*-hexane extract which contains the highest activities of releasing of growth hormone in the cell culture. Its major constituents are known as 1-monolinolein and 1'-hexadecanoic acid 2',3'-dihydroxypropylester (Fig. 1) (Kim *et al.,* 2003).

Osteoblast-like cell proliferation

NaF, a known therapeutics for osteoporosis, to increase activity of osteoblast in MG-63 cells, had a 116.7±3.2% cell proliferation effect relative to the control group at 1×10^{-7} M; $1,25(OH)_2D_3$, 130.0±7.1% ($P<0.05$) at 1×10^{-7} M; and E2, 105.3±1.5% at 1×10^{-7} M; while the two substances in question, F1 and 1-monolinolein, measured in 133.6±9.0% ($P<0.01$) at 1×10^{-5} mg/mL and 112.1±1.2% ($P=0.08$) at 1×10^{-7} M respectively (Table I). In the MTT assay using Saos-2 cells, another osteoblast-like cell, the cell proliferation rate of Saos-2 was not increased compared to the control group when herbal extract F1 and 1-monolinolein, NaF, $1,25(OH)_2D_3$, and E2 were used at 1×10^{-5} mg/mL or 1×10^{-7} M.

As reported in other studies (Kassem *et al.,* 1994; Sabokbar *et al.,* 1994), the ALP activity, a characteristic of osteoblasts, was not observed in MG-63; while in Saos-2, ALP activity increased to 189.4±2.0% of the control ($P<0.01$), when NaF was used at 1×10^{-7} M; and 182.7±29.1% when $1,25(OH)_2D_3$ was used at 1×10^{-7} M ($P<0.01$). However, E2 did not significantly affect ALP activity with 90.9±7.1% of the control group at 1×10^{-7} M. On the other hand, F1 did not increase ALP activity and 1-monolinolein increased to 118.4±6.7% at 1×10^{-7} M (Table I).

Osteoclast inhibition test

During the osteoclast inhibition study, control wells contained 339±29 cells/well. As shown in Fig. 2, IC_{25} of genistein was 9.55×10^{-9} M and it showed concentration-dependent inhibition. And IC_{25} of E2 was 1.51×10^{-8} M and that of F1 was 3.37×10^{-5} mg/mL.

Animal studies

During the first animal study, the sham group started showing significant weight gain 5 weeks after the operation ($P<0.01$), while the OVX control group started showing significant weight gain 2 weeks after the operation ($P<0.01$) (Fig. 3). The E2 groups weight gain due to ovariectomy slowed down relative to the OVX control group but

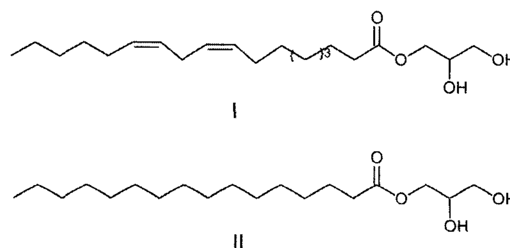

Fig. 1. Structures of 1-monolinolein (I) and 1'-hexadecanoic acid-2',3'-dihydroxy-propyl ester (II).

Table I. Osteoblast-like cell proliferation and ALP activity in MG-63 and Saos-2 cells

	MG-63		Saos-2			
	MTT assay		MTT assay		ALP activity assay	
	Concentration (M)	% of control	Concentration (M)	% of control	Concentration (M)	% of control
NaF	1×10^{-7}	116.7±3.2	1×10^{-7}	110.1±13.0	1×10^{-7}	189.4±20.0**
$1,25(OH)_2D_3$	1×10^{-7}	130.0±7.1*	1×10^{-7}	103.9± 2.9	1×10^{-7}	182.7±29.1**
E2	1×10^{-7}	105.3±1.5	1×10^{-7}	96.5± 2.2	1×10^{-7}	90.9± 7.1
F1 (mg/mL)[a]	1×10^{-5}	133.6±9.0**	1×10^{-5}	104.6± 6.2	1×10^{-5}	88.1± 8.2
1-Monolinolein	1×10^{-7}	112.1±1.2	1×10^{-7}	98.3± 1.3	1×10^{-7}	118.4± 6.7

[a]unit: mg/mL

MG-63 or Saos-2 cells were incubated for 3 days with NaF, $1,25(OH)_2D_3$, E2, F1 or 1-monolinolein. In the MTT assay, MTT was added and incubated for a further 4 h. Formed formazan was dissolved with DMSO and the absorbance was measured at 550 nm. The ALP activity was measured by colorimetric method. Result was calculated as percentage of the control value. Data are expressed as mean±SEM. Compa rison to control, *P<0.05, **P<0.01.

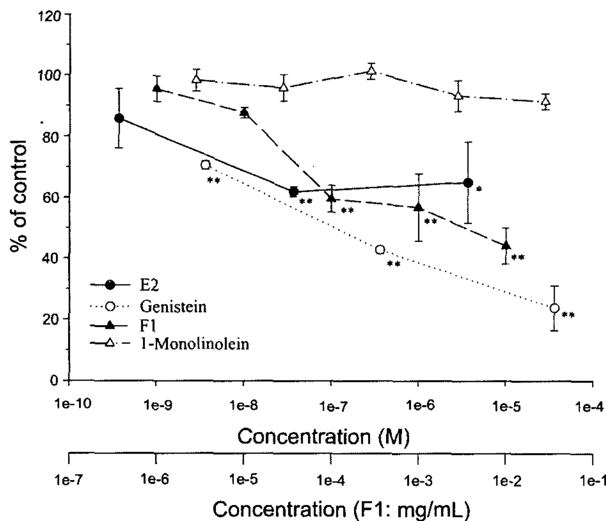

Fig. 2. Changes in number of Osteoclasts (TRAP positive multinucleated cells) after incubation with various concentrations of test compounds for 48 hr. Osteoclasts were isolated from calcium deficient mice. Symbols are estrogen (●), genistein (○), F1 (▲) and 1-monolinolein (△).

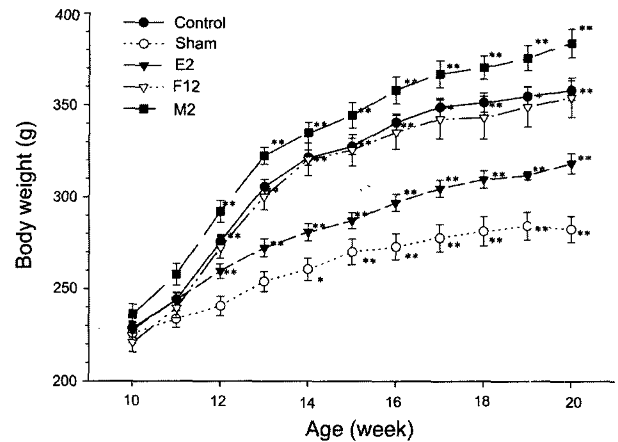

Fig. 3. Changes in body weight during treatment (10 to 20 weeks of age). Data are reported as mean±SEM. Symbols are control (●), sham (○), E2 (▼), F12 (▽), and M2 (■). Comparison to 10 weeks, * P<0.05, **P<0.01

increased compared with the sham group. When various concentrations of F1 were administered, the F11, F12, and F110 groups showed weight gain at a similar level to the OVX control group (Fig. 3). During the second animal study, the sham group started showing significant weight gain 7 weeks after the operation (P<0.05) while the OVX control group started showing significant weight gain 2 weeks after the operation (P<0.01). As in the second animal study, weight gain patterns are similar to the first study. When various concentrations of 1-monolinolein were administered, M2 and M10 groups showed similar weight gain relative to the OVX control (data not shown).

After the 9 weeks administration, the uterus of the OVX control group was 83±2 mg (wet), 20% of the sham group (414±24 mg) (P<0.01), but those of the E2 group showed 65% of the sham group (278±11 mg) (P<0.05). On the other hand, the uterus of the F11, F12 and F110 groups did not show any significant difference against those of the OVX control group (Fig. 4). Likewise, during the second animal test, the E2 group (252±9 mg) showed little difference against the sham group, but increased 304% of the OVX control group (P<0.01). The uterus of the M2 and M10 groups showed little difference in weight against those of the OVX control group (Fig. 4).

As biochemical markers, ALP activity, calcium. inorganic phosphate, urea, creatinine, and cholesterol levels (total, HDL, and LDL) in plasma were measured (Table II). ALP activity, calcium and inorganic phosphate levels decreased in plasma of all groups as time elapsed (P<0.05). However, urea and creatinine levels in plasma of all groups de-

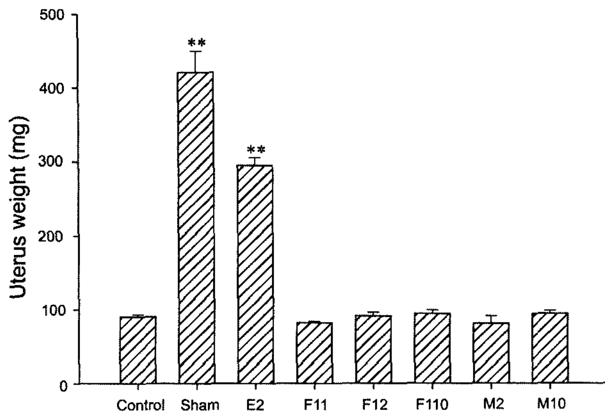

Fig. 4. Uterus weight (wet) after 9 weeks of administration of various test compounds. Comparison to control, **P<0.01.

creased 1 week after operation (at 11 weeks) compared with the levels before operation (at 10 weeks), but increased again after 9 weeks administration (at 20 weeks) (P< 0.05). Total cholesterol levels had the tendency to increase in all groups with the elapse of time, but there was no significant difference. As for HDL cholesterol levels, the F11 group showed an increase 9 weeks administration compared with the level before operation (P<0.01), but the F110 group showed a decrease (P<0.01); the M10 group showed an increase 1 week after the operation compared with the level before operation (P<0.01), but showed a decrease after 9 weeks administration (P<0.01). On the other hand, LDL cholesterol levels in all F11, F12, F110, M2 and M10 groups showed an increase after 9 weeks administration relative to the levels before the operation and 1 week after operation (P<0.01, respectively) (Table II).

Histomorphological analysis

The TBA as a proportion to the total tissue area in tibias of the sham group (bone volume fraction: BV/TV; 43.01± 2.24%) was 1.99 fold of the OVX control group (21.56± 0.54%) (P<0.01), and that of the E2 group (31.73±2.09 %) was 1.47 fold of the OVX control group (P<0.01). Among the groups administered F1, TBAs as a proportion to the total tissue area in tibias of the F12 group (29.39± 0.90%) and F110 group (29.87±2.23%) were 1.36 fold and 1.39 fold of the control, respectively (P<0.01). However, that of the F11 group (25.76±2.27%) was not significantly different from that of the control group (Fig. 5). On the other hand, TBA as a proportion to the total tissue area in lumbar of the sham group was 1.25 fold of the OVX control group (P<0.01), while those of the E2 and F11 groups were a statistically significant 1.18 fold and 1.42 fold of the OVX control group, respectively (P<0.01) (Fig. 5).

Table II. Biochemical clinical analysis in plasma during 9 weeks of F1 and 1-monolinolein administration to OVX rats

Group	Age (week)	ALP (U/dL)	Ca (mg/dL)	Inorganic phosphate (mg/dL)	Cholesterol (mg/dL)	HDL-Cholesterol (mg/dL)	LDL-Cholesterol (mg/dL)
Control	11	208.71±10.14	12.50±0.47	3.81±0.26	98.68±5.59	58.22± 2.95	40.25± 4.97
	20	142.91± 9.99##	9.78±0.24##	5.40±0.25##	112.27±4.96	51.33± 5.47	58.43± 5.03#
E2	11	236.39±15.23	12.39±0.50	6.46±0.24	93.24±5.92	56.42± 2.66	36.83± 5.30
	20	149.01±14.28##	10.17±0.27##	5.48±0.25##	111.54±4.74	45.73± 5.21	65.80± 6.18##
Sham	11	184.40±14.96	12.32±0.50	6.18±0.28	80.19±9.59	48.13± 4.26	32.06± 4.11
	20	111.09±11.98##	10.18±0.35##	5.43±0.33	81.29±4.59§§	36.90± 4.77	44.39± 7.44
F11	11	206.95±34.37	11.22±0.15	7.17±0.36	144.10±13.52§§	62.89±10.16	78.51±15.16§
	20	124.09±21.35	10.80±0.25	5.86±0.39	116.24± 7.46	76.50± 3.52§	35.23± 5.35#
F12	11	245.90±19.12	15.15±0.51§§	7.70±0.36	88.99± 3.68	54.82± 5.03	33.62± 3.58
	20	193.18±13.72	8.77±0.39##	5.13±0.19##	105.03± 5.31#	33.38± 6.46#	71.65± 8.36##
F110	11	207.59±22.98	10.15±0.30	6.35±0.41	69.24± 4.13§	53.75± 4.45	19.05± 3.49
	20	154.80±19.68	9.05±0.11§#	5.92±0.38	85.15± 3.95§#	40.31± 1.98#	44.83± 3.88#
M2	11	200.71±20.26	10.16±0.40	6.36±0.22	83.77± 5.46	50.55± 3.90	33.23± 5.58
	20	143.57±16.57	8.88±0.20§#	5.80±0.29	99.44± 5.49	40.41± 2.05	59.03± 4.49##
M10	11	194.06±17.55	12.35±0.17	5.08±0.23	93.69± 3.64	47.40± 1.55	46.29± 2.80
	20	125.75±13.73#	10.45±0.16##	4.93±0.20	98.53± 1.65	28.07± 1.19##	69.87± 1.20##

Data are expressed as mean±SEM
Comparison to 11 weeks, #; P<0.05, ##; P<0.01.
Comparison to control group, §; P<0.05, §§; P<0.01.

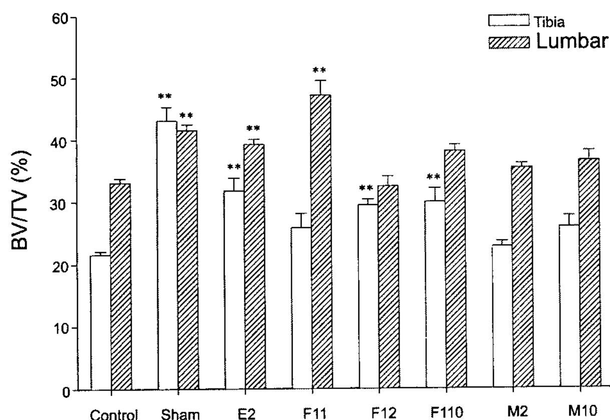

Fig. 5. Bone volume fraction (BV/TV; %) of tibia and 6th lumbar vertebra after 9 weeks of administration of various concentrations of F1 and 1-monolinolein. F11, administration of 1 mg/kg/day of F1; F12, administration of 2 mg/kg/day of F1; F110, administration of 10 mg/kg/day of F1; M2, administration of 2 mg/kg/day of 1-monolinolein; M10, administration of 10 mg/kg/day of 1-monolinolein. Data are reported as mean±SEM. Comparison to control, *P<0.05, **P<0.01.

DISCUSSION

Bone mass may be maintained at a constant level when there is a balance between activity of osteoblasts and osteoclasts. The activity of osteoblasts can be determined by measuring cell proliferation with the MTT assay (Yang *et al.,* 1996) and by measuring the activity of ALP, an enzyme characterizing osteoblasts (Sabokar *et al.,* 1994). The ximum cell proliferation of MG-63 caused by F1 at 1×10^{-5} mg/mL was approximately 133.6±9.0% compared to control (P<0.01); the value for 1-monolinolein, a major constituent of the F1 fraction, at 1×10^{-7} M was approximately 112.1±1.2% of control (P = 0.08) (Table I). The comparable values for NaF and $1,25(OH)_2D_3$, known to increase the activity of osteoblasts, were lower than the value for F1 (Table I). MG-63, an osteoblast-like cell line, was relatively good in terms of cell proliferation rate, but significantly lower in terms of ALP activity compared with other cell lines (Kassem *et al.,* 1993, 1994). This research also compared ALP activity of MG-63 and Saos-2, and found that Saos-2 was 100~200 times more active than MG-63 in terms of ALP activity. However, Saos-2 showed relatively higher ALP activity, but lower cell proliferation rate than MG-63. Thus, the cell proliferation rate of Saos-2 was not increased compared to the control group when $1,25(OH)_2D_3$ and E2 were used at 1×10^{-7} M. The values for the testing compounds including herbal extract F1 and 1-monolinolein were similar to the control at 1×10^{-5} mg/ml and 1×10^{-7} M (Table I). Conversely, as for ALP activity, NaF (Kassem *et al.,* 1994) and $1,25(OH)_2D_3$, known to increase the activity of osteoblasts, increased ALP activity by 189.4±20.0% and 182.7±29.1% compared with control, respectively. This was similar to the results reported by previous studies (Androanarivo *et al.,* 1992; Carmicheal *et al.,* 1987; Sabokbar *et al.,* 1994) where NaF increased cell proliferation to 220% and $1,25(OH)_2D_3$ increased ALP activity to 170% compared with controls in human bone marrow stromal osteoblast-like cells. However, the E2 and 1-monolinolein at 1×10^{-7} M and F1 at 1×10^{-5} mg/mL did not increased ALP activity compared to the control. $1,25(OH)_2D_3$, currently in use as an osteoporosis therapeutics, increased both cell proliferation and ALP activity, while NaF mainly increased ALP activity and E2 did increase neither cell proliferation nor ALP activity (Table I). Thus, 1-monolinolein at 1×10^{-7} M did not increased osteoblast-like cell proliferation and the ALP activity, but the F1 at 1×10^{-5} mg/mL did MG-63 cell proliferation.

Genistein (IC_{25} = 9.55×10^{-9} M), a tyrosine kinase inhibitor, was the most potent inhibitor of osteoclast activity in mouse osteoclast culture (Fig. 2). E2 (IC_{25} = 1.51×10^{-8} M) and the herbal extract F1 (IC_{25} = 3.37×10^{-5} mg/mL) were also effective inhibitors of osteoclast activity although 1-monolinolein was not (Fig. 2). Therefore, considering that 1-monolinolein and F1 were found in an *in vitro* assay to have osteoblast proliferation effects without significant difference between the two, and that F1, in particular, had similar osteoclast inhibition effects to E2, the substances may be a potential osteoporosis treatment.

The OVX animal model is a well-known osteoporosis model (Nordin *et al,* 1980), and the medication period was determined by considering various studies (Kim *et al.,* 1998). After 9 weeks of test compound administration to animals, changes in ALP, calcium, inorganic phosphate concentrations in plasma (which are biochemical markers of osteoporosis) were observed (Table II). As an indirect indicator of skeletal matrix changes, urea levels were measured, and creatinine levels were observed to determine the changes in kidney functions. In addition, it has been reported that if estrogen is administered to menopausal osteoporosis patients, LDL-cholesterol levels decrease and HDL-cholesterol levels increase (Bryant and Dere, 1998); another report states that an increase in cholesterol levels of menopausal osteoporosis patients reflects an increase in LDL-cholesterol levels (Bryant and Dere, 1998). In the test animals ALP, calcium, and inorganic phosphate levels in all groups decreased as the subjects grew older; the sham, control, E2, and M10 groups, in particular, showed a significant decrease in ALP concentrations after 9 weeks of administration relative to baseline levels before operation (Table II, P<0.05). ALP, calcium, and inorganic phosphate concentrations reflect not only the state of bones, but also the state of all other organs. Thus, it seems that the test results may not precisely reflect the changes in bone mass. And there were no changes in plasma urea and creatinine concentrations among groups and animals

ages. In addition, total cholesterol levels of the OVX control group showed no significant differences after ovariectomy, but there were significant increases in total-cholesterol and LDL-cholesterol levels relative to animals age after OVX ($P<0.05$). These were similar to those of human studies that increase in LDL-cholesterol levels resulted in increase in total cholesterol while osteoporosis in progress (Bryant and Dere, 1998). Groups administered E2 and other substances except F11 did increase in LDL-cholesterol levels compared to those before administrations ($P<0.01$). Thus F1 and 1-monolinolein did not affect cholesterol levels in this study.

Trabecular bone area (TBA) is a good indicator of changes in bone formation and resorption. The tibia TBA of the sham, E2, F12 and F110 groups increased more than the OVX control group ($P<0.01$), but the F11, M2, and M10 groups did not show differences statistical significantly. The 6th lumbar vertebra was chosen as the control for tibia TBA because the weight load of the bone is smaller, but there was a significant difference in TBA due to ovariectomy ($P<0.01$, Fig. 5). The groups receiving 1-monolinolein showed no increase in tibia and lumbar TBA (Fig. 5). However, lumbar TBA of the sham, E2, and F11 groups increased more than the OVX control group ($P<0.01$).

In the animal study, the OVX control group gained weight drastically after the operation, while the sham group gained weight relatively slowly (Fig. 3). The groups administered E2 gained weight in a pattern more similar to the sham group than to the OVX group. The F11, F12 and F110 groups and the M2 and M10 groups showed weight gain similar to the OVX group. The uterus of the OVX group atrophied more than those of the sham group due to estrogen deficiency ($P<0.01$), while the uterus of the E2 group hypertrophied as a side effect of estrogen as indicated by the increase in uterus weight (Fig. 4). However, the F11, F12, F110, M2, and M10 groups showed no weight gain of uteri similar to the OVX group (Fig. 4), suggesting that F1 and 1-monolinolein may become a candidate of SERMs.

F1 increased proliferation of osteoblasts (Table I) and inhibited the activity of osteoclasts (Fig. 2). When compared with the *in vitro* results of the E2 group, F1 seems promising as an estrogen replacement. In addition, during the *in vivo* experiment, herbal extract F1 did not cause side effects such as uterus hypertrophy. Among the F1 groups, the F12 and F110 groups show changes in tibia TBA similar to the E2 group. However, it may not be ruled out that the efficacy of F1 may be expressed at a dosage smaller than 1 mg/kg/day. For this reason, further studies need to be conducted to determine absorption behavior and stability characteristics of F1.

1-monolinolein, a major constituent of F1 isolated from the Astragali Radix in the study, showed a trend of increase in ALP activity in osteoblast cultures, however, was not effective in inhibition of osteoclast (Fig. 2). In animal studies, bone volume fractions of M2 and M10 groups were not significantly different from that of the OVX control. It may be that a sufficient amount was not used during the *in vivo* experiments or that because it is a very unstable substance, it may have been hindered from the system circulation. Another possibility is that F1 contains not only 1-monolinolein but also other active compound. 1-Monolinolein can be suspended as a stable oil-in-water (Kobayashi *et al.*, 1983), however the flavonoid could not. Administration of 1-monolinolein could not prevent the bone loss, not like F1, because 1-monolinolein may not be the active compound in F1 and may function as a helper for drug delivery of other active compound like the flavonoid in F1 fraction (Kim *et al.*, 2001). Therefore, further studies are necessary to isolate and identify active compounds of F1 and to determine the detailed mechanism of its inhibitory effects on osteoporosis.

In conclusion F1 prevents tibia and lumbar bone loss after OVX without uterine hypertrophy although its main ingredient, 1-monolinolein did not. The active compound in F1 should be isolated and identified in future and further studies are in progress.

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