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Isoflavones Extracted from *Sophorae fructus* Upregulate IGF-I and TGF-β and Inhibit Osteoclastogenesis in Rat Born Marrow Cells

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Isoflavones have been a central subject in research on the natural phytoestrogens found in *Leguminosae*. Their effects on bone formation and remodeling are important in that they can act like estrogen by binding on estrogen receptors on the target cell surface. We, therefore, believed that isoflavones may help in the treatment of patients with estrogen deficiency disease such as estrogen replacement therapy (ERT) for osteoporosis. As commonly known, osteoporosis is one of the hormonal deficiency diseases, especially in menopausal women. When estrogen is no longer produced in the body a remarkable bone remodeling process occurs, and the associated events are regulated by growth factors in the osteoblast lineage. In the present study, we investigated whether isoflavones (Isocal) extracted from *Sophorae fructus* affect the growth factors IGF-I and TGF-β that have been known to be related with bone formation. In the study, we found that the active control (PIII) effectively enhanced the level of nitric oxide (NO) and growth factors, and thereby inhibited osteoclastogenesis. The most efficient concentration was 10^{-8}% within five days, whereas the comparative control (soybean isoflavone) was not as effective even at a lower concentration. In conclusion, the products which contain enriched glucosidic isoflavone and nutrient supplements such as shark cartilage and calcium can be used for osteoporosis therapy by enhancing the production of IGF-I and TGF-β. Furthermore, the NO produced through endothelial constitutive NO synthase (ecNOS) may play a role in inhibiting bone reabsorption.

Key words: Isoflavone, *Sophorae fructus*, Osteoclastogenesis, Growth factors, Nitric oxide

INTRODUCTION

Estrogen deficiency is known to lead to an increase in bone remodeling in which resorption outstrips formation and bone mass decreases. This can be observed not only in postmenopausal women, but also in men with defects either in the estrogen receptor (ER) or in the synthesis of estrogen from testosterone (Bilezikian *et al.*, 1998). This abnormality of bone remodeling may produce a variety of skeletal disorders, such as osteoporosis, glucocorticoid osteoporosis, hyperparathyroidism, hyperthyroidism, Paget's disease, inflammation, and immobilization (Raisz, 1997). With respect to osteopetrosis, various studies have focused on phytoestrogen, which has no typical side effects but which exhibits many estrogen-like functions in cell lines as well as primary cultures (Eastel, 1998). Isoflavones are biologically regarded as active compounds found in plants, particularly in soy and in many Leguminosae plants including *Sophorae fructus*. It is recognized that the principal isoflavones generally present are genistein, daidzein, and glycitein, and that, for the most part, these aglycones usually exist as various forms of glycosidic conjugates (Setchell, 1996; Duncan *et al.*, 2003), i.e. genistin, daidzin and glycitin. In the PDR (Physicians' Desk Reference) description, soy isoflavones are regarded as phytoestrogens, which are plant-derived nonsteroidal compounds that possess estrogen-like biological activity together with lignans and coumestans (Adlercreutz, 2002). They have been found to bind to estrogen receptors-α (ER-α) and -β (ER-β), although the bind is stronger to the latter. The three main isoflavones are aglycones and the most abundant isoflavones in soy are the genistein glycosides (about 50%), followed by the daidzein glycosides (about 40%), and glycitein glycosides (about 5-10%).

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Osteoclasts, the multinucleated giant cells that resorb bone, are developed from hematopoietic cells of the monocyte and macrophage lineages. In addition, osteoblasts/ bone marrow stromal cells play an important role in the development of osteoclasts through a mechanism of cell-cell interaction between osteoclast progenitors and osteoblasts. Those cells and release factors are under control in the presence of estrogen. According to many studies, estrogen deficiency is the key initiator for the pathogenesis of osteoporosis (Riggs *et al.*, 1998) and is the most important systemic hormone in loosing normal bone turnover (Pacifici, 1998). Osteoporosis is known to be by far the most common metabolic disorder of the skeleton (Raisz, 1997) and it is known that continual loss of bone mass is a natural process of aging in the elderly (Warren, 2002). Additionally, osteoporosis can be divided into several types, including type I for postmenopausal osteoporosis and type 2 for senile osteoporosis. Bone markers which are, for example, closely related with bone formation (IGF-I and TGF-β) enhance the production of growth factors in the presence of estrogen whereas the cytokines, such as IL-1β and IL-6, promote osteoclastogenesis. This concept has been a major subject in the study of growth factors relating to bone formation when they are normally controlled by estrogen. Particularly, TGF-β is known to inhibit osteoclast function and to promote osteoclast apoptosis, resulting in an overall decrease in bone reabsorption (Mundy, 1999). A large number of hormones, growth factors, and cytokines are known to regulate osteoclast formation and function (Aubin, 2000). In turn, if the release of local factors, such as IL-1β and IL-6, is inhibited, then bone loss may be prevented by inactivating immature osteoclasts. In addition, nitric oxide (NO) can play an important role in inhibiting bone reabsorption and formation (Kanamaru *et al.*, 2001; van't Hof *et al.*, 2001).

In the last decades, there has been a veritable explosion of interest in genistein, and numerous studies have focused on the mechanisms by which genistein functions as an anticarcinogen. In addition, genistein and daidzein may play a role in the prevention of osteoporosis (Yamaguchi and Gao, 1998) and cardiovascular disorders-conditions which frequently accompany menopause in women. Interestingly, genistein has structural similarity to 17β-estradiol, but binds to the estrogen receptor with lesser affinity than estrogen itself. This weak estrogen effect may help protect osteoporosis by preventing bone reabsorption and promoting bone density by overcoming the lack of real estradiol in menopausal women. Genistein has been found to have a number of antioxidant activities, and acts as a scavenger of reactive oxygen species (ROS). It also inhibits superoxide anion generation by the enzyme xanthine oxidase, and has been found to increase the activities of the antioxidant enzymes superoxide dismutase, glutathione peroxidase, catalase and glutathione reductase. Daidzein and glycitein also appear to have reactive oxygen scavenging activity, but they have not been studied as much as genistein has. Postmenopausal women are the most vulnerable to osteoporosis due to the decline of estrogen production, which is known as a major contributing factor of osteoporosis (Amonkar, 2002). To ameliorate the loss of estrogen at menopause and thereby address the concerns regarding osteoporosis, hormone replacement therapy (HRT) is being used. Today, however, dietary alternatives to HRT, such as phytoestrogens, are typical choices for bone health (Messina, 2000). From a practical point of view, Asian women, who consume greater quantities of soy isoflavones, have a lower number of fractures than Western women do, indicating that an increased intake of isoflavone may protect individuals from osteoporosis (Duncan, 2003).

On the other hand, osteoclasts, the primary cell type responsible for bone reabsorption, are regulated by hormones, cytokines, ions, and arachidonic acid metabolites. These include glucocorticoids, vitamin D, IL-1β, IL-6, TGF-β, IGF-I, and prostaglandins for indirect stimulator (Greenfield, 1999; Swolin-Eide, 1998). Contrarily, osteoclast differentiation factor or osteoprotegerin ligand (ODF/OPGL) directly stimulates osteoclast activity through interactions with an osteoclast cell surface member of the TNF receptor superfamily known as RANK, receptor activator of NF-κB (Udagawa *et al.*, 1999; Nakagawa *et al.*, 1998). In the bone marrow, growth factors such as IGF-I and TGF-β that are produced by osteoblasts are incorporated into the bone matrix (Baylink *et al.*, 1993). Inhibitory effects on bone-resorbing cytokines and prostaglandins can decrease bone resorption, whereas stimulation of TGF-β can both decrease resorption and increase formation. Increased IGF-I could stimulate bone formation. Because the inhibition of bone resorption appears to dominate, the overall effect of estrogen is to decrease bone turnover. Bone mass increases either because the resorption space is filled in or because the indirect effects on growth factors provide for greater bone formation at local sites (Raisz, 1997).

In the present study, we wanted to investigate the effect of isoflavone complex extracted from *Sophorae fructus*, which includes genistein, and other nutrients such as shark cartilages and calcium. For the study, we hypothesized that the studied product enhance the production of growth factors, IGF-I & TGF-β, that regulate the development and function of osteoclasts and osteoblasts (Lian, *et al.*, 1999) and that are known to be the principal growth factors involving in bone remodeling. IGF-I & TGF-β present in high concentrations within the bone matrix and stimulate osteoblastic replication and enhanced bone collagen and matrix synthesis by osteoblasts (Centrella *et al.*, 1991).

Therefore, we wanted to determine whether substitutes for estrogen can induce the production of IGF-I and TGF-β, compared with control groups. Additionally, NO was detected at various concentrations for given time courses because it is known to inhibit bone resorption and formation at high levels (Aguirre *et al.*, 2001; van't Hof *et al.*, 2001). In the course of the final stage, we scrutinized the osteoclastogenesis of pre-osteoclasts using a characteristic marker of osteoclasts, tartrate-resident acidic phosphatase (TRAP) (Mundy, 1995).

MATERIALS AND METHODS

Test cell/animal

The study was performed using 10-week old Sprague Dawley (SD) rats and MG-63 osteoblast-like cell lines.

Test samples

Test samples, supplied by Rexgene Biotech, were provided at a state of end product of extracts from *Sophorae fructus*; i.e. product I (24.3%, glucosidic isoflavones; lot # 0301S2), product II (22%, aglycone isoflavones; lot # 0301S4), product III (Isocal, 47% of product I) composed of *Sophorae fructus* isoflavones and supplemented with shark cartilage and calcium, and SDB (soybean isoflavone for comparative control). All samples were dissolved in 10% FBS DMEM culture media and stored at -20°C. The concentrations for study ranged from 10^{-12}% to 10^{-4}% as appropriate for the study interests.

Reagent and assay kits

FBS (Gibco, U.S.A.), Griess Reagent (Promega, U.S.A.), HEPES (Duchefa Biochem, Netherlands), L-glutamine (Gibco, U.S.A.), Penicillin-Streptomycin (Gibco, U.S.A.), MTT reagent (Sigma Chem., U.S.A.), human IGF-I & TGF-β immunoassay kits (R&D Systems, U.S.A.), trypsin (Sigma Chem., U.S.A.), TRAP staining kit (Sigma Chem., U.S.A.) and RT & PCR premix (Bioneer, Korea) were used.

Cell culture

An osteoblast-like cell line, MG-63, was purchased from the Korean Cell Line Bank, Seoul. MG-63 was thawed in a warm water bath (37°C) for 1 min, centrifuged for 5 min. at 1250 rpm, and then suspended with 10% FBS DMEM. Cells were cultured in a 25 cm^2 culture flask at a density of 1×10^7.

Primary culture

For the study of osteoclastogenesis, we cultured bone marrow cells from femurs of 10-week old SD rats. Briefly, osteoclast cultures were prepared by excising femurs, following animal sacrifice. The excised femurs were passed though wash media (15% FBS α-MEM) several times and the final wash was performed in 30-40 mL of osteoclast medium (15% FBS α-MEM+0.28 mM L-ascorbic acid 2-phosphate and 10 mM β-Glycerophosphate). We then removed the femur's epiphyses, flushed out the marrow with 10 mL of osteoclast medium from both ends of the shaft using a 25 gauge syringe and a well mixed cell suspension, and inoculate 0.6 mL onto culture wells or 0.8 mL onto test slides. The conditions of incubation were 37°C, 5% CO_2 and 100% humidity. Simultaneously, we prepared osteoblast cultures in a similar way to osteoclast cultures except for the culture medium and some modified procedures. Briefly, the obtained cell suspension from the marrow was sieved through a 100 μm cell strainer into a conical tube and centrifuged for 10 min. at 1000 rpm. Cells were resuspended in primary culture medium (15% FBS α-MEM+0.28 mM L-ascorbic acid 2-phosphate and 10 nM Dexamethasone). We then inoculated 75 cm^2 culture flasks with 5 mL of cells and an additional 15 mL of primary culture medium, and incubated them at 37°C, 5% CO_2 and 100% humidity. Culture media were changed with 15 mL of primary culture medium per flask on days 2 and 4, and the trypsinized cells were counted on day 6. Inoculation was performed appropriately in osteoblast medium (15% FBS α-MEM+0.28 mM L-ascorbic acid 2-phosphate and 10 nM Dexamethasone without Penicillin G, Gentamycin and Amphotericin B). Media were changed every other day and cells were grown for 2 to 21 days, depending on the study design.

Osteoclastic differentiation

When ready to use for the study of osteoclastogenesis, we added 50 ng/mL of M-CSF to cell cultures for regulating osteoclastic differentiation and cultured for five days in 10% FBS α-MEM media. The degree of differentiation was evaluated by counting the TRAP positive cells.

NO assay

NO was detected according to the manufacturer's recommendation. In brief, we prepared a nitrite standard solution ranged from 1.56 μM to 100 μM for a standard reference curve. For the nitrite measurement (Griess reaction), we added 50 μL of each experimental sample to wells in triplicate, and then dispensed 50 μL of the sulfanilamide solution to all experimental samples and wells containing the dilution series for the nitrite standard reference curve. We then incubated for 10 min. at room temperature, protected from light, and dispensed 50 μL of the NED (*N*-1-napthylethylenediamine dihydrochloride) solution to all wells. After the incubation for 10 min. at room temperature while protected from light, we measured absorbance within 30 min. in a plate reader with a 540 nm filter.

RT-PCR (Reverse Transcriptase Polymerase Chain Reaction)

Total RNA was purified from MG-63 human osteoblast-like cells using Trizol reagent. cDNA was produced from sense and antisense primers and reverse transcriptase (Bioneer, Korea). The corresponding cDNAs were amplified in PCR reactions (Joo *et al.*, 2002) using the primers that correspond to the sequences.

Cytokines	Primer	Amino acid sequence
TGF-β (240bp)	5' Primer	5'-CGC CCT GTT CGC TCT GGG TAT-3'
	3' Primer	5'-AGG AGG TCC GCA TGC TCA CAG-3'
IGF-I (176 bp)	5' Primer	5'-ATG CTC TTC AGT TCG TGT GT-3'
	3' Primer	5'-AGC TGA CTT GGC AGG CTT GT-3'
ecNOS (346bp)	5' Primer	5'-AAG CCG CAT ACG CAC CCA GAG-3'
	3' Primer	5'-TGG GGT ACC GCT GCT GGG AGG-3'

PCR reaction products were analyzed by agarose gel electrophoresis, visualized by ethidium bromide staining, and quantified by measuring the cDNA density in gel slices using UVIDocMw program. The relative percentage was calculated from the sum of total density, and therefore the relative percentage represents the proportion of each cDNA targeted.

ELISA

Growth hormones released to culture media were measured by immunoassay kits for human IGF-I and human TGF-β (Quantikine®, R&D Systems), which were 3.5-h solid-phase ELISA designed to measure each growth hormone in cell culture supernates. The assay was performed in accordance with the manufacturer's protocol. We determined the optical density of each well within 30 min. at 450 nm. All absorbance data were converted to actual concentrations, i.e. ng/mL for IGF-I and pg/mL for TGF-β.

TRAP staining

For TRAP test, we prepared an acid phosphatase kit from Sigma Diagnostics and followed the manufacturer's protocol. In this method, TRAP positive cells are mostly observed in huge blue nuclei clumps.

RESULTS

In NO assay, cell response was maximum in the highest concentration (10^{-4}%) of product III, SDB and 17β estradiol, compared with the control group. Of the lower concentrations (10^{-6}%-10^{-12}%), 10^{-8}% showed the highest response, whereas SDB was almost equal to the control group, culture media (Fig. 1). These data suggested that human osteoblastic cells released NO and that the amount of NO detectable in cell supernates varied according to the concentration of each sample. In particular, PIII maintained an upper level from 10^{-6} to 10^{-12}%, compared with SDB (Fig. 1). On the other hand, after PIII, 17β estradiol unexpectedly maintained the second highest level of NO only at 10^{-6} and 10^{-8}%. Although this is somewhat weak evidence to support the hypothesis that osteoblast NO synthesis/activity is augmented by osteogenic hormones such as estrogen (Wimalawansa *et al.*, 1996) and is closely related with bone physiology (Klein-Nulend, 1998), significant differences between PIII and the control, SDB, indicate that NO may indeed play a certain role in bone cell activity, as shown in Fig. 5. A separate study, shown in Fig. 2, produced the interesting result of 17β estradiol promoting cell viability at nearly twice the levels shown in

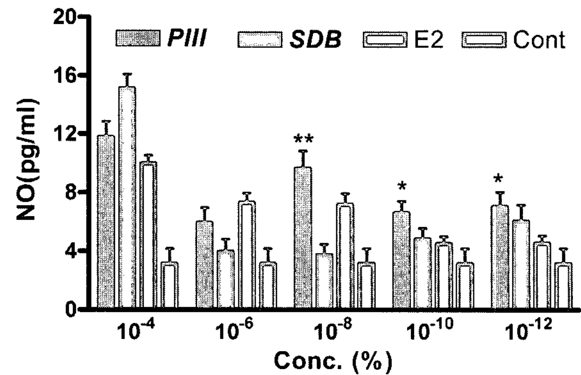

Fig. 1. Analysis of NO at various sample concentrations compared with the product. To determine the most effective experimental dose *in vitro* for 72 h, concentrations ranged from 10^{-4}% to 10^{-12}%. The cultured cells were MG-63 osteoblast-like cells and the cell number was uniformed at a density of 1×10^{-4} before adding samples. PIII, Isocal (active control); SDB, soybean isoflavone (comparative control); E2, 17β estradiol; Cont, culture media (*PIII vs. SDB, t-test, triplicates).

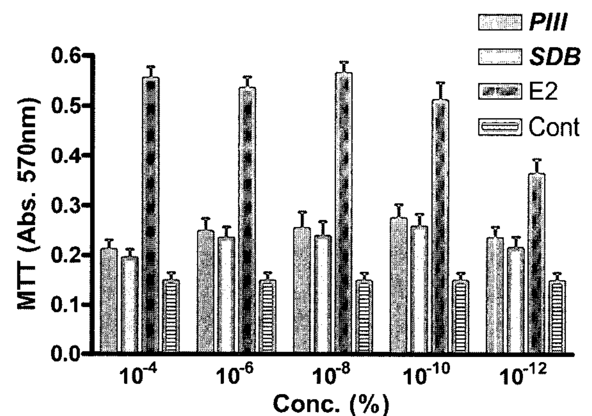

Fig. 2. MTT assay. Cell viability and proliferation were assured by MTT assay and a higher level of absorbance is proportional to the activities of live mitochondria in cytosol. Control was added for references. PIII, Isocal (active control); SDB, soybean isoflavone (comparative control); E2, 17β estradiol; Cont, culture media (No significant statistical difference was found between PIII and SDB groups).

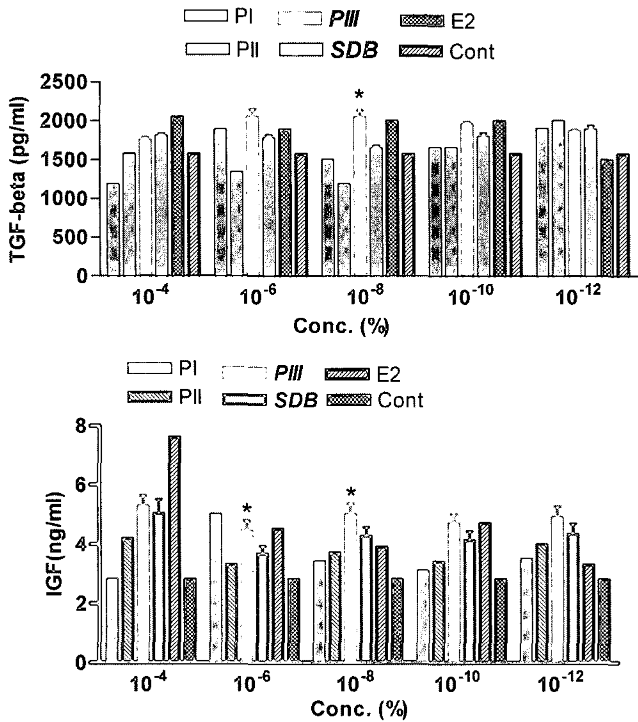

Fig. 3. Cytokine expression (IGF-I & TGF-β). Cells (MG-63) were cultured for three days and measurements were taken as described in materials and method. Cells were cultured at a density of 1×10^{-4} before samples were added. PI, glucoside; PII, aglycone; PIII, Isocal (active control); SDB, soybean isoflavone (comparative control); E2, estradiol; Cont, culture media (*PIII vs. SDB, p<0.05, triplicates).

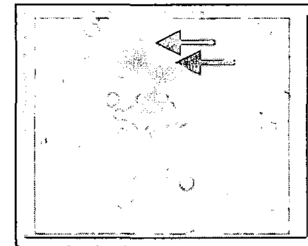

Fig. 4. Osteoclasts differentiated from bone marrow cell culture (TRAP staining). Arrows indicate osteoclasts which are differentiated under the experimental conditions *in vitro* (×200).

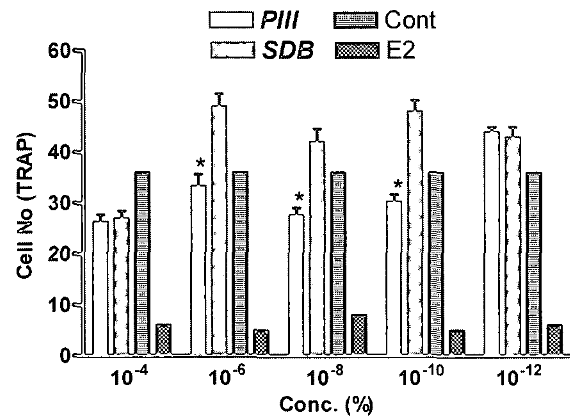

Fig. 5. Cell counts from TRAP staining. Cells were cultured for five days in combination with M-CSF and osteoblast cells in all culture wells. PIII, Isocal (active control); SDB, soybean isoflavone (comparative control); E2, 17β-estradiol; Cont, control (culture medium) (*PIII vs. SDB, p<0.05, triplicates).

the other groups.

The principal growth factors, IGF-I and TGF-β, were investigated in RT-PCR and ELISA assay. As described earlier, IGF-I and TGF-β are factors regulating osteoblast formation, differentiation and matrix formation, which play an important role in bone formation and reabsorption. For RT-PCR, we tried to avoid any undesirable result by maintaining constant experimental conditions that are required for the process of RT or PCR. In PCR reactions, we found that PIII enhanced the production of IGF-I and TGF-β from MG-63 cells, and that at a concentration of 10^{-8}% the enhancement was maintained compared with that at other concentrations (Fig. 6). These results were compared with those from ELISA assay (Fig. 3), which indicated that PIII, at least, was as effective as estradiol in the regulation of IGF-I and TGF-β. Fig. 5 shows the level

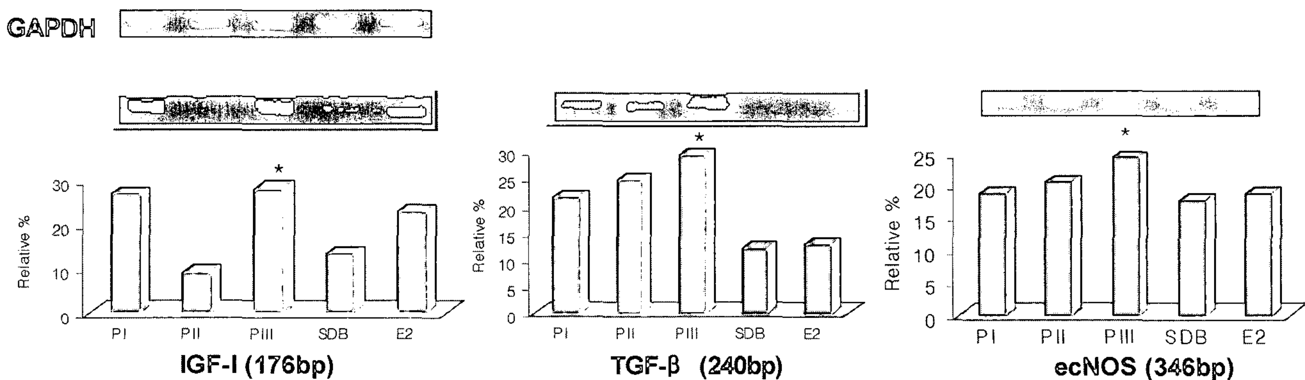

Fig. 6. mRNA expression of growth factors, IGF-I, TFG-β and ecNOS. Cells were cultured for three days at a concentration of 10^{-8}% in each group (*Prod vs. SDB, p<0.05).

of osteoclastogenesis in each group showing TRAP positive osteoclasts. 17β estradiol remarkably controlled osteoclastogenesis, whereas PIII and SDB maintained TRAP positive counts five to ten-fold greater than the E2 group. In this assessment, we found that the PIII group was not a significant suppressor or promoter of osteoclastogenesis when compared to SDB and the control group. There were no critical differences across the concentration range, but overall 10^{-8}% seemed to be the most applicable concentration.

CONCLUSION

In the present study, we found that the product, *Sophorae fructus* isoflavones, tended to stimulate NO, at a pico gram level, at a lower percentage concentration (10^{-8}%) than SDB, which showed the highest value at a concentration of 10^{-4}% (Fig. 1). This suggests that an appropriate level of NO may play a positive role in inhibiting bone resorption rather than in leading to cell death through its biphasic effect (Brandi *et al.*, 1995). Regardless of the low concentration of NO in the estradiol group, differentiation of osteoclasts was greatly inhibited, to a level less than one fourth that of PIII and SDB (Fig. 5). Notwithstanding, it was interesting that PIII stimulated ecNOS and the synthesis of NO and mRNA levels in cultured osteoblast-like cells in the same manner that has been reported in previous studies using estrogens (Armour, 1998). We further found that the production of the growth factors, IGF-I and TGF-β, was enhanced in comparison with 17β estradiol- and SDB-treated groups, in a certain time frame (72 h). In both RT-PCR and ELISA assay, we found equivalent results in gene and protein levels (Fig. 6). From the two tests methods, we confirmed that the most effective concentration for the product was 10^{-8}%, but no particular dose dependency was established. In the present study, IGF-I production was relatively well enhanced over the concentration range of 10^{-6}% to 10^{-8}%, whereas TGF-β production was more broadly enhanced up to a concentration of 10^{-10}%, when treating with PIII compared with SDB and the other derivatives (glucosidic and aglycone type). However, production of those growth factors was maintained at upper levels when compared to control and the SDB group, although they remained underneath the estradiol group (Figs. 3 and 4).

From both methods, we can conclude that the most effective concentration for the product was from 10^{-6}% to 10^{-8}%, and that there was no significant difference in concentration variation and no particular dose dependency. In PIII, we also found that the level of IGF-I and TGF-β was slightly higher than that of 17β estradiol at concentrations close to 10^{-8}%. In MTT assay, the E2 group showed the most effective result in cell viability, being almost three-fold higher than that of the other groups (Fig. 2). On the other hand, PIII and SDB maintained higher absorbance levels than that of the control group, indicating that isoflavone promotes osteoblastic proliferation *in vitro*. Finally, we found that the product inhibited osteoclastogenesis, although not to the extent of the E2 group (Fig. 5).

In conclusion, this study demonstrated that the product which contains enriched glucosidic isoflavone and nutrient supplements such as shark cartilage and calcium can be used for the treatment of osteoporosis based on its role in enhancing the production of growth factors, IGF-I and TGF-β. Furthermore, NO produced through ecNOS may play a role in inhibiting bone reabsorption and supporting evidence for this is presented in Fig. 5, in which cells were allowed to differentiate to osteoclasts (Fig. 4). These results indicate that isoflavones and shark cartilage and calcium are a synergic effector for bone formation. The present study may be a key initiation study for future investigation into *Sophorae fructus* isoflavones as our findings should arouse increased interest in glycosidic isoflavones *in vitro* and bone related nutrients for both bone remodeling and bone formation. As described in the materials and method, PIII is not a highly purified pharmacological drug but it nevertheless includes glucosidic-enriched isoflavones as an active ingredient. The data obtained suggest that PIII stimulates ecNOS, IGF-I and TGF-β, suppresses osteoclastogenesis, although this was not statistically proved when compared to the control group, and is not cytotoxic. Therefore, in spite of the limitations of its use for first choice ERT therapy, the results from this study are valuable as they confirm that, as a minimum, PIII can be used in the treatment of osteoporosis in patients, such as the elderly, who are deficient in estrogen levels after menopause and who need estrogen supplements.

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