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Inhibition of IL-1β and IL-6 in Osteoblast-Like Cell by Isoflavones Extracted from *Sophorae fructus*

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Osteoporosis is recognized as one of the major hormonal deficiency diseases, especially in menopausal women and the elderly. When estrogen is reduced in the body, local factors such as IL-1β and IL-6, which are known to be related with bone resorption, are increased and promote osteoclastogenesis, which is responsible for bone resorption. In the present study, we investigated whether glucosidic isoflavones (Isocal, PIII) extracted from *Sophorae fructus* affect the proliferation of osteoblasts and prevent osteoclastogenesis *in vitro* by attenuating upstream cytokines such as IL-1β and IL-6 in a human osteoblastic cell line (MG-63) and in a primary osteoblastic culture from SD rat femurs. Interestingly, IL-1β and IL-6 mRNA were significantly suppressed in osteoblast-like cells treated with 17β-estradiol (E2) and PIII when compared to positive control (SDB), and this suppression was more effective at 10^{-8}% than at the highest concentration of 10^{-4}%. In addition, these were confirmed in protein levels using ELISA assay. In the cell line, the cells showed that E2 was the most effective in osteoblastic proliferation over the whole range of concentration (10^{-4}%-10^{-12}%), even though PIII also showed the second greatest effectiveness at 10^{-8}%. Nitric oxide (NO) was significantly ($p<0.05$) upregulated in PIII and E2 over the concentration range 10^{-6}% to 10^{-8}% when compared to SDB, without showing any dose dependency. In bone marrow primary culture, we found by TRAP assay that PIII effectively suppressed osteoclastogenesis next to E2 in comparison with SDB and culture media (control). In conclusion, these results suggest that local bone-resorbing cytokines can be regulated by PIII at lower concentrations and that, therefore, PIII may preferentially induce anti-osteoporosis response by attenuating osteoclastic differentiation and by upregulating NO.

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

INTRODUCTION

Isoflavones are biologically active compounds found in plants, particularly in soy and in many Leguminosae plants including *Sophorae fructus*, and are known as one of the natural phytoestrogens whose effect on bone remodeling is an important research interest in terms of estrogen replacement therapy (ERT). It has been recognized that the principal isoflavones present in those plants are genistein, daidzein, and glycitein, and that, for the most part, these aglycones usually exist as various forms of glycosidic conjugates (Setchell, 1996), 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 better to the latter than the former. 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%) (Table I)

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.

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Table I. Summary of major soy isoflavones*

Aglycones[1]	Structure	Molecular ormula	Glucosidic forms[2]
Genistein	R_1-OH, R_2-H R_3-OH, R_4-OH	$C_{15}H_{10}O_5$	Genistin
Daidzein	R_1-H, R_2-H R_3-OH, R_4-OH	$C_{15}H_{10}O_4$	Daidzin

[1] the principal isoflavones present in soybeans and soyfoods.
[2] for the most part, aglycones usually exist in soy as various forms of glycosidic conjugates.
*there are 12 isoflavone isomers: 3 aglycones (genistein, daidzein, glycitein) and 9 glucosides (genistin, daidzin glycitin, acetlygenistin, acetyldaidzein, acetylglycitin, malonylgenistin, malonyldaidzin, and malonylglycitin).

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 resorption and promoting bone density by compensating for 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. Post-menopausal 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 larger quantities of soy isoflavones, have a lower number of fractures than Western women do (Duncan, 2003), indicating that an increased intake of isoflavone may protect individuals from osteoporosis.

Osteoclasts, highly specialized multinucleated cells capable of bone resorption (Fujikawa *et al.*, 2001) can be 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). In addition, macrophage-colony stimulating factor (M-CSF), one of a family of growth factors for cells of the mononuclear phagocyte system (Stanley and Heard, 1977), has been shown to play a key role in the process of osteoclast formation and bone-resorption (Felix *et al.*, 1990). In conjunction with M-CSF, 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 many reports, the importance of cytokines on osteoclast formation has been investigated and the results of those studies have shown that IL-1β and TNF-α promote osteoclastic bone-resorbing activity and osteoclast formation (Lader and Flanagan, 1998) and that these effects are mediated through the osteoblasts (Fujikawa *et al.*, 2001). From the finding that IL-6 can influence the formation of osteoclasts in bone marrow cultures (Tamura *et al.*, 1993), we came to believe that a cellular interaction between osteoblastic cells and osteoclastic progenitors is necessary for IL-6 to stimulate the formation of osteoclasts in the bone. Moreover, in most physiological systems, IL-1β, IL-6 and TNF-α function together in networks in which the stimulators often act synergistically (Ragab *et al.*, 2002). Recently, it was proposed that submaximal levels of IL-1 and IL-6 act cooperatively to stimulate osteoclast differentiation (Tai *et al.*, 1997).

In the present study, we wanted to investigate whether glucosidic isoflavone complex extracted from *Sophorae fructus*, which includes genistein, daidzein, and other nutrients such as shark cartilages and calcium. For the study, we hypothesized that this product attenuates the production of IL-1β and IL-6 in osteoblasts that decrease osteoclastogenesis. Along with this hypothesis, we tried to compare the level of nitric oxide (NO) release at various concentrations for a given incubation time course because NO is known to inhibit bone resorption and formation at high level (vant Hof *et al.*, 2001).

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 IL-1β & IL-6 immunoassay kits (Titerzyme", Assay Designs, 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 mins 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 10 μm cell strainer into a conical tube and centrifuged for 10 mins 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 manufacturers 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 minutes 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 minutes at room temperature while protected from light, we measured absorbance within 30 minutes 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 TRI 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
IL-1β (290 bp)	*5"Primer*	*5'ACT ACA GCA AGG GCT TCA GG--3'*
	3' Primer	*5'-CAT ATC CTG TCC CTG GAG GT-3'*
IL-6 (546 bp)	*5' Primer*	*5'-ATG AAC TCC TTC TCC ACA AG-3'*
	3' Primer	*5'-GTG CCT GCA GCT TCG TCA GCA-3'*
ecNOS (346 bp)	*5' Primer*	*5'-AAG CCG CAT ACG CAC CCA GAG-3'*
	3' Primer	*5'-TGG GGT ACC GCT GCT GGG AGG-3'*
GAPDH (576 bp)	*5' Primer*	*5'-CCA TCA CCA TCT TCC AGG AG-3'*
	3' Primer	*5'-CCT GCT TCA CCA CCT TCT TG-3'*

PCR reaction products were analyzed by 1.8% 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 IL-1β and human IL-6 (TiterZyme®, Assay Designs), which were 3.5-h solid-phase ELISA designed to measure each cytokine in cell culture supernates. The assay was performed in accordance with the manufacturers protocol. We determined the optical density of each well within 30 minutes at 450 nm. All absorbance data were converted to actual concentrations, i.e. pg/mL for both IL-1β and IL-6.

TRAP staining

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

Statistical analysis

The data was analyzed as a means±SEM. Statistical significance was determined using a Students *t*-test. Significant differences were found by using SPSS software (ver. 10)

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. 2. A separate study as shown in the same figure showed the interesting result of 17β estradiol promoting cell viability at nearly twice the levels shown in the other groups.

Local bone-resorbing cytokines, IL-1β and IL-6, were investigated in RT-PCR and ELISA assay. As aforementioned, IL-1β and IL-6 play an important role in osteoclast formation. In ELISA assay, we found that PIII attenuated the production of IL-1β and IL-6 from MG-63 cells, and

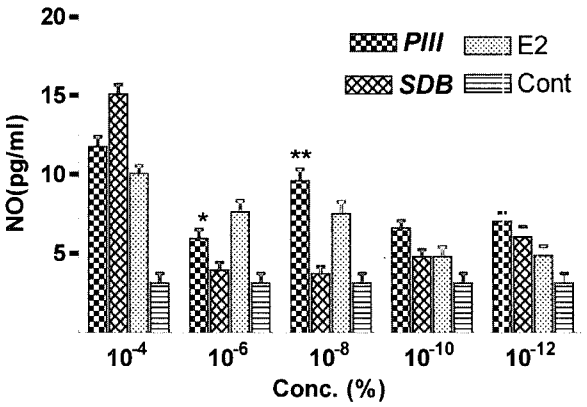

Fig. 1. Analysis of NO at various sample concentrations compared with the product. To determine the most effective experimental dose *in vitro* for 72 h, concentrations ranged from 10^{-4}% to 10^{-12}%. The cultured cells were MG-63 osteoblast-like cells and the cell number was uniformed at a density 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, $p<0.05$; **$p<0.01$, *t*-test, triplicates).

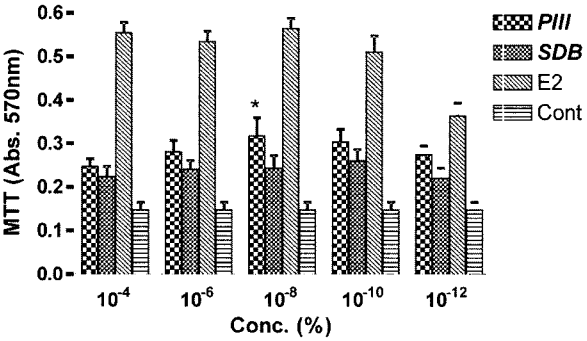

Fig. 2. MTT assay. Cell viability and proliferation were assured by MTT assay and a higher level of absorbance is proportional to the activities of live mitochondria in cytosol. Control was added for references. PIII, Isocal (active control); SDB, soybean isoflavone (comparative control); E2, 17β estradiol; Cont, culture media (*PIII vs. SDB, $p<0.05$, t-test, triplicates).

that at concentrations of 10^{-6}% and 10^{-8}% for IL-1β, and at 10^{-8}% for IL-6, significant attenuations ($p<0.05$) were detected compared with SDB and Control (Fig. 3). These results were compared with those from PCR reaction (Fig. 4C), which indicated that PIII, at least, was as effective as estradiol in the action on IL-1β and IL-6. Figs. 4A and 4B show the level of osteoclastogenesis in each group showing TRAP positive osteoclasts. 17β estradiol remarkably controlled osteoclastogenesis, whereas PIII and SDB maintained TRAP positive counts at a level five to ten-fold greater than the E2 group. There were no critical differences across the concentration range, but the most applicable concentrations seemed to be 10^{-8}% and 10^{-10}%. At a concentration of 10^{-8}%, TRAP and RT-PCR results showed that PIII well inhibited osteoclastogenesis, as well as the

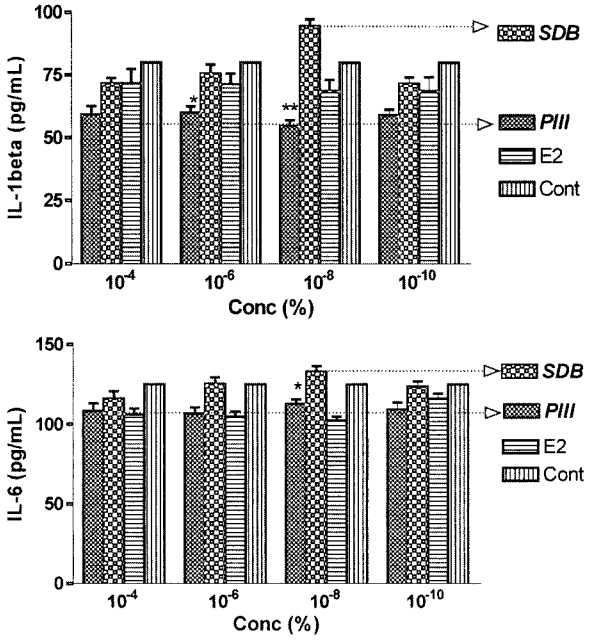

Fig. 3. Cytokine expression (IL-1β and IL-6). Cells (MG-63) were cultured for three days and measurements were performed as described in materials and method. The cell number was uniformed at a density of 1×10^{-4} before adding samples. PI, glucoside; PII, aglycone; PIII, Isocal (active control); SDB, soybean isoflavone (comparative control); E2, estradiol; Cont, culture media. (PIII vs. SDB, *p<0.05; **p<0.01, *t*-test, triplicates).

expression of IL-1β and IL-6 mRNA, compared with PI, PII, and SDB (Fig. 4B and C). Interestingly, ecNOS mRNA was expressed at the highest level.

CONCLUSION

Osteoporosis is known to be by far the most common metabolic disorder of the skeleton (Raisz, 1997), and can be divided into several types such as type I for postmenopausal osteoporosis and type 2 for senile osteoporosis. 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). Bone markers, which are, for example, closely related with bone resorption (IL-1β and IL-6), inhibit the production of the cytokines in the presence of estrogen. Therefore, we can consider that there may be a complex interaction between estrogen and multiple local factors (Eastel, 1998). In turn, if local factors, IL-1β and IL-6, are inhibited, then bone loss may be prevented by inactivating immature osteoclasts. In addition, NO can play an important role in inhibiting bone resorption and formation (vant Hof *et al.*, 2001).

The results of this study show that PIII, isoflavones extracted from *Sophorae fructus*, can play an important role in inhibiting osteoclastogenesis by downregulating IL-

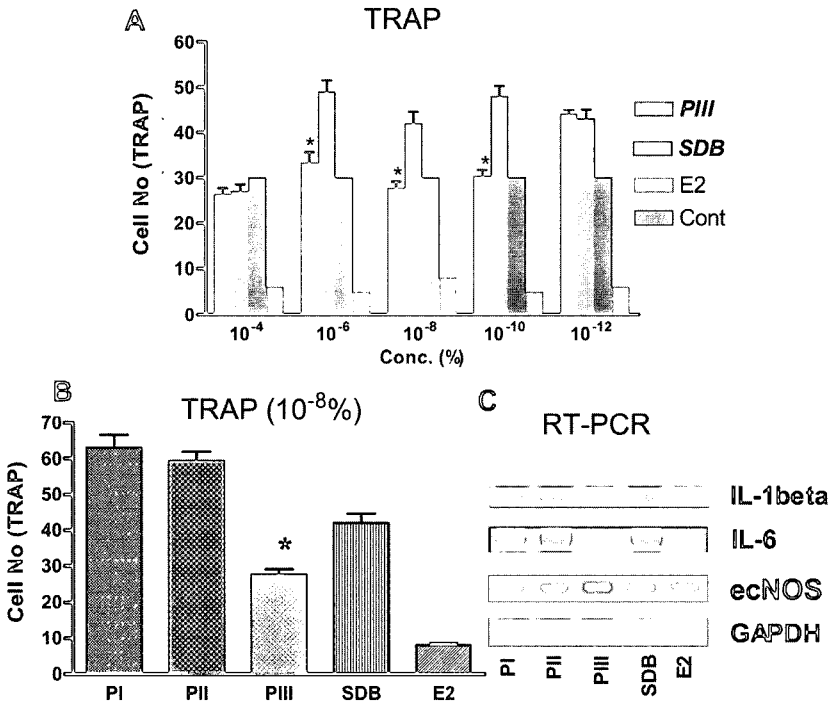

Fig. 4. Osteoclastic differentiation of rat bone marrow cells, after five-day incubation, in response to increasing concentrations in the presence of M-CSF (50 ng/mL) (A). (B) Parallel comparison of each group at the same concentration (10^{-8}%). (C) Expression of cytokines and ecNOS at 10^{-8}% in MG-63 cells. PI, glucosidic isoflavones; PII, aglycone isoflavones; PIII, Isocal (active control); SDB, soybean isoflavone (comparative control); E2, 17β-estradiol; Cont, control (culture medium). (*PIII vs. SDB, p<0.05)

1β and IL-6 and upregulating NO in osteoblast-like cells, when compared to SDB which has already been reported to have anti-osteoporosis effect. Recently, it has been reported that NO produced by osteoblasts inhibits the function of mature osteoclastic cells (MacIntyre *et al.*, 1991). This suggests that an appropriate level of NO may play a positive role in inhibiting bone reabsorption rather than in leading to cell death through its biphasic effect. 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. 4). 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 local bone-resorbing cytokines, IL-1β and IL-6, was inhibited in comparison with 17β estradiol- and SDB-treated groups, in a certain time frame (72 h) (Fig. 3). In both RT-PCR and ELISA assay, we found equivalent results in gene and protein levels (Figs. 1, 3 and 4). 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, we found that IL-1β was well regulated when treating with PIII compared with SDB and controls (Fig. 3). Contrary to expectations, IL-6 was maintained at a slightly higher concentration than other controls, whereas SDB maintained the highest levels. No clear evidence has yet been published confirming whether or not IL-6 promotes bone formation, but some studies have indicated that IL-6 can act as a promoter of bone formation by a mitogenic effect on osteoblasts (Swolin-Eide and Ohlsson, 1998). However, according to our present study, it is possible to consider that IL-6 may be a multifunctional cytokine in both bone resorption and formation. In MTT assay, the E2 group showed the most effective result in cell proliferation, being almost three-fold higher than that of the other groups (Fig. 2). On the other hand, the product and comparative control maintained their absorbance at a level higher than that of the control group, indicating that isoflavone promotes osteoblastic proliferation *in vitro*, even though there were no particular features in the product compared with SDB. Finally, we found that PIII inhibited osteoclastogenesis, although not to the extent of the E2 group (Fig. 4).

In conclusion, this study demonstrated that PIII, 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 inhibiting the production of local factors, IL-1β and IL-6. NO produced through ecNOS may play a role in inhibiting bone resorption and supporting evidence for this is presented in Fig. 4, in which cells were allowed to differentiate to osteoclasts.

Finally, it is clear from these studies that IL-1β, IL-6, and NO exert an interesting physiological tonic restraint on osteoclastic bone resorption and that PIII can play a certain role in regulating such local factors and NO, and possibly in preventing osteoporosis in menopausal women and the elderly.

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