

Genistein modulates the effects of parathyroid hormone in human osteoblastic SaOS-2 cells

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Genistein and parathyroid hormone (PTH) are anabolic agents that stimulate bone formation through their direct actions in osteoblastic cells. In the present study, we aimed to determine whether genistein modulates the actions of PTH in human osteoblastic SaOS-2 cells in an oestrogen-depleted condition. The present results showed that genistein (10^{-8} to 10^{-6} M) induced alkaline phosphatase (ALP) activity and osteoprotegerin (OPG) expression in SaOS-2 cells in a dose-dependent manner. These effects could be completely abolished by co-treatment with oestrogen antagonist ICI 182780 (7 α -[9-[(4,4,5,5,5-pentafluoropentyl)sulfonyl]nonyl]-estra-1,3,5(10)-triene-3,17 β -diol). Genistein (at 1 μ M) could stimulate the mRNA expression of receptor activator of NF- κ B ligand (RANKL). As OPG and RANKL are known to modulate osteoclastogenesis, the ability of genistein to modulate OPG and RANKL expression in SaOS-2 cells suggested that it might modulate osteoclastogenesis through its direct actions on osteoblastic cells. PTH (at 10 nM) stimulated ALP activity, induced RANKL mRNA expression and suppressed OPG mRNA expression in SaOS-2 cells, confirming its bi-directional effects on osteoblastic cells. Pre-treatment of SaOS-2 cells with genistein and oestrogen not only enhanced PTH-induced ALP activity, but also attenuated PTH up regulation of RANKL mRNA expression and PTH down regulation of OPG mRNA expression. Taken together, the present study provides the first evidence that genistein could modulate the actions of PTH in human osteoblastic SaOS-2 cells in an oestrogen-depleted condition.

Genistein: Parathyroid hormone: Alkaline phosphatase: Osteoprotegerin: Receptor activator of nuclear factor- κ B ligand

In Asia, the incidence of osteoporosis-related fractures is low as compared with Western countries (Ross *et al.* 1991; Anonymous, 1994; Lauderale *et al.* 1997). High phyto-oestrogen intake has been considered to be one of the contributing factors for the reduced risk of fracture among Asian women (Goldwyn *et al.* 2000; Valachovicova *et al.* 2004). Clinical trials have confirmed that dietary supplementation with isoflavone-enriched soya protein may improve the bone mass of both peri- and postmenopausal women (Potter *et al.* 1998; Alekel *et al.* 2000). Animal studies have shown the positive effects of treatment with isoflavones such as genistein and daidzein on bone loss induced by oestrogen deficiency (Anderson *et al.* 1998; Mei *et al.* 2001; Greendale *et al.* 2002; Morabito *et al.* 2002). The cellular mechanisms by which genistein may prevent bone loss are not fully understood.

Genistein is considered to act via a classical oestrogen receptor (ER)-mediated pathway. Genistein may stimulate ER β expression (Anderson *et al.* 2000; Cusack *et al.* 2001) and down regulate IL-6 secretion in osteoblastic cells (Chen & Anderson, 2002). Studies have shown that genistein mimics oestrogen in stimulating the expression of osteoprote-

gerin (OPG), a member of the TNF receptor superfamily, in cultured bone cells (Yamagishi *et al.* 2001; Chen *et al.* 2003). The balance of OPG and the ligand for receptor activator of NF- κ B (RANKL), another peptide expressed on the surface of stromal osteoblasts, is critical in the bone resorption process. RANKL stimulates osteoclast differentiation through its receptor (receptor activator of NF- κ B; RANK) located on osteoclasts, whereas OPG acts as a decoy soluble receptor for RANKL and prevents its interaction with RANK (Bucay *et al.* 1998; Lacey *et al.* 1998; Kong *et al.* 1999). Thus OPG neutralises RANKL functions and prevents osteoclast differentiation, activity and survival. The RANKL–OPG system plays a crucial role in osteoclastogenesis and the balance of bone remodelling. It appears that the positive effects of genistein on bone might be, at least in part, mediated by the modulation of this cytokine system.

Parathyroid hormone (PTH) plays an important role in Ca homeostasis and in regulating the rate of bone turnover. Several studies support a possible relationship between PTH and accelerated bone loss following the menopause (Khosla *et al.* 1997; Masiukiewicz *et al.* 2000). In postmenopausal women,

Abbreviations: ALP, alkaline phosphatase; E₂, 17 β -oestradiol; ER, oestrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-[4, 5-dimethylthiazol 2-yl]-2, 5-diphenyltetrazolium bromide; OPG, osteoprotegerin; PTH, parathyroid hormone; RANK, receptor activator of NF- κ B; RANKL, receptor activator of NF- κ B ligand.

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serum PTH concentration and markers of bone resorption increase with advancing age (Khosla *et al.* 1997). Women receiving oestrogen therapy can prevent increases in PTH levels and reverse virtually all age-related increases in bone resorption (Khosla *et al.* 1997; Masiukiewicz *et al.* 2000). *In vivo* and *in vitro* studies have shown that PTH causes biphasic effects on bone. Intermittent administration of PTH increases bone mass, whereas continuous infusion of PTH reduces it (Dempster *et al.* 1993). The anabolic action of PTH is the result of its positive effect on osteoblast differentiation. The bone resorptive effect of PTH is currently believed to be mediated by cytokines such as RANKL, OPG, colony-stimulatory factor 1 and IL-6 (Greenfield *et al.* 1996; Onyia *et al.* 1997; Huang *et al.* 1998; Yao *et al.* 1998; Yasuda *et al.* 1998).

In vivo and *in vitro* studies have shown that oestrogen can modulate the action of PTH on osteoblast function in an oestrogen-deficient state. Oestrogen can attenuate the PTH-induced IL-6 production (Masiukiewicz *et al.* 2000) and PTH can stimulate osteoblast function in the presence of oestrogen pre-treatment (Nasu *et al.* 2000). Although oestrogen replacement therapy has been used to prevent or treat postmenopausal osteoporosis, the potential side effects, i.e. increased risk of endometrial, breast and ovarian cancers, have limited the long-term use of oestrogen replacement therapy (Deady, 2004). Thus, alternative approaches are needed for the prevention and treatment of osteoporosis. The study of the possible skeletal benefits of soyabean isoflavones will provide support for its use in prevention of osteoporosis.

The present study aims to characterise the effects of isoflavone genistein and/or PTH on human osteoblastic SaOS-2 cells in an oestrogen-depleted condition and to determine if genistein modulates the action of PTH on osteoblasts.

Materials and methods

Culture of human osteoblastic cell line (SaOS-2)

SaOS-2 cells (ATCC no. HTB-85) were cultured in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) (Invitrogen, Carlsbad, CA, USA) at a density of 1×10^4 cells/cm², with medium changes every 3 d. On the 6th day, cells were transferred to phenol red-free Dulbecco's Modified Eagle medium supplemented with 1% charcoal-stripped fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) for 24 h. Then cells were treated with or without 17β-oestradiol (E₂; Sigma, St Louis, MO, USA) (10^{-8} M) or genistein (10^{-8} to 10^{-6} M) (Sigma) for 24 or 48 h. A high but physiological concentration (10^{-8} M) of E₂ was used as a positive control, while the dietary achievable concentration range (10^{-8} to 10^{-6} M) of genistein was chosen for investigation in the study. For anti-oestrogen treatment, SaOS-2 cells were exposed to E₂ or genistein in the presence or absence of oestrogen antagonist ICI 162780 (7α-[9-[(4,4,5,5,5-pentafluoropentyl)sulfonyl]nonyl]-estra-1,3,5(10)-triene-3,17β-diol); 10^{-6} M; Tocris, Bristol, UK) for 24 or 48 h. For combined treatment, cells were pre-treated with or without E₂ (10^{-8} M) or genistein (10^{-8} to 10^{-6} M) for 24 h, followed by treatment with (1–84) human PTH (10^{-8} M; Sigma) for another 24 or 48 h.

Cell proliferative assays

For the growth study, SaOS-2 cells were seeded in ninety-six-well plates (3×10^3 cells/well) in phenol red-free Dulbecco's Modified Eagle medium supplemented with 1% charcoal-stripped fetal bovine serum for 1 d and then treated with E₂ (10^{-8} M), genistein (10^{-8} to 10^{-6} M) or PTH (10^{-8} M) for 48 h. For the combined treatment, cells were pre-treated with or without E₂ (10^{-8} M) or genistein (10^{-8} to 10^{-6} M) for 24 h, followed by treatment with (1–84) human PTH (10^{-8} M) for another 24 h. As an indirect measure of growth, the 3-[4, 5-dimethylthiazol 2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay was used as described previously (Denizot & Lang, 1986). Briefly the medium was removed and replaced with 100 µl MTT (5 mg/ml; Sigma) in PBS. The plates were incubated for 4 h at 37 °C, followed by the addition of 100 µl lysis buffer (0.04 M-HCl in propan-2-ol). The multi-well plates were shaken for 1 h at room temperature. The signals were detected by using a microplate reader at a wavelength of 595 nm.

Alkaline phosphatase activity

Quantitative alkaline phosphatase (ALP) activity of the culture was determined by an assay based on the hydrolysis of *p*-nitrophenylphosphate to *p*-nitrophenol by using an ALP detecting kit (Sigma). Treated cells were rinsed three times with PBS and harvested with 100 µl Nonidet P-40 lysis buffer (20 mM-tri(hydroxymethyl)-aminomethane-HCl, pH 7.5, 150 mM-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂, 10% glycerol, 1% Nonidet P-40). The buffer was supplemented with protease inhibitors (2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mM-phenylmethylsulfonyl fluoride). Lysates were put on ice for 20 min and subjected to centrifugation at 14 000 rpm for 30 min at 4 °C. Supernatant fractions were used for ALP activity detection. The ALP activity was determined according to the manufacturer's instructions (Massion & Frankenfeld, 1972). Briefly, 1.0 ml ALP reagent was mixed with a 20 µl sample and incubated for 30 s. Absorbance (A) of the tested sample at 405 nm using water as reference was first recorded as INITIAL A. The absorbance recorded exactly after 2 min was FINAL A. ALP activity was determined as follows:

$$\text{ALP (U/l)} = ((\text{FINAL} - \text{INITIAL}) / 2 \times \text{TV} \times 1000) / (18.45 \times \text{SV} \times \text{LP}),$$

where TV was the total volume (1.02 ml), SV was the sample volume (0.02 ml) and LP was the lightpath (1 cm).

To normalise matrix protein expression to total cellular protein, a fraction of the reaction solution was used in a Bradford protein assay (Bio-Rad Laboratory, Hercules, CA, USA) (Bradford, 1976).

Real-time reverse transcriptase polymerase chain reaction analysis

Total RNA was isolated from cells using Trizol reagent according to the standard protocol. Approximately 2 µg total RNA was used to generate first strand cDNA in each sample using SuperScript II RT with oligo(dT) 12–18 primers

(Invitrogen, Carlsbad, CA, USA). Samples of total cDNA were amplified in each PCR reaction mixture of selected genes (Table 1). PCR amplification and analysis were achieved using a Smart Cycler thermal cycler (Cepheid, Sunnyvale, CA, USA) instrument. Thermocycling was done in a final volume of 25 μ l containing 0.5 μ l cDNA sample or calibrator; 0.3 μ M of each primer; 12.5 μ l $2 \times$ iO™ SYBR Green Supermix (Bio-Rad Laboratory). After denaturing of cDNA at 95°C for 3 min, the cycling conditions were as follows: forty cycles consisting of denaturation at 95°C for 12 s, annealing at 56°C (for ALP), 58°C (for OPG), 56°C (for RANKL) or 55°C (for glyceraldehyde-3-phosphate dehydrogenase; GAPDH) for 12 s, and extension at 72°C for 18 s. A melting curve analysis was performed after the completion of Smart Cycler analysis and the PCR products were subjected to electrophoresis on a 1% agarose gel, visualised, and photographed under UV illumination. Expected band lengths were 597 bp for ALP, 441 bp for OPG, 396 bp for RANKL and 452 bp for GAPDH. GAPDH was chosen as the reference gene for RT-PCR analysis. For quantification of RT-PCR product, standard curves of the threshold cycle against the amount of input cDNA for both target and reference gene were generated by dilution of cDNA extracted from an untreated control sample (a calibrator). The relative abundance of RT-PCR products in each unknown sample was then derived from these standard curves based on the measured threshold cycle levels for both target and reference genes, and was expressed as the target:reference ratio.

Immunoblotting

Conditioned media from cultured cells were collected and concentrated with Microcon-10 (Millipore Corp., Bedford, MA, USA). Equal amounts of concentrated proteins (20 μ g) were separated by SDS-PAGE on 10% reducing gels and transblotted to polyvinylidene fluoride membranes (Immobilin-P; Millipore Corp.). Immunoblotting was performed after blocking non-specific binding on the membrane with 5% non-fat milk. The blots were probed firstly with polyclonal rabbit antihuman OPG (1:1000; Peprotech EC Ltd, London, UK) as the primary antibody, followed by incubation with the goat anti-rabbit antibody conjugated with horseradish peroxidase (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h. Finally, the antigen-antibody complexes were detected using an enhanced chemiluminescence reagent and visualised by the Lumi-Imager with the software Lumi

Analyst version 3.10 (Roche, Mannheim, Germany). Transferred membrane was also stained with Coomassie brilliant blue after immunodetection. The intensities of the protein bands for different lanes were compared to ensure that samples under different treatment were equally loaded.

Statistical analysis

Data are reported as mean values with their standard errors. The non-paired Student's *t* test was used to calculate statistical significance between the control group and each treatment group. Statistical analysis of dose responses was performed by one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Effects of genistein and parathyroid hormone on cell proliferation

The effects of genistein, E₂ or PTH on cell proliferation were determined using the MTT assay. Treatment of human osteoblastic SaOS-2 cells with vehicle, genistein (10^{-8} to 10^{-6} M), E₂ (10^{-8} M) or PTH (10^{-8} M), for 48 h did not alter the cell proliferation rate (Fig. 1 (a); $P > 0.05$). These results are in agreement with those reported previously, i.e. that E₂ has no effect on the proliferation of SaOS-2 cells (Slootweg *et al.* 1992; Rao *et al.* 1994; Nasu *et al.* 2000). Similarly, the cell proliferation rate was not altered in SaOS-2 cells that were co-treated with PTH and 10^{-8} M-E₂ or varying concentrations of genistein (Fig. 1 (b); $P > 0.05$).

Regulation of alkaline phosphatase activity and mRNA expression by genistein

SaOS-2 cells predominantly express the ER β isoform and produce ALP in culture. ALP activity can be considered as an osteoblastic phenotype marker and thus an osteoblastic differentiation index in SaOS-2 cells (Murray *et al.* 1987). Preliminary experiments indicated that ALP activities increased in a time-dependent manner (24–72 h), and could be detected optimally at 72 h (data not shown). As shown in Fig. 2 (a), E₂ (10^{-8} M) had no effect on the ALP activity of SaOS-2 cells ($P > 0.05$), while genistein (10^{-8} to 10^{-6} M) increased the ALP activity in a dose-dependent manner ($P < 0.05$). Co-treatment of SaOS-2 cells with oestrogen antagonist ICI 182780 (10^{-6} M) completely inhibited the increase of ALP activity induced by genistein

Table 1. Primers used for reverse transcriptase polymerase chain reactions and real-time reverse transcriptase polymerase chain reactions

Primer	Sequence	Orientation	Product size (bp)	Temperature (°C)
Alkaline phosphatase	GTGGACTATGCTCACAACAA	Sense	597	56
	GGAGAAATACGTTTCGCTAGA	Antisense		
Osteoprotegerin	GAACCCAGAGCGAAATACA	Sense	441	58
	CGCTGTTTTCACAGAGGTCA	Antisense		
RANKL	ATGGTGTACGTCCTAAAAC	Sense	396	56
	GTGTTCTCTACAAGGTCAAG	Antisense		
GAPDH	ACCACAGTCCATGCCTACAC	Sense	452	55
	TTCACCACCCTGTTGCTGTA	Antisense		

RANKL, receptor activator of NF- κ B ligand; GAPDH; glyceraldehyde-3-phosphate dehydrogenase.

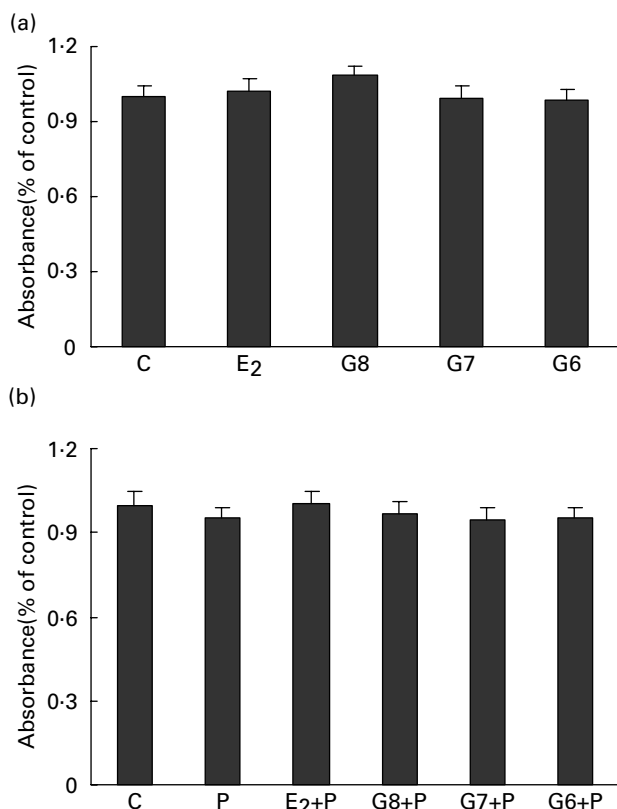


Fig. 1. Effects of 17 β -oestradiol (E₂), genistein and parathyroid hormone (PTH) on cell proliferation in human SaOS-2 cells. (a) SaOS-2 cells were treated variously with vehicle (control; C), 10⁻⁸ (G₈), 10⁻⁷ (G₇) or 10⁻⁶ (G₆) M-genistein or 10⁻⁸ M-E₂ for 48 h. Cell proliferation rate was determined by 3-[4,5-dimethylthiazol 2-yl]-2,5-diphenyltetrazolium bromide assay. This result is representative of three independent experiments. Values are means, with their standard errors represented by vertical bars. No significant change was found in the cell proliferation rate of SaOS-2 cells in response to treatment with genistein or E₂. (b) SaOS-2 cells were treated with vehicle (control; C), 10⁻⁸ M-E₂ or 10⁻⁸ (G₈), 10⁻⁷ (G₇) or 10⁻⁶ (G₆) M-genistein for 24 h, followed by treatment with PTH (P) for another 24 h. This result is representative of three independent experiments. Values are means, with their standard errors represented by vertical bars. No significant change was found in the cell proliferation rate of SaOS-2 cells in response to the combined treatment.

(Fig. 2 (b); $P < 0.05$). The results indicated that genistein could stimulate SaOS-2 cell differentiation at dietary-achievable concentrations and these effects were ER-dependent. To determine whether E₂ or genistein could regulate ALP expression transcriptionally, SaOS-2 cells were treated either with vehicle, E₂ (10⁻⁸ M), or genistein (10⁻⁸ to 10⁻⁶ M) for 48 h. The results shown in Fig. 3 indicate that the mRNA expression of ALP did not change in response to treatment with either E₂ or genistein ($P > 0.05$). Thus, it appears that genistein regulated ALP activity post-transcriptionally.

Modulating action of genistein on parathyroid hormone-induced alkaline phosphatase activity

To determine if genistein and E₂ modulated the effects of PTH on osteoblastic differentiation, their combined effects on ALP activities were determined in SaOS-2 cells. ALP activities

were assayed upon pre-treatment of SaOS-2 cells with either genistein, E₂, or its vehicle for 24 h, followed by co-incubation with PTH for another 48 h so that the activities could be detected in SaOS-2 cells at the optimal duration of exposure (72 h). As shown in Fig. 4, PTH (10⁻⁸ M) alone significantly increased ALP activity ($P < 0.05$). The increase in ALP activity by PTH was augmented by pre-treatment of SaOS-2 cells with genistein in a dose-dependent manner ($P < 0.05$). PTH-induced ALP activity was maximally augmented by 2.7 times following pre-treatment with 10⁻⁷ M-genistein. Pre-treatment with E₂ (10⁻⁸ M) also has positive effects on the PTH-induced ALP activity (Fig. 4; $P < 0.05$). The observed positive effect of E₂ and PTH is consistent with the results reported in a previous study (Rao *et al.* 1994). The combined effect of genistein and PTH on ALP gene expression was also determined. ALP gene expression was not modulated by PTH in the presence or absence of E₂ or genistein pre-treatment (data not shown).

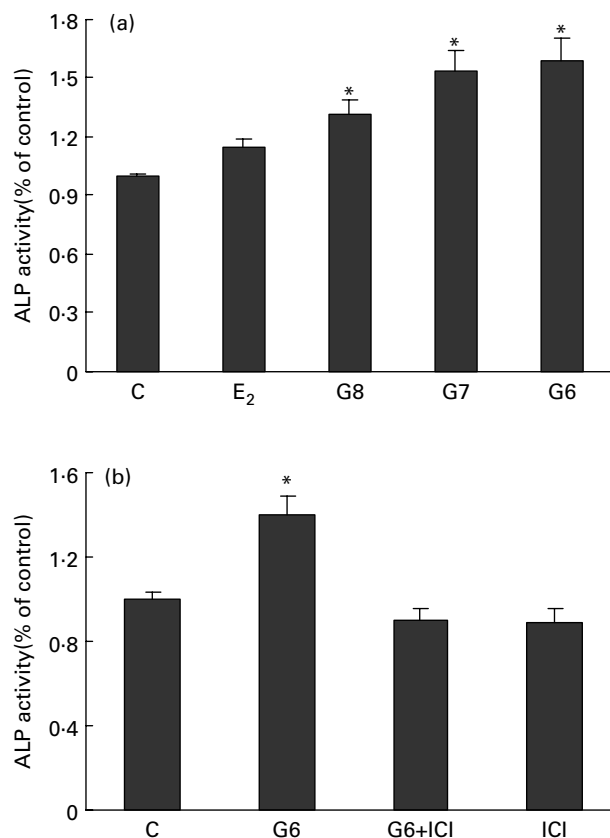


Fig. 2. Effects of 17 β -oestradiol (E₂) and genistein on alkaline phosphatase (ALP) activity in human SaOS-2 cells. (a) SaOS-2 cells were cultured and treated variously with vehicle (control; C), 10⁻⁸ (G₈), 10⁻⁷ (G₇) or 10⁻⁶ (G₆) M-genistein or 10⁻⁸ M-E₂ for 72 h. (b) SaOS-2 cells were cultured and treated with vehicle (control; C) or 10⁻⁶ (G₆) M-genistein in the presence of 10⁻⁶ M-ICI 182780 (7 α -[9-[(4,4,5,5,5-pentafluoropentyl)sulfonyl]nonyl]-estra-1,3,5(10)-triene-3,17 β -diol). Then the cell lysates were used for ALP activity detection. Results were obtained from four independent experiments. Values are means, with their standard errors represented by vertical bars. Genistein, but not E₂, increased ALP activity in a dose-dependent manner. ICI 182780 completely abolished the induction of ALP activity by genistein. * Mean value was significantly different from that for the control ($P < 0.05$).

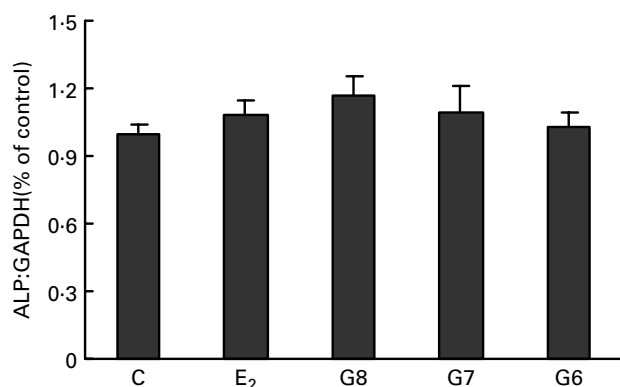


Fig. 3. Effects of 17β -oestradiol (E_2) and genistein on alkaline phosphatase (ALP) mRNA expression in human SaOS-2 cells. SaOS-2 cells were cultured and treated variously with vehicle (control; C), 10^{-8} M (G_8), 10^{-7} M (G_7) or 10^{-6} M (G_6) m-genistein or 10^{-8} M- E_2 . Total RNA were isolated and subjected to real-time RT-PCR analysis for detection of ALP, and the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript. The mRNA expression level of ALP is shown as a ratio to the expression of GAPDH. Results were obtained from three independent experiments. Values are means, with their standard errors represented by vertical bars. ALP mRNA expression level was not altered in SaOS-2 cells in response to treatment with E_2 or genistein.

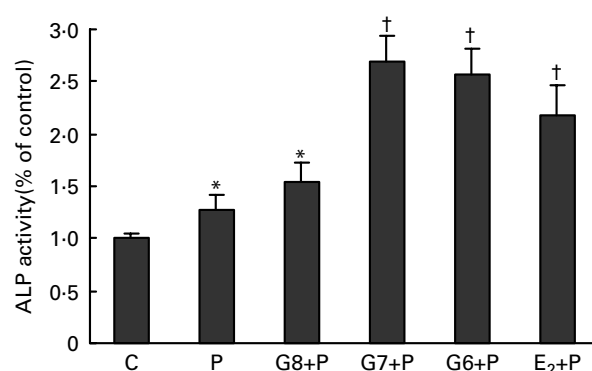


Fig. 4. Modulating action of genistein or 17β -oestradiol (E_2) on parathyroid hormone (PTH)-induced alkaline phosphatase (ALP) activity. SaOS-2 cells were cultured and treated variously with vehicle (control; C), 10^{-8} M (G_8), 10^{-7} M (G_7) or 10^{-6} M (G_6) m-genistein or 10^{-8} M- E_2 for 24 h, followed by treatment with 10^{-8} M-PTH (P) for another 48 h. Cell lysates were then used for ALP activity detection. Results were obtained from three independent experiments. Values are means, with their standard errors represented by vertical bars. PTH treatment increased ALP activity. Pre-treatment with genistein augmented PTH-induced ALP activity in a dose-dependent manner. * Mean value was significantly different from that for the control ($P < 0.05$). † Mean values were significantly different from the group of C, P and G8 + P ($P < 0.05$).

Effects of genistein on osteoprotegerin protein expression

OPG is a secretory protein produced by osteoblastic cells. The effect of genistein on its expression in SaOS-2 cells was therefore determined by measuring the amount of OPG secreted in the culture medium. The effects of genistein on OPG protein expression in SaOS-2 cells are shown in Fig. 5. E_2 (at 10 nM) failed to alter the OPG protein expression in SaOS-2 cells, while genistein stimulated OPG protein secretion in a dose-dependent manner (Fig. 5 (a); $P < 0.05$). The stimulation of OPG protein secretion by 10^{-7} M- and 10^{-6} M-genistein was 1.8- and 1.6-fold, respectively. The up regulation of OPG protein expression was completely blocked by oestrogen antagonist ICI 182780 (Fig. 5 (b); $P < 0.05$), suggesting that the effects of genistein on OPG protein expression were ER-dependent. The immunoblots were stained with Coomassie brilliant blue to ensure that samples were equally loaded, and the differences observed between the samples were due to different treatment (data not shown).

Effects of genistein on osteoprotegerin and receptor activator of nuclear factor- κ B ligand mRNA expression

OPG and RANKL are both expressed by osteoblastic cells and are involved in modulating osteoclastic cell activities. The secretion of OPG by osteoblastic cells could decrease the interactions of RANKL with RANK located on the osteoclastic cell surface, thereby preventing the induction of osteoclastic activities. Thus, the RANKL:OPG mRNA expression ratio could be used as an indirect index to evaluate the potential effects of different treatments on bone remodelling. An increase in the RANKL:OPG ratio suggests an increase in ability of the osteoblastic cells to recruit osteoclasts, whereas a decrease in the ratio suggests the reduction of osteoclastic cell recruitment. To determine if genistein could modulate osteoclastic cell activities, the effects of different concentrations of genistein

on OPG and RANKL mRNA expression in SaOS-2 cells were determined. The expression of OPG and RANKL mRNA were analysed by quantitative real-time RT-PCR. As shown in Fig. 6 (a), treatment of SaOS-2 cells with E_2 significantly increased OPG mRNA expression ($P < 0.05$) but did not significantly alter the level of RANKL mRNA expression (Fig. 6 (b); $P > 0.05$). The differential effects of E_2 on RANKL and OPG mRNA expression in SaOS-2 cells resulted in a significant decrease in the RANKL:OPG ratio (Fig. 6 (c); $P < 0.05$), suggesting that E_2 exerts inhibitory effects on the recruitment of osteoclastic cells. The effect of genistein on OPG mRNA expression was similar to E_2 , in which treatment of SaOS-2 cells with 10^{-6} M-genistein resulted in a 1.4-fold increase in OPG mRNA expression ($P < 0.05$). However, 10^{-6} M-genistein also increased RANKL mRNA expression by more than 1.3-fold (Fig. 6 (b); $P < 0.05$). As a result, at 10^{-6} M, the effect of genistein on induction of OPG mRNA expression in SaOS-2 cells was counteracted by its inductive effects on RANKL mRNA expression. Thus, genistein (10^{-8} to 10^{-6} M) did not alter the RANKL:OPG ratio in SaOS-2 cells (Fig. 6 (c); $P < 0.05$).

Modulating action of genistein on parathyroid hormone-induced osteoprotegerin and receptor activator of nuclear factor- κ B ligand mRNA expressions

To determine if genistein could modulate the effects of PTH on osteoclastogenesis, the expressions of OPG and RANKL mRNA in SaOS-2 cells co-treated with PTH and genistein were determined. The present results indicate that genistein can alter OPG and RANKL expressions only at higher concentration (10^{-7} and 10^{-6} M). Thus, SaOS-2 cells were pre-treated with either genistein at higher concentrations, E_2 (10^{-8} M) or its vehicle for 24 h, followed by co-treatment with 10^{-8} M-PTH for another 24 h. Fig. 7 (a) and Fig. 7 (b) show that treatment of SaOS-2 cells with PTH alone resulted in a significant

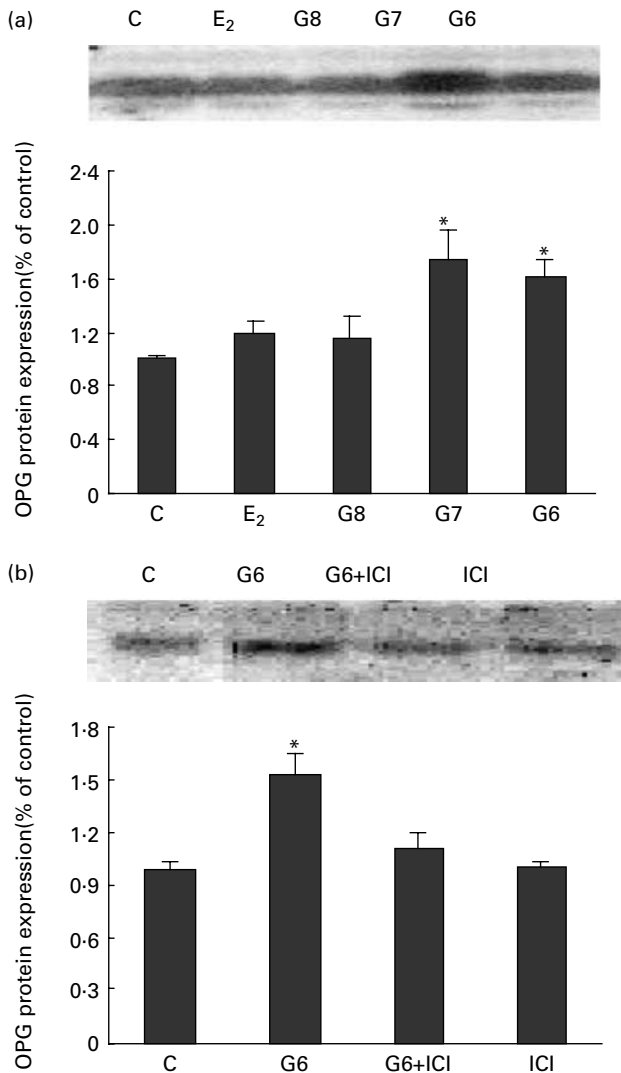


Fig. 5. Effects of 17 β -oestradiol (E₂) and genistein on osteoprotegerin (OPG) protein expression in human SaOS-2 cells. Proteins were isolated from the conditioned media and fractionated using 10% SDS-PAGE and immunoblotted with antibody against OPG. (a) SaOS-2 cells were cultured and treated variously with vehicle (control; C), 10⁻⁸ (G8), 10⁻⁷ (G7) or 10⁻⁶ (G6) m-genistein or 10⁻⁸ M-E₂. (b) SaOS-2 cells were cultured and treated with vehicle (control; C) or 10⁻⁶ (G6) m-genistein in the presence of 10⁻⁶ M-ICI 182780 (7 α -[9-[(4,4,5,5,5-pentafluoropentyl)sulfonyl]nonyl]-estra-1,3,5(10)-triene-3,17 β -diol). The results shown are representative of three independent experiments. Values are means, with their standard errors represented by vertical bars. Genistein stimulated OPG protein secretion in a dose-dependent manner. E₂ failed to alter the OPG protein expression. ICI 182780 completely abolished the induction of OPG protein expression by genistein. * Mean value was significantly different from that for the control ($P < 0.05$).

decrease in the expression of OPG mRNA as well as a significant increase in the expression of RANKL mRNA ($P < 0.05$). The resulting RANKL:OPG ratio was significantly increased upon treatment with PTH in SaOS-2 cells (Fig. 7 (c); $P < 0.05$), confirming the action of PTH in modulating osteoclastic activities. Pre-treatment of SaOS-2 cells with genistein or E₂ could reverse the inhibitory effect of PTH on OPG gene expression (Fig. 7 (a)). In contrast, pre-treatment of SaOS-2 cells with E₂, but not genistein, completely attenuated the induction of RANKL mRNA expression by PTH

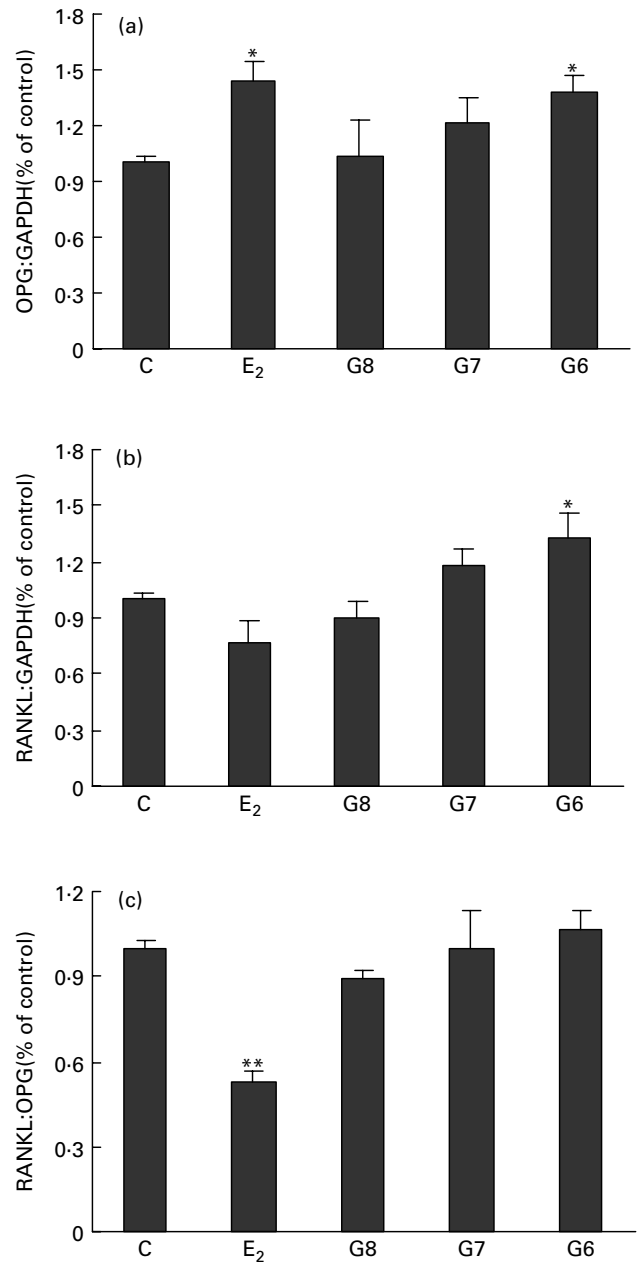


Fig. 6. Effects of 17 β -oestradiol (E₂) and genistein on osteoprotegerin (OPG) and receptor activator of nuclear factor- κ B ligand (RANKL) mRNA expressions in human SaOS-2 cells. SaOS-2 cells were cultured and treated variously with vehicle (control; C), 10⁻⁸ M-E₂, or 10⁻⁸ (G8), 10⁻⁷ (G7) or 10⁻⁶ (G6) m-genistein. Total RNA were isolated and subjected to real-time RT-PCR analysis for detection of OPG (a), RANKL (b) and the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript. The mRNA expression level of OPG and RANKL are shown as a ratio to the expression of GAPDH. The graphical results shown are representative of three independent experiments. Values are means, with their standard errors represented by vertical bars. (c) The graphical representation of the RANKL:OPG ratio. Genistein increased the mRNA expression of OPG and RANKL in a dose-dependent manner. E₂ significantly increased OPG mRNA expression. Mean value was significantly different from that for the control: * $P < 0.05$, ** $P < 0.01$.

(Fig. 7 (b)). Taken together, it appears that genistein and E₂ can prevent the up regulation of the RANKL:OPG ratio by PTH in osteoblastic cells (Fig. 7 (c); $P < 0.05$). These results suggest that the beneficial effect of genistein and E₂ on

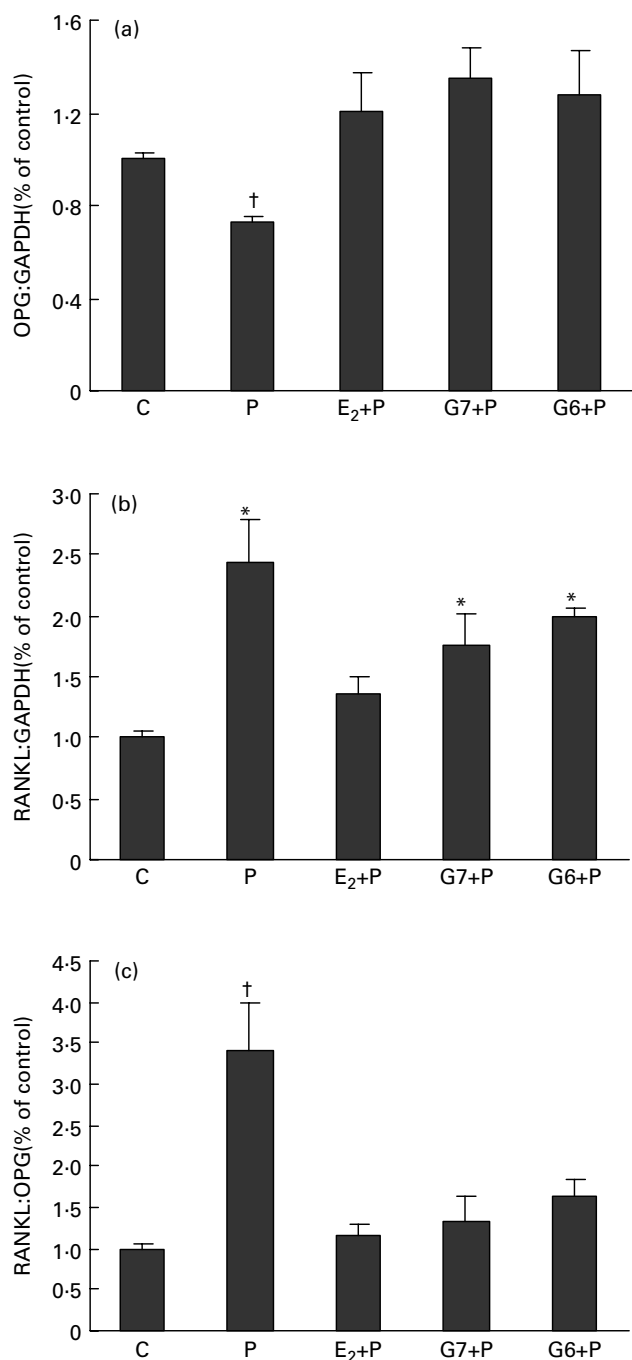


Fig. 7. Modulating action of 17 β -oestradiol (E₂) or genistein on osteoprotegerin (OPG) and receptor activator of nuclear factor- κ B ligand (RANKL) mRNA expressions regulated by parathyroid hormone (PTH) in human SaOS-2 cells. SaOS-2 cells were cultured and treated variously with vehicle (control; C), 10⁻⁸ M-E₂, or 10⁻⁷ (G7) or 10⁻⁶ (G6) M-genistein for 24 h, followed by treatment with 10⁻⁸ M-PTH (P) for another 24 h. Total RNA were isolated and subjected to real-time RT-PCR analysis for detection of the OPG (a), RANKL (b) and the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript. The mRNA expression levels of OPG and RANKL are shown as ratios to the expression of GAPDH. The graphical results shown are representative of three independent experiments. Values are means, with their standard errors represented by vertical bars. (c) The graphical representation of the RANKL:OPG ratio. Pre-treatment with genistein or E₂ could completely reverse the PTH-induced increase in the RANKL:OPG ratio. * Mean value was significantly different from that for the control ($P < 0.05$). † Mean value was significantly different from that for the control and the other treated groups ($P < 0.05$).

bone health is partially mediated by modulating the effects of PTH on OPG and RANKL expression in osteoblastic cells.

Discussion

Several phenotypic markers of osteoblastic cells, including cell proliferation (MTT) and ALP, were used in the present study to determine the effects of genistein and PTH on human osteoblastic SaOS-2 cells. The results indicate that genistein, but not E₂, significantly increased ALP activity in a dose-dependent manner, supporting its role in modulating osteoblastic cell differentiation. It appears that the stimulatory effect of genistein on ALP activity is ER-dependent and that cell proliferation as determined by the MTT assay is not affected by genistein or E₂ treatment. The present results are consistent with those reported by others (Rao *et al.* 1994; Nasu *et al.* 2000), that E₂ has no significant effect on ALP activity or cell proliferation and that neither E₂ nor genistein modulate ALP gene expression in SaOS-2 cells. These results indicate that cell proliferation and ALP gene expression might not be involved in the anabolic effects of E₂ and genistein in this bone cell model system.

Previous studies showed that serum genistein and isoflavone levels increased in a dose-dependent manner in response to *in vivo* administration of genistein in animals (Santell *et al.* 2000) as well as soya food consumption in human subjects (King & Bursill, 1998). However, the plasma level of genistein was seldom over 1 μ mol/l even in a population with high traditional soya food intake. Thus, in the present study, the dietary achievable concentrations of genistein ranging from 10⁻⁸ to 10⁻⁶ M were chosen in order to mimic these *in vivo* conditions.

The equilibrium of RANKL and OPG expression plays an important role in controlling bone remodelling. To our knowledge, only three studies have looked at the effect of genistein on RANKL gene expression in osteoblastic cell lines (Chen & Anderson, 2002; Viereck *et al.* 2002; Chen *et al.* 2003). However, mRNA signals of RANKL were not detectable in these cell model systems. The present study is the first to report that RANKL gene expression is detectable in SaOS-2 cells and could be regulated by genistein. Genistein (at 1 μ M) produced a 1.3-fold increase in RANKL gene expression. However, the stimulatory effect on RANKL gene expression did not occur in SaOS-2 cells in response to treatment with 10⁻⁸ and 10⁻⁷ M-genistein, indicating that the effect of genistein on RANKL expression was dose-dependent.

Genistein was recently reported to have stimulatory effects on OPG mRNA levels and protein secretion in several different osteoblastic cell lines, including OB, hFOB and MC3T3 cells (Chen & Anderson, 2002; Viereck *et al.* 2002; Chen *et al.* 2003). In the present study, the effects of genistein on the steady-state level of OPG mRNA and protein production in human SaOS-2 cells were assessed. Genistein significantly increased OPG protein and mRNA expression in a dose-dependent manner. The increase in OPG protein secretion induced by genistein appears to be ER-dependent, as co-treatment of SaOS-2 cells with ICI 182780 completely abolished the induction of OPG protein secretion by genistein.

PTH was shown to act bi-directionally on osteoblastic SaOS-2 cells in the present study. PTH (10⁻⁸ M) treatment

significantly increased ALP activity, suggesting that PTH could modulate osteoblastic cell differentiation and has an anabolic effect on bone. On the other hand, PTH significantly decreased OPG mRNA expression and stimulated RANKL mRNA expression. The increase in the RANKL:OPG ratio in SaOS-2 cells by PTH as demonstrated in the present study is in agreement with the well-known role of PTH in inducing bone resorption. The results indicate that PTH could potentially increase osteoclastic cell recruitment by increasing the RANKL:OPG ratio in osteoblastic cells.

Increasing evidence suggests that oestrogen might modulate the action of PTH in bone (Masiukiewicz *et al.* 2000). An association between oestrogen deficiency and the rise in serum PTH was reported previously (Khosla *et al.* 1997). For women, receiving oestrogen therapy could prevent the increase in PTH levels associated with the menopause and reverse virtually all age-related increase in bone resorption induced by PTH (Khosla *et al.* 1997; Masiukiewicz *et al.* 2000). The present results are consistent with previous reports (Rao *et al.* 1994; Nasu *et al.* 2000), i.e. that the PTH-induced ALP activity was further increased in response to pre-treatment with E₂. At the same time, E₂ can exert its anabolic effect on bone through the up regulation of OPG gene expression and the down regulation of the RANKL:OPG ratio in this cell model system. Most importantly, E₂ pre-treatment can completely reverse the induction of RANKL gene expression and the inhibition of OPG gene expression by PTH. These results indicate as a possible mechanism that E₂ could modulate the effect of PTH by stimulating osteoblastic cell differentiation and inhibiting osteoclastogenesis, thereby promoting bone formation in osteoblastic cells.

The present results also demonstrate that genistein could modulate the action of PTH in osteoblastic cells. PTH-induced ALP activity was further increased in SaOS-2 cells in response to pre-treatment with genistein. Pre-treatment of SaOS-2 cells with 10⁻⁷ and 10⁻⁶ M-genistein modestly attenuated PTH-induced RANKL mRNA expression and significantly reversed the inhibition of OPG mRNA expression by PTH. Thus, genistein mimics the effect of E₂ in modulating the stimulatory effects of PTH on ALP expression as well as reversing the inductive effects of PTH on osteoclastogenesis via its modulation of the RANKL:OPG ratio. The present findings suggest beneficial effects of genistein on bone health associated with an oestrogen-deficient state.

In conclusion, the present data demonstrate the anabolic effects of phyto-oestrogens in human SaOS-2 osteoblastic cells. PTH acted bi-directionally on osteoblastic SaOS-2 cells by stimulating ALP activity, inducing RANKL gene expression and inhibiting OPG gene expression. In addition, the present study demonstrates that the anabolic effect of oestrogen in osteoblastic SaOS-2 cells was at least partly mediated through the modulation of the RANKL-OPG system. Furthermore, both genistein and oestrogen can augment PTH-induced ALP activity and attenuate the induction of osteoclastogenesis by PTH in SaOS-2 cells. The results of the present study provide new insights for understanding the molecular actions of genistein in osteoblastic cells. Most importantly, as genistein is a major type of dietary phyto-oestrogens commonly found in soya products, the present study provides the mechanistic basis for supporting the observed

bone-protective effects reported previously in both clinical and epidemiological studies. Thus, a diet high in phyto-oestrogen content might be helpful for postmenopausal women with high circulating PTH levels to protect against bone loss and prevent the development of osteoporosis.

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