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Zinc modifies the effect of phyto-oestrogens on osteoblast and osteoclast differentiation *in vitro*

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Abstract

Osteoblast and osteoclast activity is disrupted in post-menopausal osteoporosis. Thus, to fully address this imbalance, therapies should reduce bone resorption and promote bone formation. Dietary factors such as phyto-oestrogens and Zn have beneficial effects on osteoblast and osteoclast activity. However, the effect of combinations of these factors has not been widely studied. We therefore examined the effect of coumestrol, daidzein and genistein in the presence or absence of zinc sulphate (Zn) on osteoclast and osteoblast activity. Osteoclast differentiation and bone resorption were significantly reduced by coumestrol (10^{-7} M), daidzein (10^{-5} M) and genistein (10^{-7} M); and this direct anti-osteoclastic action was unaffected by Zn (10^{-5} M). In addition, Zn augmented the inhibitory effect of phyto-oestrogens on the osteoblast-derived stimulus for osteoclast formation, significantly reducing the ratio of receptor activator of NF-κB ligand (RANKL)-to-osteoprotegerin mRNA expression in human osteoblast. We then examined the effect of these compounds on osteoblast activity. Mineralisation was enhanced by coumestrol (10^{-5} to 10^{-7} M), daidzein (10^{-5} to 10^{-6} M) and genistein (10^{-5} M); and Zn significantly augmented this response. Zn and phyto-oestrogens also significantly enhanced alkaline phosphatase activity and Runt-related transcription factor 2 (*Runx2*) mRNA expression. On the other hand, Zn blunted phyto-oestrogen-induced type I collagen and osteocalcin expression and suppressed coumestrol and daidzein-stimulated osterix expression. Zn may therefore modify the anabolic action of phyto-oestrogens, promoting characteristics associated with early rather than late stages of osteoblast differentiation. Our data suggest that while Zn enhances the anti-osteoclastic effect of phyto-oestrogens, it may limit aspects of their anabolic action on bone matrix formation.

Key words: Osteoblasts: Osteoclasts: Differentiation: Zinc: Phyto-oestrogens

The skeleton constantly remodels in response to changes in mechanical load, serum Ca and micro-damage[1,2]. This dynamic process generates a bone mass and structure optimised to current physical and mineral requirements. At a cellular level, remodelling is performed by osteoblasts that secrete and mineralise new bone matrix and osteoclasts that resorb bone. Osteoblast and osteoclast activity is tightly regulated such that during each remodelling cycle osteoblast formation is temporally coupled to resorption, ensuring that there is little net bone loss. However, this balance is disrupted in many skeletal disorders such as post-menopausal osteoporosis and osteomyelitis[3,4]. In post-menopausal women, the reduction in circulating oestrogen increases bone turnover and skews remodelling in favour of osteoclastic resorption[4]. The resulting bone loss increases fracture risk at elements with a high trabecular content such as the femoral neck and distal radius and ulna.

Hormone replacement has been shown to prevent the increase in osteoclast formation and thereby reduce fracture risk[5]. Hormone replacement also has an anabolic action, increasing bone formation and volume in rats and humans[6,7]. This contrasts with other antiresorptive drugs, such as bisphosphonates, which typically only suppress osteoclast activity. However, the widespread use of hormone replacement has been re-assessed in the light of large-scale clinical trials that showed a substantial increase in the risk of breast cancer and CHD in older women prescribed combination hormone replacement[5]. Therefore, several alternative compounds with oestrogenic actions have been examined for their antiresorptive and anabolic potential. These include phyto-oestrogens, a diverse group of plant-derived factors with a structure and function similar to oestradiol. Some epidemiological studies suggest that diets with high phyto-oestrogen content, such as soya-rich diets, may generate a

Abbreviations: β-GP, β-glycerophosphate; ALP, alkaline phosphatase; L-AA, L-ascorbic acid; OPG, osteoprotegerin; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; Runx2, Runt-related transcription factor 2; TRAP, tartrate-resistant acid phosphatase.

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more robust skeleton. A positive association between soya consumption and bone mineral density has been noted in Asians[8–10] and supplements have also been shown to have beneficial effects on bone mineral density[11–14]. However, not all studies note a positive effect at all skeletal sites and efficacy varies depending on the phyto-oestrogen and dose studied[15].

The protective effect of phyto-oestrogens is thought to occur through a combination of osteoclast- and osteoblast-mediated actions. Several studies note decreases in resorption markers following phyto-oestrogen supplementation[9,16] and *in vitro* studies show a direct suppressive effect of phyto-oestrogens on cytokine-induced osteoclast differentiation[17,18]. In addition to suppressing resorption, phyto-oestrogens have also been shown to increase bone formation markers such as serum alkaline phosphatase (ALP) and osteocalcin levels in post-menopausal women[12,19]. Genistein has also been shown to increase mineral apposition and bone formation rates in ovariectomised rats[20] and phyto-oestrogens stimulate osteoblast differentiation and mineralisation *in vitro*[21–23].

In addition, other nutritional factors have been shown to influence remodelling activity. Zn promotes osteoblast activity *in vitro*[24], Zn deficiency is associated with osteopenia in men[25] and Zn supplements prevent exercise-induced falls in long bone mass in rats[26]. Osteoclast activity is also decreased by Zn[27,28]. The ability of dietary factors to not only prevent further bone resorption but also replace bone already lost is desirable; however, studies have not fully examined the effect of combinations of dietary factors on bone cell differentiation and activity. Similarly, the effect of these factors on osterix mRNA expression which regulates the formation of mature osteoblasts has not been investigated. We therefore examined the effect of genistein, coumestrol and daidzein in the presence of Zn on osteoblast and osteoclast function *in vitro*.

Methods

Media and reagents

Saos-2 human osteoblast-like cells were obtained from ECACC (catalogue no. 89050205) and cultured in phenol red-free Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% charcoal-stripped fetal calf serum (Autogen Bioclear) 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin (all from Sigma). RAW264.7 monocytes (American Type Culture Collection, catalogue no. TIB-71) were incubated in phenol red-free Dulbecco's minimum essential medium supplemented with 10% charcoal-stripped fetal calf serum (Autogen Bioclear), 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin (all from Sigma). All incubations were performed at 37°C in 5% CO_2, and cultures fed every 2–3 d by replacing half of the culture volume with fresh medium. Zinc sulphate heptahydrate (Zn) was obtained from Sigma. The non-selective oestrogen antagonist ICI 182780 was obtained from Tocris Biosciences. Recombinant murine TNF-α was purchased from Insight Biotechnology. All other reagents and kits were obtained from Sigma unless stated.

Measurement of mineralisation and alkaline phosphatase activity

Preliminary studies established that concentrations of Zn below 10^{-5} M showed no interaction with phyto-oestrogens; and all subsequent studies therefore used Zn at a concentration of 10^{-5} M. The effect of coumestrol (10^{-5} to 10^{-9} M), daidzein (10^{-5} to 10^{-9} M) and genistein (10^{-5} to 10^{-9} M) in the presence or absence of Zn (10^{-5} M) on ALP activity was assessed as follows. Saos-2 cells (1×10^4 per well) were incubated in ninety-six-well plates for 24 h to enable cells to adhere. Cultures were then incubated in relevant phyto-oestrogen and Zn concentrations in the presence of β-glycerophosphate (β-GP; 10 mM) and L-ascorbic acid (L-AA; 50 mg/l) for 4 d. ALP activity was measured by staining cultures with *p*-nitrophenyl phosphate (1 mg/ml) in 0·2 M-Tris buffer at 37°C for 30 min[29]. Absorbance was measured at 405 nm and the results were then normalised to total cell number and expressed as the amount of ALP required to liberate 1 mmol of *p*-nitrophenol/min per 10^4 cells.

Mineralisation was assessed using a modification of Hale's methodology[30]. This enables the rapid and direct quantification of mineralisation by measuring calcein incorporation into mineralised nodules. Cells were treated with β-GP (10 mM) and L-AA (50 mg/l) to initiate mineralisation and the medium supplemented with genistein, daidzein or coumestrol (10^{-5} to 10^{-9} M) with or without Zn (10^{-5} M). After 18 d of incubation, the culture medium was aspirated, the monolayer washed with PBS and incubated in culture medium containing 1 μg/ml calcein for 4 h at 37°C. Cultures were then washed three times in PBS and the fluorescence measured by a cytofluor II fluorescence multi-well plate reader (PerSeptive Biosystem) at 485 nm excitation and 530 nm emission.

Proliferation

Saos-2 cells were cultured in ninety-six-well plates at a density of 1×10^4 cells per well in the presence of coumestrol (10^{-5} to 10^{-9} M), daidzein (10^{-5} to 10^{-9} M) or genistein (10^{-5} to 10^{-9} M) with or without Zn (10^{-5} M) for 4 d. Proliferation was then assessed using a commercial AQueous one solution cell proliferation assay (Promega) according to the manufacturer's instructions.

Real-time quantitative PCR analysis

Saos-2 cells (5×10^5 per well) were incubated in six-well plates for 24, 48 or 96 h with coumestrol (10^{-7} M), genistein (10^{-7} M) or daidzein (10^{-5} M) with or without Zn (10^{-5} M). Total RNA was extracted from these cultures using a Sigma genelute RNA isolation kit and reverse-transcribed with Moloney Murine Leukemia Virus RT using random nonamer primers. Real-time PCR was performed on a StepOne PCR system (Applied Biosystems) using the DNA-binding dye SYBR green for detection of PCR products. A total of 2 μl of external plasmid standard or complementary DNA were added to a final reaction volume of 25 μl containing 0·05 U/μl Taq, SYBR green and specific primers (0·2 μM).

Primers for genes were as follows: human osteocalcin forward CCCAGCGGTGCAGAGTCCAG, reverse CCTCCCTCCTGGGCTCCAGG; human Runt-related transcription factor 2 (*Runx2*) forward AGACCCCAGGCAGGCACAGT, reverse GCGCCTAGGCACATCGGTGA; human osterix forward GCACCCTGGAGGCAACTGGC, reverse GAGCTGGGTAGGGGGCTGGA; human type I collagen forward CCTGGCAGCCCTGGTCCTGA, reverse CTTGCCGGGCTCTCCAGCAG; human receptor activator of NF-κB ligand (*RANKL*) forward ACAGGCCTTTCAAGGAGCTGTGC, reverse ACCAGATGGGATGTCGGTGGC; human osteoprotegerein forward AATCGCACCCACAACCGCGT reverse AGCAGGAGACCAAAGACACTGCA; human *β-actin* forward GCGCGGCTACAGCTTCACCA, reverse TGGCCGTCAGGCAGCTCGTA. For the generation of standard curves, the corresponding complementary DNA was cloned into pGEM-T Easy (Promega). The concentration of DNA plasmid stock was determined by the optical density at 260 nm. Copy number for each plasmid was calculated from these measurements. The linear range of the assay was determined by the amplification of log serial dilutions of external plasmid standard from 500 to 5×10^6 copies. The progress of the PCR amplification was monitored by real-time fluorescence emitted from SYBR Green during the extension time. Reaction conditions were 94°C for 2 min, followed by thirty-five cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. At the end of each PCR run, a melt curve analysis was performed to show the absence of non-specific bands. For each sample, mRNA levels were expressed as an absolute copy number normalised against the *β-actin* gene. The mRNA copy number was calculated for each sample from the standard curves by the instrument's software. The samples were analysed in triplicate.

Osteoclast differentiation and bone resorption assays

To examine the direct effect of Zn on the anti-osteoclastic action of phyto-oestrogens, RAW264.7 cells were transferred to ninety-six-well plates at a density of 1×10^4 cells per well. The effect of Zn on the anti-resorptive action of phyto-oestrogens was assessed by seeding 10^4 RAW264.7 cells onto 20 mm^2 slices of devitalised bovine bone in ninety-six-well plates. Cells and bone slices were then incubated with combinations of TNF-α (50 ng/ml), genistein (10^{-5} to 10^{-9} M), daidzein (10^{-5} to 10^{-9} M) or coumestrol (10^{-5} to 10^{-9} M) with or without Zn (10^{-5} M) for 4 d for assessment of tartrate-resistant acid phosphatase (TRAP)-positive osteoclast formation or 8 d for bone resorption.

Assessment of tartrate-resistant acid phosphatase-positive osteoclast formation

Osteoclast formation was evaluated by staining for the specific osteoclastic marker TRAP using a modification of the method of Burstone[31] using naphthol AS-BI phosphate as a substrate. The number of TRAP-positive cells was counted using an

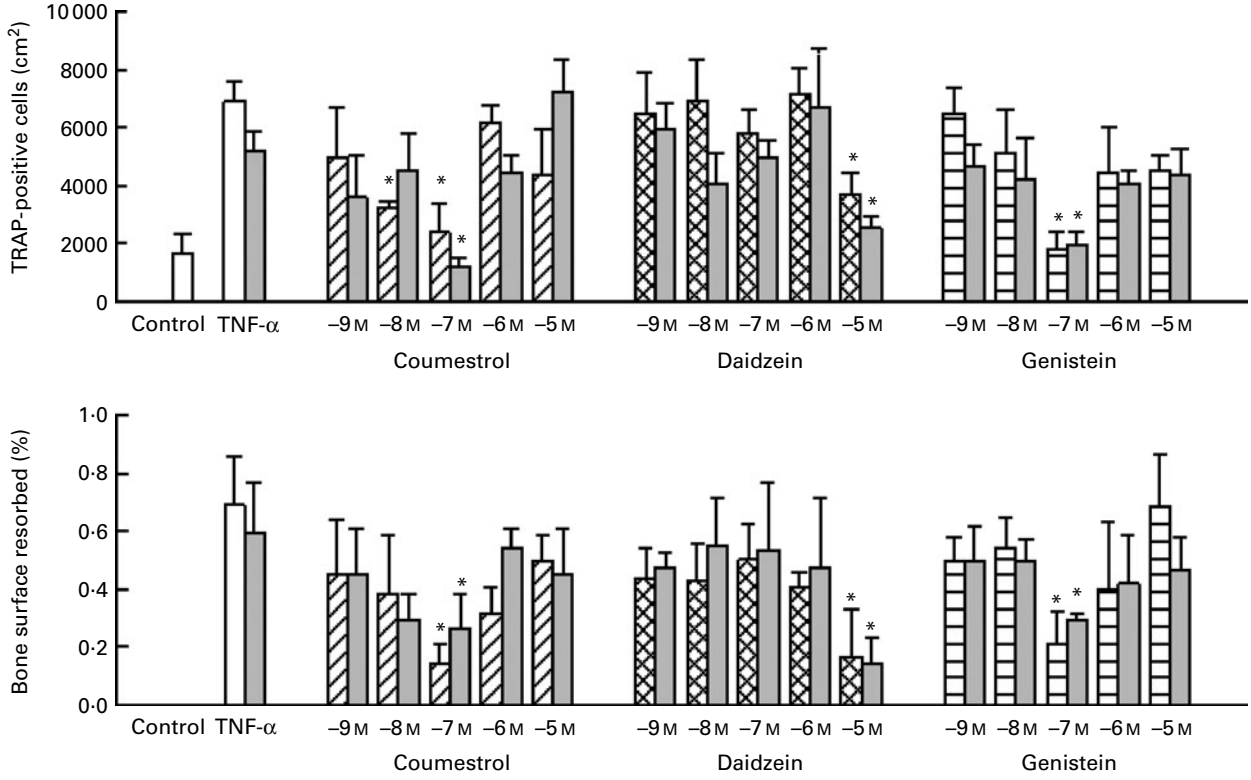

Fig. 1. Zinc has no effect on the direct anti-osteoclastic action of phyto-oestrogens. RAW264.7 cells were incubated in the presence of TNF-α (50 ng/ml) for 4–8 d plus coumestrol (10^{-5} to 10^{-9} M, ▨), daidzein (10^{-5} to 10^{-9} M, ⊠) or genistein (10^{-9} to 10^{-5} M, ▤) in the presence or absence of zinc (10^{-5} M, ■). Osteoclast formation was assessed by tartrate-resistant acid phosphatase (TRAP) staining and bone resorption was determined by the percentage of bone surface displaying resorption pits analysed by reflected light microscopy. Values are means of three separate experiments, with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Mean values were significantly different from those of the TNF-α-treated group ($P<0·05$).

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eyepiece graticule at a magnification of ×100 and the results expressed as the number of cells/cm^2. All experiments were performed in triplicate.

Bone resorption

After incubation, cells were removed from the surface of bone slices by immersion in 10% (v/v) receptor activator of NF-κB (RANK) sodium hypochlorite for 10 min, followed by washing in distilled water and dehydration in 70% ethanol. After drying, slices were mounted onto glass slides and stained with 1% toluidine blue for 2 min, washed in distilled water and then dehydrated in ethanol to enable visualisation of resorption pits. The percentage of bone surface resorbed was quantified by reflected light microscopy using an eyepiece graticule and magnification of ×100 on an Olympus BHB microscope with a Schott KL1500 light source.

Statistical analysis

Differences between groups were assessed using Fisher's one-way ANOVA (Statview; Abacus Concepts). A difference of $P<0{\cdot}05$ was considered statistically significant.

Results

Zinc has no effect on the direct anti-osteoclastic action of phyto-oestrogens

Excessive resorption is central to bone loss in several skeletal disorders including post-menopausal osteoporosis. We and others have previously shown that phyto-oestrogens possess antiresorptive actions, directly suppressing cytokine-induced osteoclast differentiation and bone resorption[18]. However, the effect of combinations of phyto-oestrogens and Zn on this direct anti-osteoclastic action has not been examined. As shown previously[18], coumestrol, daidzein and genistein, all significantly suppressed TRAP-positive osteoclast formation and bone resorption (Fig. 1). The dose–response for the phyto-oestrogens matched previous results with maximal suppression noted with coumestrol (10^{-7} M), daidzein (10^{-5} M) and genistein (10^{-7} M). In contrast, TRAP-positive osteoclast formation and bone resorption were unaffected in the presence of Zn alone. Furthermore, the anti-osteoclastic action of all phyto-oestrogens was not affected by the addition of Zn (Fig. 1).

Zinc augments the suppressive action of phyto-oestrogens on osteoblastic receptor activator of NF-κB ligand:osteoprotegerin ratio

Phyto-oestrogens may also indirectly suppress osteoclast formation by modifying expression of the key osteoblast-derived regulators of osteoclastogenesis RANKL and osteoprotegerin (OPG). Osteoclast formation is dependent on a balance between two osteoblast-derived cytokines, RANKL which induces osteoclast differentiation after binding to its receptor receptor activator of NF-κB (RANK) and OPG, a soluble decoy receptor which sequesters RANKL, preventing RANK activation[32]. To assess the effect of phyto-oestrogens and Zn on *RANKL* and *OPG* expression, we incubated osteoblasts with Zn and the concentrations of phyto-oestrogens shown to have the maximal suppressive effect on osteoclastogenesis. We found that the *RANKL:OPG* gene expression ratio was significantly reduced by Zn (4·16-fold), coumestrol (1·88-fold) and genistein (3·57-fold) in comparison to control (Fig. 2). Furthermore, combinations of Zn and genistein or coumestrol further reduced *RANKL/OPG* gene expression in comparison to Zn or phyto-oestrogens alone (Fig. 2). In contrast, although daidzein lowered *RANKL/OPG* expression, this did not reach significance and in the presence of daidzein the suppressive action of Zn was not noted (Fig. 2).

Zinc augments the stimulatory effect of phyto-oestrogens on osteoblast mineralisation

To examine the potential interaction between phyto-oestrogens and Zn on osteoblast differentiation and activity, we utilised human Saos-2 osteoblast-like cells which readily form mineralised nodules in the presence of L-AA and β-GP. Genistein, daidzein and coumestrol all enhanced mineralisation, with coumestrol having the most pronounced effect (Fig. 3(a)). Zn alone had no significant effect on mineralisation as assessed by calcein incorporation into mineralised nodules. Coumestrol (10^{-5} to 10^{-7} M) significantly enhanced osteoblastic mineralisation, with maximal effects noted at 10^{-6} M which induced a 1·62-fold increase in mineralisation. Daidzein (10^{-5} to 10^{-6} M) also significantly enhanced mineralisation with a maximal 1·43-fold increase noted at 10^{-5} M. Genistein stimulated a significant 1·39-fold increase in calcein incorporation at the highest dose studied (10^{-5} M). The addition of Zn augmented the anabolic effect of all phyto-oestrogens, significantly increasing mineralisation compared to cultures treated

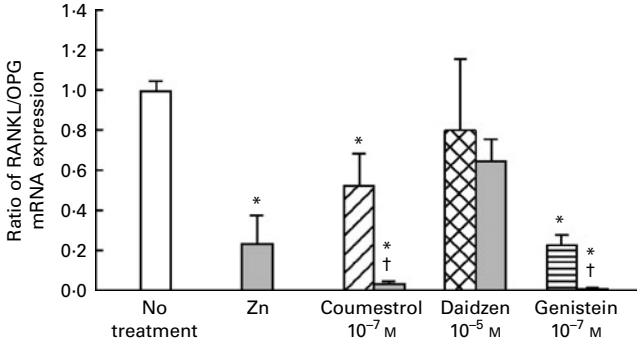

Fig. 2. Phyto-oestrogens and zinc suppress the osteoblast-derived stimulus for osteoclast formation. Human osteoblast-like Saos-2 cells were incubated with zinc plus coumestrol (10^{-7} M, ▨), daidzein (10^{-5} M, ⊠) or genistein (10^{-7} M, ▤) in the presence or absence of zinc (10^{-5} M, ▩). Total RNA was extracted and the expression of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) quantified by real-time PCR. Data were normalised to β-actin and expressed as a ratio of RANKL-to-OPG expression in comparison to control. Values are means of two separate experiments, with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Mean values were significantly different from those of the control group ($P<0{\cdot}05$). † Mean values were significantly different from those of the zinc- or relevant phyto-oestrogen-treated group alone ($P<0{\cdot}05$).

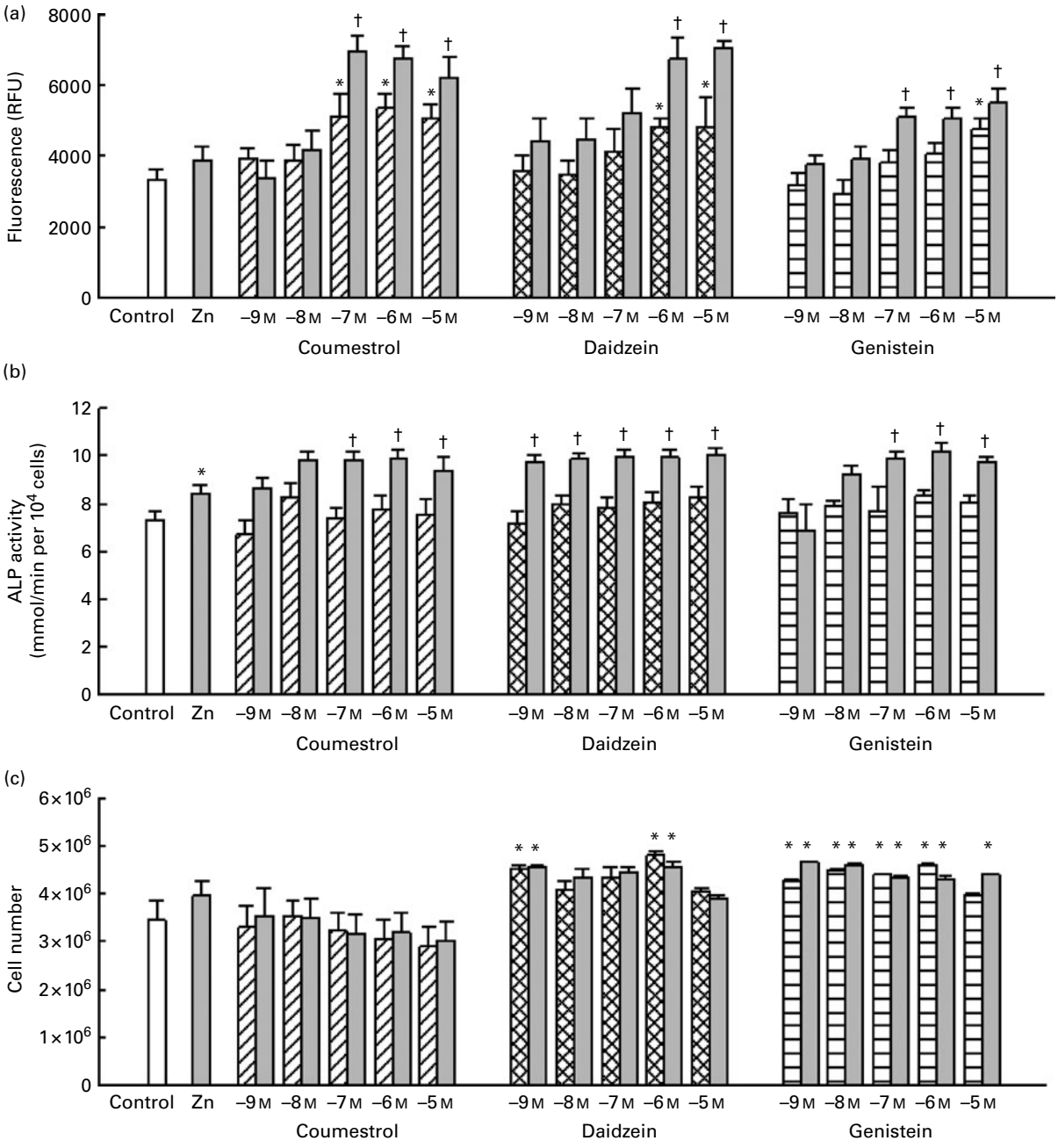

Fig. 3. Zinc augments the effect of phyto-oestrogens on osteoblast mineralisation and differentiation. Saos-2 cells were incubated with coumestrol (10^{-5} to 10^{-9} M, ▨), daidzein (10^{-5} to 10^{-9} M, ⊠) or genistein (10^{-9} to 10^{-5} M, ▤) with or without zinc (10^{-5} M, ▥) for 4–18 d. Mineralisation, osteoblast differentiation and proliferation were then assessed using (a) calcein incorporation, (b) alkaline phosphatase (ALP) activity and (c) 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assays. Values are means of three separate experiments, with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. *Mean values were significantly different from those of the control group ($P<0{\cdot}05$). †Mean values were significantly different from those of the zinc- or relevant phyto-oestrogen-treated group ($P<0{\cdot}05$).

with phyto-oestrogen alone, coumestrol (10^{-5} to 10^{-7} M), daidzein (10^{-5} to 10^{-6} M) and genistein (10^{-5} to 10^{-7} M). To assess whether the augmentative action of genistein, coumestrol and daidzein was mediated by an oestrogen-dependent mechanism, we cultured cells with concentrations of phyto-oestrogens shown to enhance mineralisation in the presence or absence of the oestrogen antagonist ICI 182780 (10^{-6} M). The antagonist had no effect on mineralisation in control cultures, but prevented the augmentative effect of genistein, daidzein or coumestrol in the presence or absence of Zn (Fig. 4), suggesting that phyto-oestrogens directly enhance osteoblastic mineralisation by an oestrogen receptor-dependent mechanism.

To determine the cellular mechanism by which Zn and phyto-oestrogens enhanced mineralisation, we examined their effect on osteoblast proliferation and ALP expression. As shown previously, Saos-2 cells constitutively express detectable levels of ALP in the presence of L-AA and β-GP,

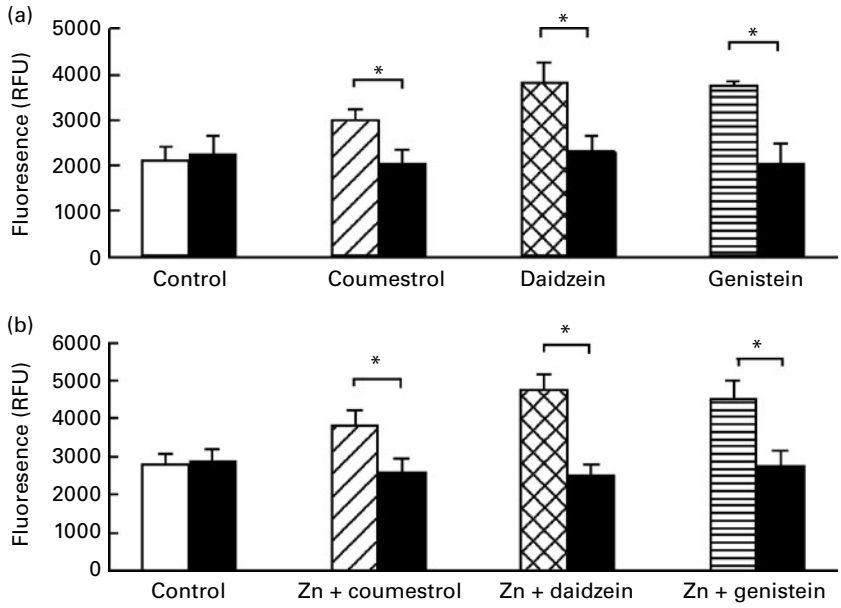

Fig. 4. The oestrogen antagonist ICI 182780 abolished the augmentative effect of phyto-oestrogens on mineralisation in the presence or absence of zinc. (a) Saos-2 cells were cultured with phyto-oestrogen concentrations shown to promote mineralisation and suppress osteoclast formation coumestrol (10^{-7} M, ▨), daidzein (10^{-5} M, ⊠), genistein (10^{-7} M, ▤) with or without ICI 182780 (10^{-6} M, ■). (b) Saos-2 cells were cultured with zinc (10^{-5} M) plus phyto-oestrogen concentrations shown to promote mineralisation coumestrol (10^{-7} M, ▨), daidzein (10^{-5} M, ⊠), genistein (10^{-7} M, ▤) with or without ICI 182780 (10^{-6} M, ■). Values are means of three separate experiments, with their standard errors represented by vertical bars. Differences between groups were assessed by one-way ANOVA. * Mean values were significantly different from the indicated group ($P<0{\cdot}05$). RFU, related fluorescence ratio.

which was significantly elevated in the presence of Zn for 4 d (Fig. 3(b)). The augmentative action of Zn was further enhanced in the presence of coumestrol (10^{-5} to 10^{-7} M), daidzein (10^{-5} to 10^{-9} M) or genistein (10^{-5} to 10^{-7} M). Peak interactions were noted at 10^{-5} M daidzein, 10^{-6} M coumestrol and 10^{-6} M genistein which induced a significant 1·34-, 1·24- and 1·21-fold increase in ALP activity compared to Zn-treated cultures.

While phyto-oestrogens and Zn clearly enhanced osteoblast differentiation, they had little effect on proliferation. Zn alone had no effect on osteoblast number (Fig. 3(c)). Similarly, coumestrol on its own or in combination with Zn had no proliferative effect although there was a trend towards lower osteoblast number in all groups. Concentrations of daidzein shown to enhance mineralisation also had no effect on proliferation although daidzein 10^{-6} and 10^{-9} M with or without Zn increased osteoblast number. Genistein (10^{-6} to 10^{-9} M) induced a modest but significant increase in cell number and Zn increased the effect of the highest genistein concentration examined (10^{-5} M).

Zinc blunts the augmentative action of phyto-oestrogens on type I collagen and osteocalcin expression

Bone matrix comprises two major elements, inorganic hydoxyapatite and a range of organic constituents. The organic component consists primarily of type I collagen and several non-collagenous proteins such as osteocalcin. During formation, osteoblasts first secrete organic elements to form osteoid which is subsequently mineralised during maturation. Aberrant osteoid formation or inadequate mineralisation, as occurs in vitamin D associated rickets or osteomalacia, compromises skeletal integrity and can lead to an increased fracture risk. Thus, to assess the effects of compounds on bone quality, both the level of mineralisation and organic constituents has to be considered. We therefore examined the effect of Zn and phyto-oestrogen concentrations shown to augment mineralisation and suppress osteoclast formation on type I collagen and osteocalcin expression. Zn alone had no effect on type I collagen or osteocalcin mRNA expression (Table 1). In contrast, all phyto-oestrogens significantly enhanced type I collagen mRNA expression, a marker that is expressed from progenitor stages of osteoblast differentiation; coumestrol (10^{-7} M) stimulated a 12·04-fold increase, daidzein (10^{-5} M) a 14·39-fold increase and genistein (10^{-7} M) a 7·35-fold increase (Table 1). Phyto-oestrogens also enhanced osteocalcin expression, a marker of mature osteoblast differentiation; coumestrol induced a 2·1-fold increase, daidzein a 16·7-fold increase and genistein a 3·1-fold increase.

Interestingly, the addition of Zn blunted the stimulatory effect of phyto-oestrogens on type I collagen and osteocalcin expression, although mRNA levels were still significantly above that of control. Thus, in contrast to a positive interaction with phyto-oestrogens on mineralisation, Zn appears to blunt the stimulatory action of phyto-oestrogens on organic components of bone matrix.

Zinc augments the effect of phyto-oestrogens on Runx2 expression but suppresses osterix expression

Osteoblast differentiation is regulated by a network of transcription factors that control gene expression. These include

Table 1. Zinc blunts the stimulatory effect of phyto-oestrogens on the expression of organic components of bone matrix

(Mean values and standard deviations)

	Type I collagen mRNA copies per 10^6 β-actin copies		Osteocalcin mRNA copies per 10^6 β-actin copies	
	Mean	SD	Mean	SD
Control	35 048	13 799	64	19
Zn	20 017	8045	41	11
Coumestrol (10^{-7} M)	422 137*	127 678	136*	19
Coumestrol (10^{-7} M) and Zn	86 339*†	2529	92*†	9
Daidzein (10^{-5} M)	504 560*	11 805	1066*	389
Daidzein (10^{-5} M) and Zn	119 396*†	9980	321*†	13
Genistein (10^{-7} M)	257 731*	86 054	200*	34
Genistein (10^{-7} M) and Zn	51 177†	12 014	96*†	9

* Mean values were significantly different from those of the control group ($P<0.05$).
† Mean values were significantly different from those of the relevant phyto-oestrogen alone ($P<0.05$).

Table 2. Zinc augments the effect of phyto-oestrogens on Runx2 expression

(Mean values and standard deviations)

	Runx2 mRNA copies per 10^6 β-actin copies		Osterix mRNA copies per 10^6 β-actin copies	
	Mean	SD	Mean	SD
Control	7152	2320	9037	862
Zn (10^{-5} M)	8952	1377	7693	362
Coumestrol (10^{-7} M)	23 114*	1678	13 856*	1505
Coumestrol (10^{-7} M) and Zn	34 591*†	7006	10 291†	443
Daidzein (10^{-5} M)	19 705*	1599	39 273*	1498
Daidzein (10^{-5} M) and Zn	44 631*†	8695	17 651†	3610
Genistein (10^{-7} M)	7148	778	11 049	1492
Genistein (10^{-7} M) and Zn	18 179*	1169	10 107	12

Runx2, Runt-related transcription factor 2
* Mean values were significantly different from those of the control group ($P<0.05$).
† Mean values were significantly different from those of the relevant phyto-oestrogen treatment alone ($P<0.05$).

Runx2 and osterix which are expressed in a temporally defined manner. Runx2 expression is elevated during the early stages of osteoblast differentiation, when it promotes the formation of immature osteoblasts from mesenchymal stem cells. Runx2 expression is then down-regulated during the formation of mature osteoblasts[33], whereas osterix expression is restricted to mature osteoblasts[33]. To determine the potential molecular mechanism mediating the effect of Zn and phyto-oestrogens on osteoblast differentiation, we examined the effect of phyto-oestrogens and Zn on Runx2 and osterix mRNA expression.

In keeping with their lack of effect on mineralisation, Zn or genistein (10^{-7} M) alone had no effect on Runx2 or osterix gene expression (Table 2). Similarly, coumestrol (10^{-7} M) and daidzein (10^{-5} M) enhanced Runx2 and osterix expression. Interestingly, the addition of Zn had a differential effect on phyto-oestrogen-induced Runx2 and osterix mRNA expression. Phyto-oestrogen-induced Runx2 expression was significantly enhanced by Zn, whereas Zn suppressed the augmentative effect of coumestrol and daidzein on osterix expression.

Discussion

Bone remodelling, the coupled process of osteoclastic bone resorption and osteoblastic bone formation, generates a skeleton optimised to current mechanical and mineral requirements. During a normal remodelling cycle, bone resorption and formation are balanced such that there is little net bone loss. However, this balance is disrupted in disorders associated with an increased fracture risk such as post-menopausal osteoporosis and osteomyelitis. Numerous studies suggest that dietary factors such as Zn and phyto-oestrogens have a positive impact on bone cell activity[18,34–36]; however, the cellular mechanism mediating this action is unclear and few studies have examined the effect of combinations of these factors on osteoclast and osteoblast activity *in vitro*. Our present study further clarifies the cellular mechanism through which these dietary factors may interact and suggests that appropriate combinations of Zn and phyto-oestrogens may augment mineralisation and further suppress bone resorption. These results strengthen the data for the use of combinations of Zn and phyto-oestrogens in the treatment of skeletal disorders.

Bone resorption is regulated by osteoblast/stromal-derived signals that stimulate osteoclast differentiation from monocytic precursors. Resorptive stimuli such as a fall in circulating calcium increase the expression of osteoblastic RANKL while decreasing OPG expression, a soluble decoy receptor for RANKL[32]. The subsequent binding of RANKL to its receptor RANK on the surface of non-committed monocytes activates a network of intracellular signals that induce the expression of osteoclastic genes such as TRAP and cathepsin K. In the absence of pro-osteoclastic stimuli, osteoblastic RANKL expression decreases and OPG concentrations rise; OPG then sequesters RANKL and thereby prevents RANK activation and osteoclast differentiation. Elevated RANKL levels and the presence of pro-osteoclastic inflammatory cytokines such as TNF-α are hallmarks of many osteolytic disorders[37,38]. At a cellular level, it is therefore possible to suppress osteoclast formation by either directly inhibiting the osteoclast precursor response to cytokine activation or alternatively by an indirect action on osteoblasts to lower RANKL:OPG ratios.

Previous studies suggest that phyto-oestrogens suppress osteoclast formation through both mechanisms. Coumestrol, daidzein and genistein directly inhibit osteoclast formation in response to pro-osteoclastic cytokines *in vitro*[18,39] and decreased osteoblastic RANKL:OPG ratios have been noted following treatment with a range of phyto-oestrogens[22,40]. Genistein and daidzein also reduce osteoblastic expression of other inducers of osteoclast differentiation including IL-6[41]. Zn has also been shown to reduce osteoclast formation *in vitro*[28,42,43], whereas Zn deficiency is associated with increased levels of osteoclast formation and bone resorption[44]. However, the cellular mechanism by which Zn suppresses osteoclast differentiation is unclear, as previous studies have used heterogeneous bone marrow cultures[28] or have shown inconsistent osteoclastic responses to changes

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in Zn status[44–46]. Similarly, interactions between Zn and phyto-oestrogens have not been widely investigated.

To help clarify this, we examined the direct effect of Zn on homogeneous RAW264.7 monocytic cultures and the indirect action on osteoblastic RANKL:OPG ratios. We noted no direct effect of Zn on TNF-α-induced osteoclast formation in RAW264.7 cells and Zn also had no effect on the anti-osteoclastic action of coumestrol, daidzein or genistein in these cultures. This differs from results using mouse bone marrow cultures where Zn significantly suppressed parathyroid hormone-induced osteoclast formation and combinations of Zn and genistein decreased RANKL-induced osteoclastogenesis[28]. The suppressive action noted in these studies may be due to an indirect effect of Zn mediated through stromal cells present in bone marrow cultures which are absent from RAW264.7 cultures. In keeping with this, we noted that Zn alone suppressed osteoblastic *RANKL:OPG* gene expression ratio and also augmented the suppressive effect of phyto-oestrogens. This assertion is strengthened by the studies of Holloway in which Zn suppressed bone resorption in the presence of added osteoblasts[42]. Thus, Zn-associated changes in osteoclast number are most probably mediated through an indirect action on osteoblasts rather than a direct effect on monocyte differentiation and appropriate concentrations of Zn and coumestrol or genistein may have a more pronounced anti-osteoclastic effect than either alone.

Serum phyto-oestrogen concentrations differ between populations and are dependent on an individual's diet. Asians who typically have a soya-rich diet have significantly higher phyto-oestrogen concentrations compared to Westerners[47]. The range of phyto-oestrogen concentrations examined in the present study reflects those measured in Asians (10^{-6} to 10^{-7} M) and Westerners (10^{-8} M). The anti-osteoclastic concentrations of genistein and coumestrol seen in this study are similar to those shown previously[18] and are in the range of levels measured in Asian populations but higher than those achieved by Western diets[47]. In contrast, typical Asian and Western diets are unable to generate serum concentrations of daidzein similar to those shown to suppress TNF-α-induced osteoclastogenesis in our studies. However, these concentrations could be achieved with daidzein supplementation which generates tissue levels several orders of magnitude higher than dietary sources[48]. Serum Zn concentration also varies between populations with diets that lack animal-sourced foods, leading to a high risk of Zn deficiency[49]. The Zn concentration used in our studies is similar to previous *in vitro* experiments, and is within normal serum reference ranges reflecting those achieved by healthy Western diets[50,51]. Thus, Zn tissue levels of this concentration could augment the anti-osteoclastic effect of coumestrol and genistein, which in turn could limit the excessive resorption associated with post-menopausal osteoporosis and osteomyelitis.

However, antiresorptives such as bisphosphonates only address part of the underlying pathology as they have little effect on the reduction in osteoblast function[52]. Antiresorptives also fail to restore bone that has already been lost and may in the long term lead to atypical fractures, as normal remodelling rates are required to repair micro damage[53]. An ideal therapeutic strategy would therefore rectify defects in both osteoclast and osteoblast activity. Osteoblastic bone formation is a tightly regulated process in which an organic extracellular matrix, consisting primarily of type I collagen and other non-collagenous proteins such as osteocalcin and osteonectin, is initially secreted[54]. Non-collagenous proteins may then have a role in controlling the subsequent mineralisation of the matrix regulating the nucleation and appropriate growth of hydroxyapatite crystals within osteoid. We found that Zn enhanced the stimulatory effect of coumestrol, daidzein and genistein on osteoblast mineralisation *in vitro*. Combinations of coumestrol and Zn and genistein and Zn had the most potent effect (10^{-7} M), whereas effects with diaidzein were only noted at 10^{-6} M and above.

In contrast to the beneficial action on mineralisation, Zn partly blunted the stimulatory effect of coumestrol, daidzein and genistein on type I collagen and osteocalcin mRNA expression. However, expression levels were still significantly above control, indicating that matrix formation was still enhanced. Thus, appropriate combinations of Zn, coumestrol, daidzein or genistein augment osteoblast function *in vitro,* enhancing the expression of components of the organic matrix and stimulating mineral deposition.

The augmentative action of Zn would at least in part appear to be due to increased expression of the marker of osteoblast differentiation ALP. Osteoblast-derived matrix vesicles contain high ALP levels and mutations in ALP lead to the genetic disorder hypophosphatasia which is characterised by poorly mineralised bone[55]. ALP promotes the initial stage of mineralisation by hydrolysing inhibitory pyrophosphate to generate inorganic phosphate needed for the initiation of hydroxyapatite deposition[54]. Thus, elevated ALP activity would be expected to enhance mineral formation. In contrast, the augmentative effect of phyto-oestrogens alone would not appear to be mediated through an effect on ALP as levels remained near control. Similarly, although daidzein and genistein stimulated a modest increase in osteoblast number, this was only seen at concentrations other than those shown to enhance mineralisation.

To further examine the mechanism by which phyto-oestrogens and Zn augment osteoblast function, we examined the expression of key intracellular regulators of osteoblast differentiation. Osteoblastogenesis is a sequential process involving multiple transcription factors that stimulate mesenchymal precursors to form immature pre-osteoblasts and ultimately mature osteoblasts[56]. The initial stage of osteoblastic lineage commitment is controlled by the selective expression of Runx2, which promotes the formation of immature osteoblasts characterised by the production of organic extracellular matrix components including type I collagen and osteocalcin. Homozygous loss of Runx2 is lethal due to the lack of osteoblasts and skeletal elements in mice[57], whereas heterozygous loss leads to cleidocranial dysplasia in humans and is associated with abnormal osteoblast development in mice[57,58]. With the formation of mature osteoblasts capable of mineralising osteoid, Runx2 expression falls whereas levels of osterix increase[59].

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Previous studies have shown that Zn enhances Runx2 expression[51], but to date no study has examined the effect of Zn on osterix expression. We found that anti-osteoclastic concentrations of coumestrol (10^{-7} M) and daidzein (10^{-5} M) significantly enhanced Runx2 expression and Zn significantly augmented Runx2 in the presence of all phyto-oestrogens. On the other hand, Zn blunted the stimulatory action of coumestrol and daidzein on osterix expression. Therefore, Zn appears to promote the expression of a transcription factor profile typical of early mature osteoblast with high Runx2 and low osterix levels. This profile is likely to explain the observed changes in organic matrix protein expression. The studies of Liu *et al.*[60] showed that Runx2 maintains osteoblasts in an immature state, with transgenic Runx2 expression suppressing type I collagen and osteocalcin production and preventing the formation of mature osteoblasts. Osteocalcin levels are comparatively low in pre-osteoblasts when Runx2 expression peaks, and osteocalcin levels subsequently rise as Runx2 expression decreases in mature osteoblasts. Thus, the blunting of type I collagen and osteocalcin expression is in keeping with Zn promoting the formation of early rather than late stages of mature osteoblast differentiation.

Our data show that Zn augments the indirect anti-osteoclastic action of coumestrol and genistein at concentrations typically generated by soya-rich diets. Interactions between Zn and anti-osteoclastic phyto-oestrogen concentrations were also noted for osteoblast differentiation and function. Appropriate combinations of Zn and phyto-oestrogens increased ALP activity, extracellular matrix expression and mineralisation. This effect may be due to Zn inducing the formation of an early mature stage of osteoblast differentiation. These results strengthen data for the use of combinations of Zn and phyto-oestrogens in the treatment of skeletal disorders.

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