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The Effect of Phytoestrogens on Bone and T Cells Differentiation and Activity

By

Sahar Saadi Karieb

A thesis submitted to the Plymouth University in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biomedical and Biological Sciences
Faculty of Science and Technology

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Dedicated to my beloved mother, Ashwaq Khairallah

The effect of phytoestrogens on bone and T cells differentiation and activity

Sahar S. Karieb

Abstract

The fall in circulating oestrogen (E2) after the menopause leads to an increased rate of bone remodelling, excessive osteoclast activity and a greater fracture risk. Until recently hormone replacement therapy (HRT) was prescribed to post-menopausal women to prevent bone loss, however HRT is associated with an elevated incidence of cardiovascular disease, stroke and cancer. These side-effects led to an interest in naturally occurring compounds with oestrogenic action such as phytoestrogens (PEs), which are non-steroidal-plant derived compounds. Human trials and animal studies suggest a beneficial effect of PEs on bone mass, although their ability to modify osteoclast formation in response to key inflammatory cytokines has not been examined. The aim of the following studies was to determine the effect of physiologically relevant concentrations of genistein, coumestrol and daidzein on TNF-α-induced osteoclast formation, osteoblasts differentiation and T cell activity.

Genistein (10^{-7} M), daidzein (10^{-5} M), and coumestrol (10^{-7} M) significantly reduced TNF-α-induced TRAP positive osteoclast formation and bone resorption, which was prevented by the E2 antagonist ICI 182,780. The suppressive action on osteoclast formation was associated with a significant reduction in TNF-α-induced *c-fos* and *NFATc1* mRNA expression and NFATc1 nuclear translocation. Constitutive c-fos expression prevented the inhibitory action of PEs on osteoclast differentiation, resorption and *NFATc1* expression.

The effect of PEs, in the presence or absence of the anabolic nutritional factor zinc, on osteoblasts differentiation and bone nodule formation was examined *in-vitro*.

Coumestrol (10^{-5} to 10^{-7} M), daidzein (10^{-5} to 10^{-6} M) and genistein (10^{-5} M) enhanced bone nodule formation and ALP activity in human osteoblasts, and this effect was significantly augmented in the presence of zinc (10^{-5} M). Furthermore, PEs and zinc increased *Runx2* mRNA expression and Zn^{2+} augmented the inhibitory effect of PEs on RANKL/OPG ratio. This suggests that in addition to the direct inhibitory effect on osteoclast formation PEs also in-directly reduce the osteoblastsic stimulus for osteoclast formation and promote bone formation.

E2 deficiency is thought to promote osteoclastogenesis by modifying Thelper1 (Th1) cell proliferation and inflammatory cytokine production in particular TNF-α. I therefore examined the effect of PEs on T cell proliferation and inflammatory cytokine production. All PEs prevented the augmentative effect of con A stimulated T cells on osteoclast formation in co-culture. However the mechanism of action varied, genistein reduced con A stimulated TNF-α, IL-1β and RANKL expression with little effect on viability, coumestrol decreased cell viability and TNF-α expression whereas the inhibitory effect of daidzein was mediated via suppression of viable T cell number.

This study provides novel evidence that PEs have multiple effects on bone cell activity, directly inhibiting TNF-α-induced osteoclast formation, reducing the osteoblasts and T cell derived stimulus for osteoclast formation and augmenting osteoblasts differentiation and bone formation. Thus, PEs have a potential role in the treatment of post-menopausal osteoporosis and inflammatory skeletal disorders and that the beneficial effect noted in previous studies is mediated through multiple mechanisms.

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Abbreviations

Abbreviation	Glossary
1,25(OH)D3	Vitamin D3
ALP	Alkaline phosphatase
AP-1	Activator protein
APC	Antigen-presenting cell
AR	Androgen receptor
ATF4	Activating transcription factor 4
ATJ18	zing finger-containing factor
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
CBfβ	Core-binding factor
CHD	Coronary heart disease
Con A	Concanavalin A (a lectin from *Canavalia ensiformis*)
CTR	calcitonin receptor
DAP12	DNAX-activating protein of 12 kDa
DC-STAMP	Dendritic specific transmembrane protein
DKK1	Dickkopf-related protein 1
Dlx5	Distal-less homeobox
DNA	Deoxyribonucleic acid
E2	Oestrogen
EC_{50}	Half maximal effective concentration
EDTA	trypsin-ethylenediaminetetraacetic acid
EREs	Oestrogen response elements
ERK	extracellular-signal-regulated kinases
ERα	Oestrogen receptor-alpha

ERβ	Oestrogen receptor-beta
FCRγ	Fc-gamma receptor
FZD	7-transmembrane-spanning Frizzled
Gla	Carboxyglutamic acid
GSK	Glycogen synthase kinase
HDL	High density lipoprotein
HRT	Hormone replacement therapy
IFNGR1	Interferon-gamma receptor 1
IFN-β	Interferon-beta
IFNγ	Interferone-gamma
IGF	Insulin-like growth factor
IGF-I	Insulin-like growth factor 1
IKKα	inhibitor of kB kinase alpha
IKKβ	inhibitor of kB kinase beta
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-7	Interleukin-7
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun NH_2-terminal kinase
LDL	Low-density lipoprotein
LEF	lymphoid enhancer-binding protein family transcription factors
LRP	LDL-receptor protein
MAPK	Mitogen-activated protein kinases
M-CSF	Macrophage-colony stimulating factor
MGP	matrix Gla protein
MHC II	Major histocompatibility complex class II
MSX2	Msh homeobox 2

NFAT	Nuclear factor activated T cells
NFATc1	Nuclear factor activated T cells, cytoplasmic 1
NFκB	Nuclear factor-keppa light-chain-enhancer of activated B cells
NIK	*NF-κB* binding kinase
NTX	Carboxyl-telopepetide of type 1 collagen
OCN	Osteocalcin
ODF	Osteoclast differentiation factor
O-DMA	O-desmethylangolsin
OPG	Osteoprotegerin
OPN	Osteopontin
OSCAR	Osteoclast-associated receptor
P1PP	Amino-terminal propeptide of type 1 collagen
PBMC	Peripheral blood mononuclear
PEs	Phytoestrogesns
PGE2	Prostaglandin E2
FGF23	Fibroblast growth factor 23
PPARγ	Peroxisome proliferator-activated receptor
PSA	Prostate specific antigen
PTH	Parathyroid hormone
PTK	Protein-tyrosine kinase
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
Redox	Reduction-oxidation reaction
RGD	Arg-Gly-Asp-containing proteins osteopontin (bone sialoprotein)
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2

SERMs	Selective oestrogen receptor modulators
SHBG	Sex hormone binding globulin
Smad	The mothers against decapentaplegic
SYK	Tyrosine kinase
TCF	T-cell factor
TGF-β	Transforming growth factor-beta
TNFR	Tumour necrosis factor receptor
TNF-α	Tumour necrosis factor-α
TRAFs	TNFR-associated factors
TRAP	Tartrate resistant acid phosphatase
TREM2	Triggering receptor expressed on myeloid cells 2
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
Zap	Cytoplasmic protein tyrosine kinase

Author's Declaration

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Chapter One : Bone Anatomy, Formation and Resorption

Chapter 1

1.1 Introduction

Bone is a dynamic and specialised connective tissue that is able to resist deformation, but at the same time be flexible enough to allow energy absorption during impact loading. There are five categories of bones: long bones, short bones, flat bones, sesamoid and irregular bones. Bone has a variety of functions: firstly, protective and supportive functions shielding vital internal organs such as bone marrow and brain. Second, mechanical loading and movement. The third function of bone is in the maintenance of mineral homeostasis, as bone serves as a reservoir of calcium and phosphate (Clarke, 2008).

Bone serves as a mineral reservoir for calcium and phosphorus and releases these minerals to the blood to maintain homeostatic balance. Calcium plays an important role in skeletal mineralisation and its deficiency can lead to osteomalacia (Sambrook, 2001). Bone also plays an important role in movement providing articulation for skeletal muscles tendons, ligaments and joints. In addition, bone has a metabolic function through storage of fat via yellow bone marrow (adipose cells) which provides a local energy reservoir in the bone (Gimble et al., 1996, Lecka-Czernik, 2012).

Recent publications suggest that in addition to classic roles bone can also be considered an endocrine organ. Evidence has been accumulating that bone cell derived FGF23 and osteocalcin have important roles in regulating kidney function and glucose metabolism. Osteoblast derived osteocalcin has an endocrine action on pancreatic β-cells, stimulating proliferation and insulin production (Fukumoto and Martin, 2009). A positive relationship was found between osteocalcin levels and insulin production and glucose tolerance. Animal studies also suggest an additional peripheral effect of osteocalcin promoting insulin sensitivity and reducing the accumulation of adipose tissue. It is therefore unsurprising that lower levels of osteocalcin are found in diabetics than

healthy individuals and this may contribute to disease progression (Hwang et al., 2012, Hwang et al., 2009, Pollock et al., 2011). Furthermore, osteocytic FGF23 promotes renal phosphorus secretion by inhibiting the 1alpha hydroxylation of vitamin D.

Bones are not static entities, bone structure is constantly altered during growth, or in response to changes in mechanical stress, circulating mineral or in response to damage. This structural change occurs through a process of remodelling, which involves bone resorption followed by bone formation. Remodelling occurs through the action of osteoclast which are responsible for the production of HCl and proteases which enable the formation of resorption pits, and osteoblasts which secret the organic and inorganic components of bone matrix to form new bone. During bone formation a proportion of osteoblasts become encapsulated in the matrix becoming osteocytes which have a role in mechano-transduction (Matsuo and Irie, 2008).

1.2 Bone Structure

There are two types of bone tissue: cortical bone (also termed compact bone) that has a primary role in resisting mechanical loading and trabecular bone (also termed spongy or cancellous bone) which is the major site of calcium exchange and also has a secondary mechanical role. Cortical bone is the out layer of bone and composes 80% of the adult human skeleton; it has a low porosity and provides protection and support. Cortical bone is composed of osteons, which are the basal structural unit of bone, also called Haversian systems, these are formed from concentric lamella of bone tissue that also contain blood vessels and nerve fibres (Clarke, 2008). The outer surface of cortical bone is coved by a membrane termed the periosteum (Figure 1.1).

Trabecular bone consists of an irregular lattice of thin columns which are typically orientated in the same direction as the predominate strain direction experienced by that bone. Trabecular bone composes 20% of the human skeleton and has a number of

functions such as mineral homeostasis (Sambrook, 2001), it can also withstand compressive load better than cortical bone due to the absorbance of energy efficiently. The strength of trabecular bone depends on the extent of connectivity between adjacent trabeculae and loss of this leads to a drastic reduction in mechanical competence (Chavassieux et al., 2007).

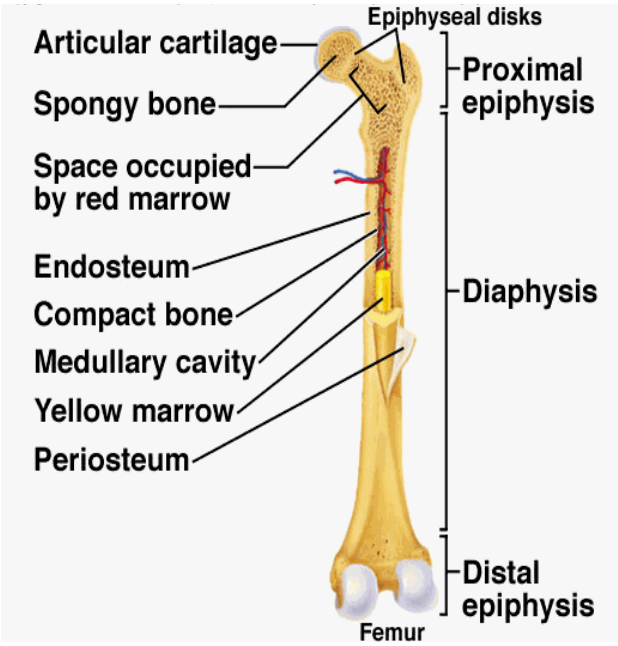

Figure 1.1. Long Bone structure and anatomy. Adapted from http://phsgirard.org/Anatomy.html.

1.3 Bone Tissue

Bone tissue has both organic and inorganic elements. The organic component of bone matrix is composed mainly from type I collagen, a range of non-collagenous proteins and proteoglycan. Type I collagen forms from two identical α1 chains and a single α2 chain which combine to form a molecule of type I collagen that provides the flexibility and strength of bone. Type I collagen then undergoes various post-translation modifications to form mature fibres. The organic component of the matrix is synthesized first and is subsequently mineralised with inorganic calcium hydoxyapatite crystals ($Ca[PO_4]_6[OH]_2$) that increase the material stiffness of bones (Follet et al., 2004). Collagen fibrils provide the flexibility and elasticity to bone whereas the bone mineral provides rigidity (Chavassieux et al., 2007). Moreover, the direction of collagen fibres is often related with the direction of load (Martin and Boardman, 1993).

In addition to collagen, there are also non-collagenous proteins which are synthesized by bone cells and make up 15% of bone protein. Osteoblasts produced several proteins that play an important role in bone matrix mineralisation including enzymes such as alkaline phosphatase (ALP), calcium binding proteins, carboxyglutamic acid (GLa), prophosphatase, ion channels, signal transduction molecules such as 14-3-3 family members and other related proteins. Modulation of these proteins' expression may effect bone formation and resorption (Xiao et al., 2007).

1.4 Osteoblasts

Osteoblasts are derived from mesenchymal stem cells and are responsible for the synthesis and deposition of the organic and inorganic components of bone matrix. The formation of a mature bone forming osteoblasts is a sequential process that involves the development of immature osteoblasts prior to the formation of mature cells. Mature osteoblasts are characterised by the expression of several markers including ALP and

type-I collagen which are both important for bone matrix synthesis and mineralisation (Murshed et al., 2005). Osteoblasts also secrete other proteins characteristic of bone including osteocalcein (OCN), osteopontin (OPN), matrix Gla proteins (MGP), bone sialoprotein (BSPs), osteonectin, proteoglycan and growth factors such as bone morphogenetic proteins (BMPs) and TGF-β (Young et al., 1992, Mundlos and Olsen, 1997, Young, 2003, Olsen et al., 2000, Harada and Rodan, 2003, Ralston and de Crombrugghe, 2006). Following the completion of bone formation, osteoblasts can either undergo apoptosis, become osteocytes by embedding in the matrix or transform into lining cells that cover inactive bone surface (Clarke, 2008). Osteoblastsic lineage cells respond to various hormonal, growth factors and cytokines such as BMPs, IGF, parathyroid hormone and prostaglandin E2 (PGE2), which modify the expression of osteoblastsic transcriptional factors such as *Runx2* and *Osterix* which are some of the earliest osteoblastsic markers and play a critical role in osteoblast differentiation (Mackie, 2003, Karsenty, 2003). Moreover, *Runx2* in turn regulates expression of genes encoding VEGF, osteocalcin, RANKL and collagen (Lian et al., 2006). In addition to their important role in bone formation, osteoblasts also play a role in osteoclast differentiation by secreting cytokines such as M-CSF, RANKL and OPG which bind to specific receptors expressed by osteoclast precursors and subsequently promoting osteoclast formation.

1.4.1 Osteoblast differentiation

Osteoblast differentiation from mesenchymal cells is under the control of numerous transcription and growth factors. Mesenchymal cells are pluripotent having the capacity to differentiate under appropriate conditions into several lineages such as osteoblasts, chondrocytes, adipocytes, myoblasts and fibroblasts (Barry and Murphy, 2004, Baksh et al., 2004) (Figure 1.2). The proliferation and differentiation of osteoblast is dependent

on the coordinated expression of multiple transcription factors such as *Runx2* and *osterix (osx)* (Nakashima et al., 2002, Karsenty and Wagner, 2002). In brief, preosteoblast reside near the bone surface and express the early marker of osteoblast differentiation ALP (Luu et al., 2007, Karsenty, 1999). Subsequently preosteoblast differentiate into active mature osteoblast which display characteristic changes: a large nucleus, extensive endoplasmic reticulum and enlarged Golgi which is important to support the secretion of matrix proteins such as type I collagen (Olsen et al., 2000).

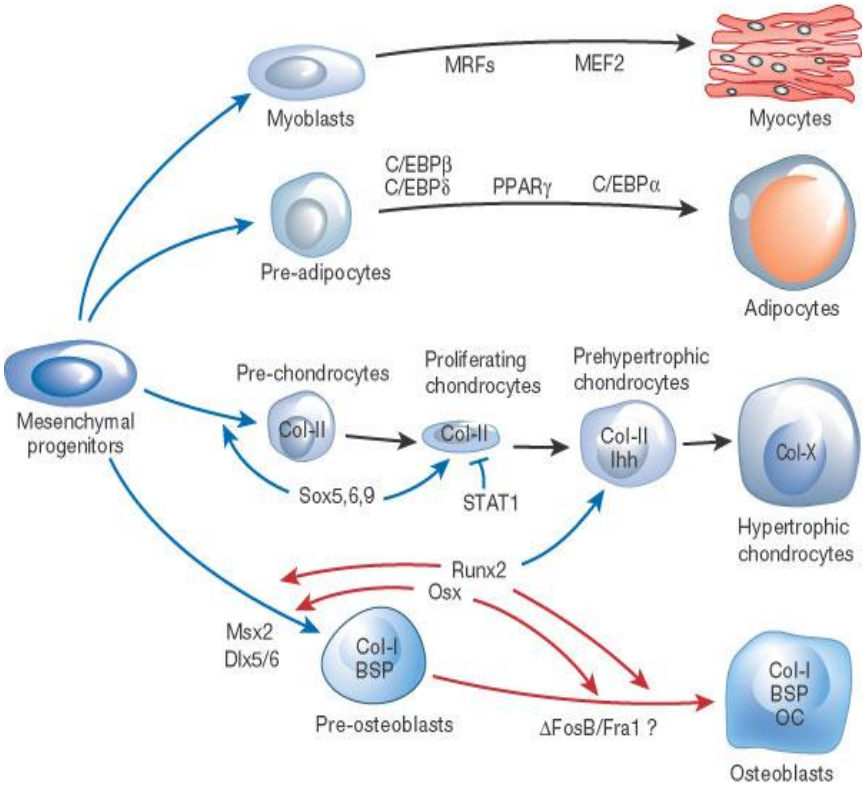

Figure 1.2. Mesenchymal cells differentiate into osteoblasts under *Runx2* and *osterix* control. Mesenchymal cells also give rise to adipocyte, myocyte and chondrocytes under the control of specific transcriptional factors. Osteoblast is differentiated under the control of *Runx2*, a key regulator of osteoblast differentiation pathway, while *osterix* is downstream of Runx2 and is important for osteoblast maturation. From (Harada and Rodan, 2003).

1.5 Key Regulators of Osteoblast Formation

1.5.1 TGF/BMP

Transforming growth factors (TGF) are a family of 30 secreted dimeric polypeptide cytokines that regulate numerous cellular functions including differentiation, proliferation and apoptosis (Blobe et al., 2000, Feng and Derynck, 2005, Mishra et al., 2005). These include TGFβ, TGFα and BMPs. With regards to TGFβ there are three major isoforms TGFβ1, 2 and 3, which are conserved in mammals (Massague, 1998). TGFβ members affect cellular function through two types of receptor I and II transmembrane serine/therionine kinase.

TGFβ has been reported to play a critical role in mammalian cellular function and the disruption of TGFβ signals has a detrimental effect on the cardiovascular, skeletal, and muscular systems (Serra and Chang, 2003). Furthermore, it has been shown that fibrodysplasia ossificans progressiva, a rare genetic disorder associated with ectopic formation of bone, is caused by a gain of function mutation in the TGFβ type I receptor ACRVI (Shore et al., 2006). Additionally, BMPs, provide further important signals that are essential for full osteoblast differentiation and maturation (Krane, 2005, Chen et al., 2004). Moreover, it has been reported that Wnt/β-catenin signalling synergises with BMP signalling to promote osteoblast differentiation (Mbalaviele et al., 2005). BMPs also have the capacity to stimulate endochondral bone formation (Okamoto et al., 2006, Cao and Chen, 2005). For instance BMPs 1-7 are expressed in skeletal tissues, while BMP 2, 4 and 6 are detected in cultures of osteoblastsic cells (Canalis et al., 2003, Anderson et al., 2000). BMP-2, 6, 7 and 9 have been shown to induce osteogenesis *in-vivo* and *in-vitro* (Kang et al., 2004, Luu et al., 2007), whereas BMP3 acts as a negative regulator of bone formation (Daluiski et al., 2001). Interestingly, BMPs induce *Runx2* in mesenchymal progenitors through the action of BR-Smad to promote osteoblast

differentiation (Maeda et al., 2004). In contrast, BMP2 has been shown to induce the expression of *osx* in chondrocytes and mouse progenitors through a *Runx2*-independent mechanism (Lee et al., 2003, Yagi et al., 2003). In addition, BMP2 can effect *osx* expression via p38 and ERK pathways in osteogenic culture (Celil et al., 2005).

The mothers against decapentaplegic (Smad) transcription factors play a critical role in TGF-β and BMP signalling (Miyazawa et al., 2002). Smad consist of three major classes: receptor-regulator-Smad (R-Smad), common partner BMP and TGF-β mediator Smad (Co-Smad), and inhibitory Smad (I-Smad). R-Smad act as activator of BMP or TGF-β thus called also (BR-Smad or TR-Smad). Co-Smad (Smad 4 and 3) act as a partner of BMP and TGF-β activity, while I-Smad (Smad 7) acts as an inhibitor of R-Smad phosphorylation.

BMP signal transduction involves both Smad-dependent and independent pathways such as ERK, JNK and MAP kinase (Derynck et al., 2001). Following receptor activation, receptor Smads are phosphorylated and then form complexes with co-Smad members (3 &4) (Dennler et al., 2002). This exposes nuclear localisation signals on the surface of the dimer which enables nuclear translocation (Moustakas et al., 2001, Li and Cao, 2006, Wrana, 2000). Inhibitory Smads (Smad 7) prevent receptor Smad binding by binding to Smad 4, to activated receptors and also promote the proteolytic destruction of activated Smad.

1.5.2 Wnt Signalling

Wnts are a family of secreted glycoproteins and are ligands for 7-transmembrane-spanning frizzled (FZD) receptors. These proteins are involved in numerous aspects of cellular biology, cell proliferation and differentiation. Wnt proteins released from cells or expressed on the surface of activated cell bind to FZD/LRP5/6 to form a complex. Wnt are important for the development of numerous tissues including bone, and its

pathways are keys to osteoblast differentiation. Aberrant signalling in this pathway causes a wide range of diseases such as cancer and degenerative diseases (Reya and Clevers, 2005, Clevers, 2006). Multiple pieces of evidence suggest that Wnt signalling plays a critical role in the promotion of osteoblast differentiation and bone development (Bergwitz et al., 2001, Fischer et al., 2002). Wnt proteins effect bone biology through effects on cell growth, proliferation, differentiation and lifespan by several pathways such as Wnt/β-catenin or canonical pathway (Westendorf et al., 2004, Rawadi and Roman-Roman, 2005).

Wnt proteins are divided into different classes: the first group activates the Wnt/β-canoncial signalling pathway that is implicated in the formation of complexes between Wnt-FZD proteins and LDL-receptor protein 5 or 6 (LRP5 or 6 receptors) (Tamai et al., 2000, He et al., 2004). Both LRP5 and 6 contain extracellular domain with a consensus with EGF (epidermal growth factor), while non-canoncial Wnt5a class binds to FZD and activates heterotrimeric G proteins, which in turn increase intracellular calcium via protein kinase C-dependent mechanisms or stimulate c-Jun N-terminal kinase (JNK)-dependent changes in cytoskeletal structure (Veeman et al., 2003) (Figure 1.3).

Canonical Wnt pathway occurs when Wnt3a bind to the receptor complex of frizzled and LRP5 or LRP6; signals are generated through dishevelled, Axin and Frat-1 proteins that disrupt the protein complex and inhibit the activity of glycogen synthase kinase 3 (Gsk3) causing hypophosphorylation of its substrate β-catenin followed by stabilization of β-catenin followed by its accumulation in the cytosol. The accumulated β-catenin translocates into nucleus and work together with TCF/LEF induces the expression target genes of osteoblast (Hay et al., 2005). Non-canonical Wnt pathway start after Wnt5a binding to Frizzled and Ror1 or Ror2 receptors which in turn activates the Planar cell polarity pathway and calcineurin-dependent mechanism (Takahashi et al., 2011) (Figure 1.3). The Wnt5a pathway may also play an important role in RANKL-induced

osteoclast formation (Maeda et al., 2012). While β-catenin decreases bone resorption by up regulating OPG expression and decreasing RANKL expression *in-vitro* (Spencer et al., 2006).

Wnt/β-catenin signalling plays an important role in osteoblast differentiation as well as in fracture healing and osteoclast differentiation. Recent studies examining the importance of Wnt/β-catenin signalling in osteoblasts formation using conditional inactivation of this pathway, it's revealed that β-catenin activity is critical for osteoblasts maturation and consequently for bone formation and development in endochonderal and intramembranous bone (Day et al., 2005, Hill et al., 2005, Hu et al., 2005). However, it has been showed that Wnt/β-catenin signalling in osteoblasts may coordinate postnatal bone formation by controlling both osteoclast and osteoblast differentiation (Holmen et al., 2005). Canonical Wnt signalling is modulated by *Runx2* and *osx*. Furthermore, β-catenin/TCF1 increases *Runx* 1 and 2 promoter activity (Gaur et al., 2005). Recent studies have shown there is a possibility to use Wnt signalling as an anabolic agent in bone. Interestingly, humanised monoclonal antibody against sclerostin improved bone anabolism in post-menopausal men and women (Padhi et al., 2011).

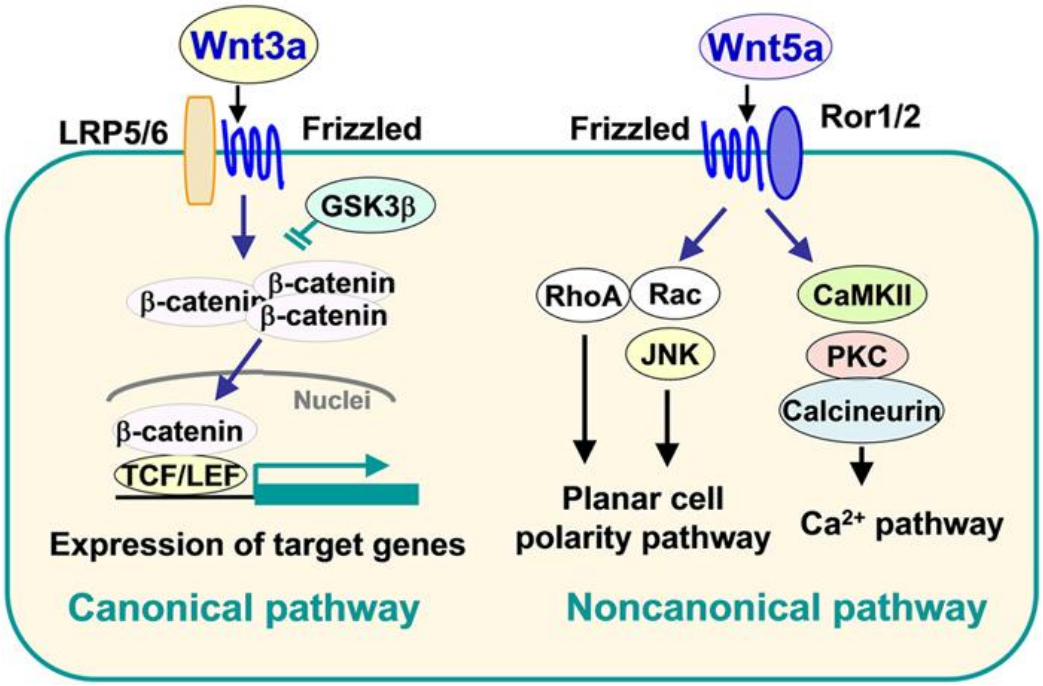

Figure 1.3. A schematic diagram of Wnt signalling pathway. The canonical pathway starts after binding of Wnt to FZD, LRP5/6 complex receptor and this activated Dsh and causing β-catenin phosphorylation. β-catenin accumulation and translocation to the nucleus and binds to transcriptional factors such as T-cell factor (TCF)- and lymphoid enhancer-binding protein (LEF)-family transcription factors mediated transcription of target genes. The noncanonical Wnt (Wnt5a) binds to of Frizzled and Ror1 or 2 receptor complex, and this binding activates planar cell polarity through RhoA Rac and JNK. From (Takahashi et al., 2011).

1.5.3 Osteoblastsic transcription factors (Runx2, Osterix, TAZ and ATF4)

Runt-related transcription factor 2 (*Runx2*) (also called cbfa1 OSF-2 and AML3) is a master transcription factor for osteoblast differentiation. *Runx2* is one of three transcriptional factors belonging to Runt transcription factor family which consists of *Runx1*(cbfa2, PEBP2AB, cbfa2 and AML1), *Runx2* (PEBP2aA, cbfa1 and AML3) and *Runx3* (PEBP2aC, cbfa3 and AML2) (Lian et al., 2003). There are two isoforms of Runx2, type I and type II, which have different N-terminal sequences. Runx2 is expressed in several organs and tissues such as thymus, bone tissues and calcified cartilage (Ogawa et al., 1993, Banerjee et al., 1997) and is essential for cartilage mineralisation and skeletal development. Runx2 contains a 128 amino acid DNA binding motif and a binding domain that is necessary for dimerization with several other transcription factors (Karsenty, 2000). Cbfβ (core-binding factor) is a co-transcription factor that modifies the DNA-binding affinity of Runx family members. Both Runx2 isoforms have a similar activity in the presence of cbfβ, while in the absence of cbfβ, Runx2-II activity was higher that Runx2-I, suggesting that Runx2 isoforms exert their activity via cbfβ-dependent mechanism (Kanatani et al., 2006).

Runx2 has been identified as a master transcription factor for controlling skeletal formation and mineralisation by promoting osteoblast differentiation at early stages of differentiation (Karsenty, 2000, Kern et al., 2001). Runx2 also plays an important role in maturation and maintenance of chondrocytes as well as vascular invasion into cartilage (Komori, 2005, Zelzer et al., 2001). Interestingly, Runx2 is also important in terminal stages of chondrocytes maturation (Drissi et al., 2002, Iwamoto et al., 2005, Dong et al., 2006). The expression of Runx2 during bone formation regulates osteoblast gene expression (Komori et al., 1997, Otto et al., 1997), and loss of Runx2 activity results in a lack of bone formation. *Runx2* regulates several genes indicative of osteoblast differentiation and function including ALP and osteocalcin (Otto et al., 1997,

Choi et al., 2001, Kim et al., 1999, Komori, 2005). Furthermore, *in-vitro* studies showed that Runx2 expression in skin fibroblast leads to osteoblast specific gene expression demonstrating that Runx2 is sufficient to induce the initial stage of osteoblast differentiation (Ducy et al., 1997).

The critical role of Runx2 in bone development was demonstrated through gene ablation experiments and Runx2-null mice die soon after birth and have reduced bone formation and mineralisation rates (Komori et al., 1997, Otto et al., 1997). Moreover, Runx2 overexpression induced a lethal skeletal phenotype including dwarfism and precocious mineralisation (Ueta et al., 2001). Over-expression of Runx2 in chondrocytes results in chondrocyte hypertrophy and endochondral ossification.

Several transcription factors co-operate with Runx2 to regulate its action on osteoblast differentiation, and some of these factors provide co-stimulatory signals while others repress Runx2 activity by affecting DNA binding activity or transactivation potential. For instance CBFβ (PEP2β) enhances Runx2 activity to induce osteoblastsic transcription genes, and lack of this protein during embryonic skeleton development blunts bone formation (Yoshida et al., 2002). Moreover, Distal-less homeobox (Dlx5) and msh homeobox 2 (Msx2) expressed in early stage of osteoblast differentiation can also interact with Runx2 activity. Dlx5 functions as a co-activator while Msx2 act as a transcriptional repressor, and they are also described as essential factors for intramembranous ossification and skeleton development (Bendall and Abate-Shen, 2000). Runx2 is also activated by MAP kinase pathways (Franceschi et al., 2003).

In contrast several proteins repress Runx2 activity via different mechanism including binding to the Runt domain to prevent DNA binding, removing cytoplasmic Runx2 or binding to the nuclear matrix targeting Runx2 domain. ATJ18 (zing finger-containing factor) was shown to reduce Runx2 during osteoblast differentiation through DNA binding competition and decreased ALP activity and Runx2-mediated osteacalcin

promoter activation (Jheon et al., 2001). Collectively, these findings indicate that Runx2 plays a critical role in bone development and expression of major bone matrix genes in early stage of osteoblastsogenesis.

Osterix (*Osx*) is a zinc-finger-containing transcription factor expressed by osteoblasts during embryonic development and is essential for bone formation (Komori, 2006, Nakashima et al., 2002). Osterix has a DNA binding domain that consists of three C_2H_2-type zinc fingers at its C-terminus. It also contains a proline and serine-rich transcription domain and activates genes, amongst others that encode for type I collagen and osteocalcein. Osx is well established as an important regulator of osteoblast differentiation during intramembranous and endochondral ossification. Osx-null mice have no cortical or trabecular bone and mesenchymal cells can not differentiate into osteoblasts in these mice (Nakashima et al., 2002), while the formation of cartilage is normal in osx-null mice (Inada et al., 1999). In addition, osx mutants exhibited a lack of osteoblast markers such as osteocalcin, osteopontin and bone sialoprotein as well as a reduction in the expression of Colla1 (Nakashima et al., 2002).

Osterix expression appears to be dependent on Runx2 during osteoblast differentiation as osx is not expressed in Runx2-null mice, whereas Runx2 is expressed in osx-deficient mice (Nakashima et al., 2002). In addition osx expression in response to BMP-2 and insulin-like growth factor I (IGF-I) in mesenchymal cells occurs via MAPK and protein kinase D (PKD) signalling (Celil and Campbell, 2005). Moreover, this study observed that Runx2 is important but not sufficient for the induction of BMP-2 mediated osx expression, suggesting that other factors may be involved. Interestingly, it has been reported that NFATc1 signalling cooperates with osx to accelerate osteoblast differentiation (Koga et al., 2005).

The transcription factor TAZ is a recently described co-activator that regulates mesenchymal differentiation into osteogenic or adipocyte lineages (Komori, 2006).

TAZ modifies various cellular functions including differentiation, cell cycle progression and apoptosis via binding to 14-3-3 proteins (Hong et al., 2005). TAZ contains a ubiquitin-associated protein (UAP)-like domain that binds to pro-pro-amino acid-Try motifs found in Runx2 and the adipogenic transcription factor PPARγ (Hong and Yaffe, 2006). TAZ has an important role in osteoblast differentiation enhancing the transcriptional activity of Runx2-driven genes in terminal stages of osteoblast differentiation, and suppressing adipocyte differentiation via inhibition of adipogenic PPARγ-induced gene transcription (Hong et al., 2005). This leads to the selective expression of genes associated with osteoblast differentiation and thereby reinforces osteoblastsic lineage switching.

Activating transcription factor 4 (ATF4) is a basic domain-leucine zipper protein (bZip) which has been shown to be a key regulator of osteoblast differentiation (Komori, 2006). ATF4 has been reported to induce the expression of osteoblast-specific genes such as type I collagen and osteocalcin (Yang et al., 2004). Furthermore, ATF4 stimulation in non-osteoblastsic cells induced osteocalcin promoter luciferase construct activity and osteocalcin expression suggesting that ATF4 similar to other osteoblastsic factors has the ability to induce osteoblast-specific gene expression in non osteoblastsic cells (Yang and Karsenty, 2004). Additionally, cooperative interaction between Runx2 and ATF4 promotes osteoblast-specific osteocalcin gene expression and this may present a novel intramembranous mechanism regulating Runx2 activity and osteoblast differentiation (Xiao et al., 2005). The activity of ATF4 is modified by the inhibitor FIAT, elevated FIAT levels reduce ATF4 activity, and transgenic FIAT mice are osteopenic and display reduced markers of osteoblastic differentiation (Yu et al., 2006). The inhibitory action of FIAT is primarily due to leucine zipper mediated dimerization with ATF4 and thereby prevents transcription of osteoblastsic genes.

In addition to the various pathways and transcription factors mentioned above other factors may also play a significant role in the regulation of osteoblast differentiation and bone formation such as c-fos, Fra1, Jun, homeobox-containing transcription factors Msx (1,2,3), Knox-20 and SP3 (Harada and Rodan, 2003, Komori, 2006). Furthermore, NFATc1 signalling may play an important role in osteoblasts formation. Interestingly NFATc1 can interact with osx and form a complex that activates osx-mediated collagen expression but not Runx2-mediated osteocalcin formation (Koga et al., 2005). Recent studies demonstrated that skull formation as well as osteoclast formation was impaired in NFATc1-deficient mice, suggesting that NFATc1 may regulate bone formation by affecting both osteoblast and osteoclast (Winslow et al., 2006).

1.6 Osteoclast

Osteoclasts are giant multinucleated cells that form from haematopoietic stem cells and are responsible for bone resorption. Osteoclast differentiation and activation is regulated by local and systemic factors such as RANKL, OPG, TNF-α, IL-1, IL-6, M-CSF, parathyroid hormone and 1,25(OH) D3. These stimuli are synthesised by a range of cell types including osteoblasts, stroma and lymphocytes (Teitelbaum and Ross, 2003, Blair and Athanasou, 2004). Activated osteoclast expresses multiple markers which enable their identification such as TRAP, Cathepsin K and calcitonin receptors.

Resorption begins when mature osteoclast become polarized and attach to the bone surface via $\alpha_v\beta_3$integrin interactions with RGD containing proteins in the bone matrix to create a sealed zone (Ross and Teitelbaum, 2005). Subsequently, the ruffled border which is a complex structure of folds of the plasma membrane secretes a range of factors that breakdown the organic and inorganic components of the matrix. The release of HCl via the action of V class-ATPase proton pumps and chloride channels acidifies the sealed zone to pH 4.5 which helps solubilise bone mineral (Blair et al., 2002)

(Figure 1.4). Then, osteoclast produce TRAP, cathepsin K and metalloproteinase 9 (MMP-9) to digest the organic matrix (Boyle et al., 2003). TRAP (tartrate-resistant acid phosphatase) is a metalloenzyme that plays an important role in bone resorption. TRAP is proposed to dephosphorylate bone matrix proteins including osteopontin and bone sialoprotien, and generates reactive oxygen species (ROS). Although, transgenic mice overexpressing TRAP developed a mild osteoporosis with increased osteoblasts activity (Angel et al., 2000). Phosphorylation of osteopontin facilitates osteoclast migration and may promote bone resorption (Ek-Rylander and Andersson, 2010). TRAP also generates ROS due to its redox activity which is required for bone desorption and degradation. Once the osteoclast has finished forming a 4-5μm resorption pit, it detaches from the bone surface and either moves onto a new surface to start the process again or undergoes apoptosis.

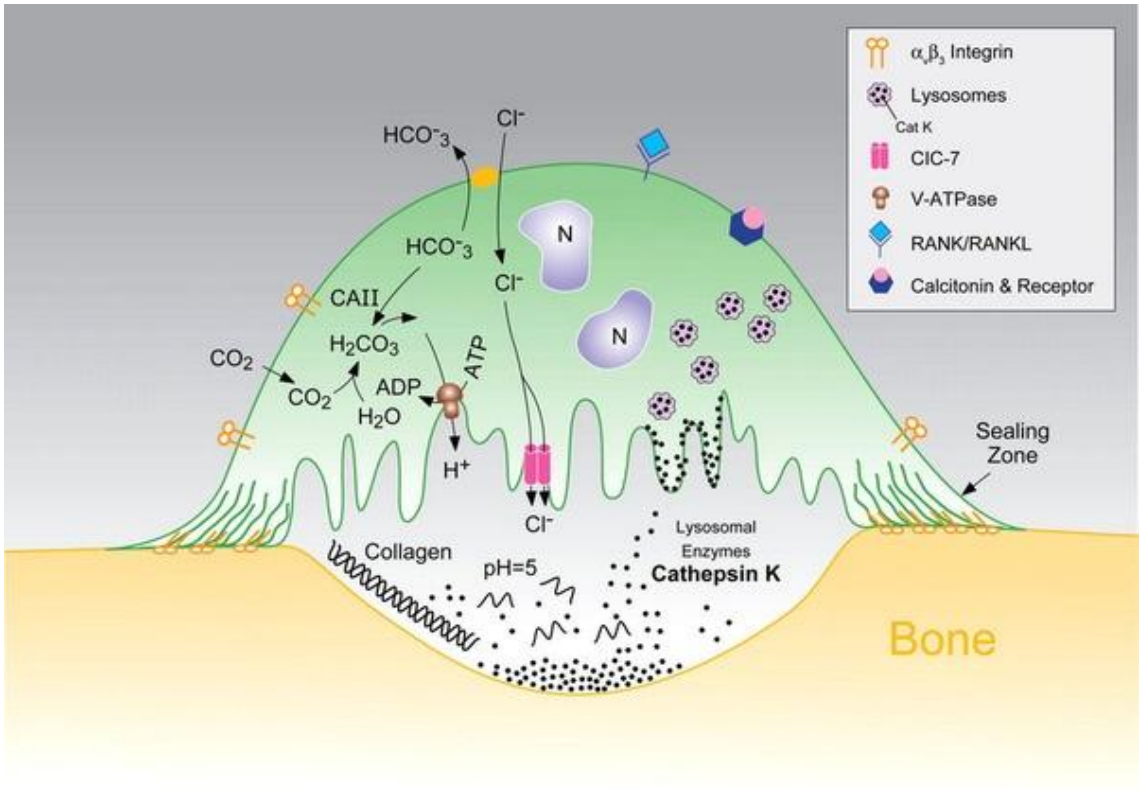

Figure 1.4. Bone matrix degradation mechanism by osteoclast. Osteoclast attach to bone matrix and create a sealed zone via integrin interactions. Osteoclast acidifies the resorption lacunae by secreting H^+ and Cl^- ions via osteoclast-specific V-ATPase. Chloride Channel 7 (CLCN-7) for demineralisation and Cathepsin K starting the dissolution of bone components. From (Rodan and Duong, 2008).

The regulation of bone resorption is a complex process controlled by various local and systemic factors. The major day to day systemic regulator of osteoclast formation is parathyroid hormone, but other steroid and polypeptide hormones also regulate osteoclast formation and bone resorption. Many systemic factors regulate osteoclast differentiation by modifying the expression of cytokines which in turn directly or indirectly effect osteoclast formation and activity including TNF-α, IL-1, IL-6, RANKL and OPG.

1.6.1 Systemic Regulators of Osteoclastogenesis

1.6.1a Parathyroid hormone (PTH)

PTH is a hormone produced from the parathyroid glands in response to hypocalcaemia and acts as a potent stimulator of bone resorption, renal calcium reabsorption and calcium uptake from the gut. This acts to restore circulating serum calcium levels. PTH has a dual effect on bone metabolism depending on the administration profile, continuous PTH administration stimulates resorption while intermittent PTH exerts an anabolic effect on bone formation (Frolik et al., 2003, Li et al., 2007). PTH was found to induce osteoclast formation from murine haematopoietic cells by an in-direct effect mediated via stromal or osteoblastsic cells (Mcsheehy and Chambers, 1986, Fuller et al., 1998). This indirect action involves the modification of osteoblastsic receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) expression. PTH may also increase osteoclastic receptor activator of nuclear factor kappa-B (RANK) mRNA expression (Lee and Lorenzo, 1999). In co-cultures of murine bone marrow osteoblasts, PTH treatment increases osteoblastsic RANKL expression and inhibits osteoblastsic OPG expression and this change in RANKL/OPG ratio is associated with increased osteoclast formation (Huang et al., 2004). All these findings suggest that the

pro-osteoclastic action of PTH occurs indirectly through an effect on the regulation of osteoblastsic modification of RANKL and OPG expression.

1.6.2b Calcitonin

Calcitonin is a polypeptide hormone secreted by the parafollicular cells of the thyroid gland in response to elevated calcium levels and has an inhibitory action on bone resorption but no effect on bone formation. The physiological effect of calcitonin is mediated by calcitonin receptors (CTR). Calcitonin inhibits osteoclast formation via binding to CTR, stimulating cAMP accumulation which reduces osteoclast motility and induces the retraction of osteoclast from the bone surface (Gorn et al., 1995). In addition, CTR-knockout mice have a significant increase in osteoclast number (Kauther et al., 2011). Calcitonin's action on Ca^{2+} homeostasis in humans however is secondary to that of PTH.

1.6.1c 1,25-Dihydroxyvitamin D3 (1,25-$(OH)_2D_3$)

The active form of vitamin D3 (1,25-dihydroxyvitamin D3 (1,25-$(OH)_2D_3$) acts as a potent stimulator of bone formation and resorption and has a stimulatory effect on osteoclast differentiation *in-vitro* and *in-vivo*. This is mediated via vitamin D receptors (VDR) expressed by osteoblasts and osteoclasts. Interestingly, vitamin D3 is important for bone growth and mineralisation and has a regulatory effect on calcium and phosphate levels.

$1,25(OH)_2D_3$ plays an important role in osteoclast formation. In contrast, $VDR^{-/-}$ mice failed to induce osteoclastogenesis in the presence of $1,25(OH)_2D_3$ (Takeda et al., 1999). Moreover, *in-vitro* studies using SaoS2 osteoblastsic cells treated with $1,25(OH)_2D_3$ showed an up-regulation of *RANKL* mRNA expression, and also increased osteoclastogenesis from peripheral monocyte, indicating that the RANKL promoter contains vitamin D responsive elements (Kitazawa et al., 2003). Similar results were

detected in co-culture of bone morrow macrophage and ST2 stromal cells (Kitazawa and Kitazawa, 2002). Furthermore, $1,25(OH)_2D_3$ can modify the production of cytokines such as IL1α, M-CSF and TGF-β which are implicated in osteoclastogenesis (Lee et al., 2002, Rubin et al., 1996).

1.6.2 Local Regulators of Osteoclastogenesis

1.6.2a RANK /RANKL and OPG axis

The effect of many pro-resorptive factors such as PTH is mediated through modification of RANKL/OPG expression. RANKL also known as osteoprotergerin ligand (OPG-L), TNF-related activation-induced cytokine (TRANCE) and osteoclast differentiation factor (ODF), is a 38 kDa protein belonging to the tumour necrosis factor (TNF)-superfamily and is encoded by the *TNFSF11* gene (Lacey *et al.*, 1998). RANKL is primarily expressed by osteoblasts but is also expressed by activated T cells, fibroblasts and mammary tissue (Anderson *et al.*, 1997) (Figure 1.5). RANKL exists as a membrane-bound protein and also as a secreted soluble C-terminal form. RANKL binds to its receptor RANK expressed on the surface of monocytic osteoclast precursors and mature osteoclasts to stimulate osteoclast differentiation and bone resorption (Lee et al., 2006a). In addition to the critical role of RANKL in osteoclastogenesis it is also plays a pivotal role in the regulation of dendritic cell survival, lymphocyte development, and lymph node organogenesis (Kong *et al.*, 1999). RANK deficiency causes severe osteopetrosis due to an absence of osteoclast. Furthermore, osteoclast precursors in these mice are unable to differentiate to osteoclast *in-vitro* in the presence of RANKL and M-CSF (Dougall *et al.*, 1999). The treatment of RANK mutant mice with IL-1, 1,25(OH)D3 did not induce osteoclast formation, suggesting that the resorptive activity of these factors is mediated through the RANK signalling pathway, while TNF-α

induced osteoclast formation is still relatively unaffected suggesting that TNF-α may act through alternative pathway to induce osteoclast differentiation (Li et al., 2000).

The binding of RANKL to RANK initiates a complex network of intracellular signal transduction cascades that stimulate the expression of a number of osteoclastic genes (Boyle et al., 2003). This initially involves the recruitment of TNFR-associated proteins 2, 5 & 6 (TRAFs) to RANK which in turn activate mitogen-activated protein kinases ERK, JNK (c-jun N-terminal kinases), *AP-1* transcription factors, *NFκB* and *NFATc1* (Lee and Kim, 2003, Leibbrandt and Penninge, 2008). Loss of many of these factors prevents osteoclast differentiation but key to the differentiation process is NFATc1 which is considered by many to be a master regulator of osteoclast genes.

Osteoprotegerin (OPG) is a 110kDa secreted glycoprotein that is a member of the TNF receptor superfamily (Hofbauer and Heufelder, 1997). OPG consists of 401 amino acids residues, it is different from other members of TNF-receptor superfamily because it lacks a tansmembrane domain (Suda et al., 1999). OPG acts as a decoy receptor for RANKL and blocks the interaction between RANKL and RANK thereby inhibiting osteoclast differentiation and activation (Lacey et al., 1998, Tsuda et al., 1997). OPG administration to ovx mice significantly impaired bone loss and increased trabecular bone density and prevents bone loss after ovariectomy (Shimizu-Ishiura et al., 2002) suggesting that OPG could be used in the treatment of osteoporosis (Bekker et al., 2001). Histological analysis of OPG treated mice showed that OPG significantly inhibited osteoclast number and rescued the osteoporotic defect in OPG deficient mice (Min et al., 2000). Furthermore, OPG prevents ovariectomy-induced osteoclast formation and bone resorption in rats (Simonet et al., 1997).

In addition to RANK derived signals further co-stimulatory inputs from immunoreceptor tyrosine-based activation motifs (ITAM) containing immunoglobulin like receptors are also required for osteoclast formation. These include osteoclast-

associated receptors (OSCAR) and Triggering receptor expressed on myeloid cells 2 (TREM2) which interact with immunoreceptor tyrosine-based activation motifs (ITAM)-bearing adaptors FcRγ and DAP12 respectively (Paloneva et al., 2003, Kim et al., 2002b). Phosphorylation of these ITAM residues by RANK or unknown ligands leads to activation of SYK, Zap70 and phospholipase Cγ (Kaifu et al., 2003, Merck et al., 2004, Koga et al., 2004, Kim et al., 2002b, Lanier et al., 1998, McVicar et al., 1998). This in turn leads to an increase in intracellular Ca^{2+} levels which regulate the nuclear accumulation of key osteoclastic transcription factor *NFATc1* via stimulation of calmodulin and calcineurin (Kim *et al*., 2008) (Figure 1.6). The nature of the ligands for TREM2 and OSCAR is still open to debate, although it has been suggested that specific motifs within fibrillar collagen on bone surfaces may represent one potential stimulus (Barrow et al., 2011).

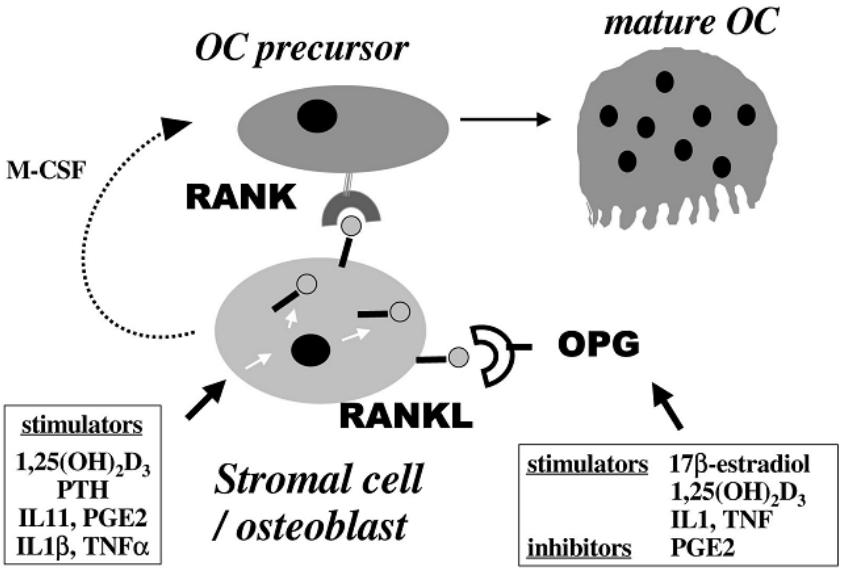

Figure 1.5. Role of RANKL and its receptor RANK in osteoclast differentiation. From (Roux and Orcel, 2000).

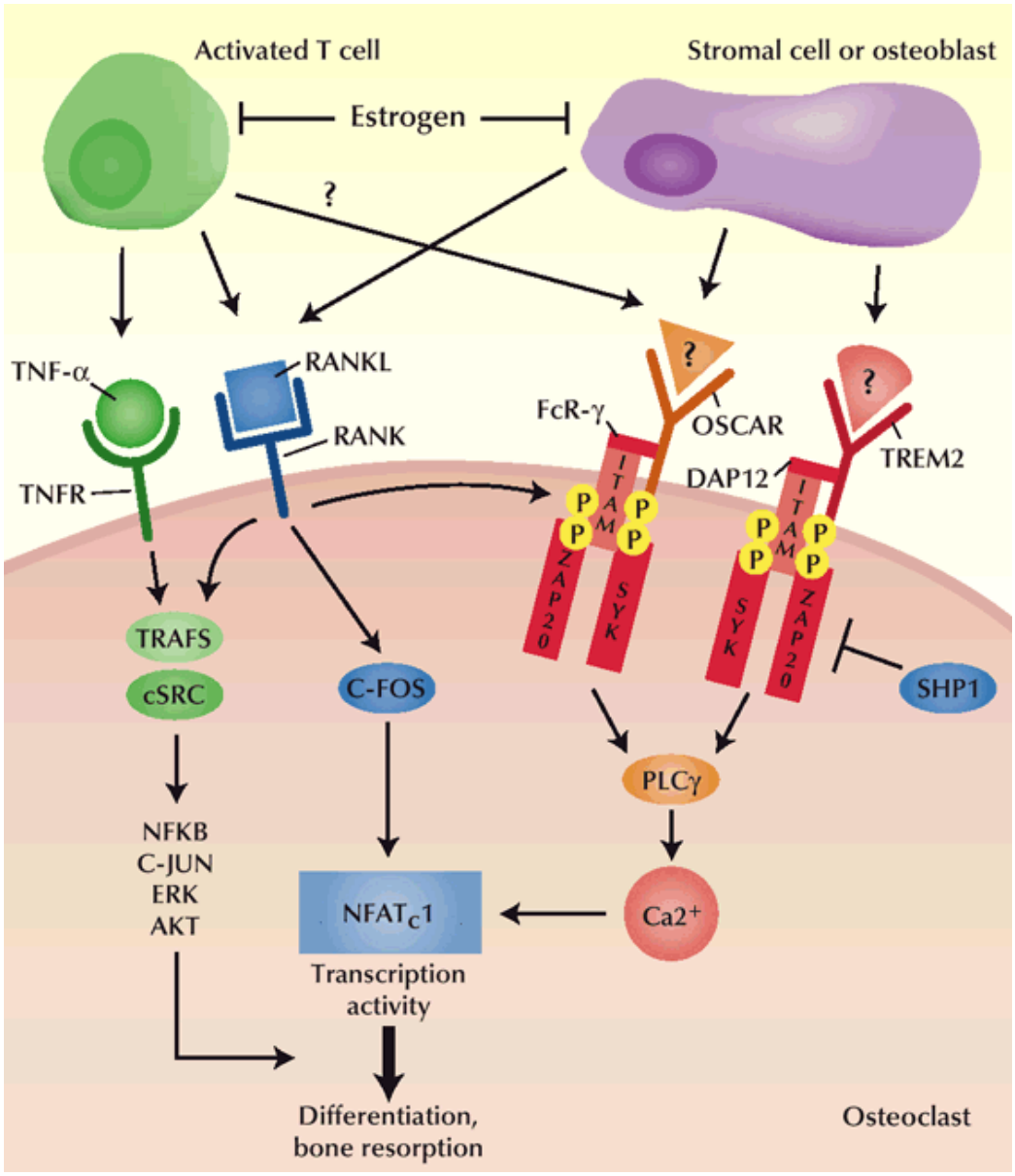

Figure 1.6. TNF-α receptor, RANK/RANKL and ITAM motifs signalling in osteoclast differentiation pathway. The binding of RANKL to RANK activates several transcription factors including NFATc1 expression, a master key regulator of osteoclast differentiation. Another co-stimulatory ITAM pathways are required for calcium-dependent NFATc1 activation. These co-stimulatory pathways are mediated through FcRγ or DAP12 and their co-receptor OSCAR and TREM2. After the interaction of RANK/RANKL and undefined ligands, ITAM motifs are phosphorylated and this phosphorylation increased NFATc1 nuclear accumulation and lead to stimulate osteoclast differentiation. From (Baron, 2004).

1.6.2b Macrophage-colony stimulating factor (M-CSF)

Macrophage-colony stimulating factor (M-CSF) is haematopoietic growth factor for differentiation of monocytes and macrophage lineages. It is produced by stromal cells, macrophages and T lymphocyte and acts on cells expressing the M-CSF receptor called c-fms (Douglass *et al*., 2008). M-CSF can affect osteoclast precursors directly or indirectly via stromal cells. Its primary mode of action occurs at the inception of osteoclast formation where it directly acts on non-committed monocytes to enable RANK expression and promote survival. Thus, it is essential for the initial stages of osteoclast formation. Fuller *et al*. (1993) also demonstrated that M-CSF modulates osteoclast survival and motility at later stages of differentiation. In addition, M-CSF plays a central role in TNF-α-induced osteoclastogenesis and bone resorption (Kitaura *et al*., 2007).

1.6.2c Transforming growth factor-beta (TGF-β)

Transforming growth factor-beta (TGF-β) belongs to a superfamily of related proteins that include bone morphogenic proteins (BMPs). It controls proliferation, cellular differentiation and other functions in many cell types. In the mononuclear phagocyte system, TGF-β helps maintain precursor responsiveness to activators of osteoclast formation, facilitates migration and exerts an anti-inflammatory effect (Fuller *et al*., 2000). Thus, TGF-β1 may act as a commitment factor in osteoclast differentiation, priming precursors for osteoclast formation, but once this priming stimulus has been received no further inputs are required other than activation stimuli (Koseki *et al*., 2002). This facilitative action would appear to be dependent on the ability of TGF-β to antagonise a range of inflammatory cytokines that prime precursors to alternate macrophage lineages, thereby maintaining a RANKL responsive pool (Fox *et al*., 2000). In addition to this action on osteoclast precursors, TGF-β has been shown to modify

osteoblastsic RANKL/OPG expression. This effect appears to be dose dependent with low levels elevating RANKL expression whereas higher concentrations decrease RANKL and promote OPG expression (Karst et al., 2004). In addition this response may be skewed to elevated RANKL expression in osteoporotic women (Jurado et al., 2010). Thus the effect of TGF-β on osteoclast formation is complex and it is likely that the precise effect on osteoclast formation is dependent on concentration, the primary site of synthesis/release and stage of the remodelling cycle, with higher levels likely to be present at the end of the osteoclastic phase feeding back on osteoblasts to limit the resorptive stimulus and thereby prevent excess resorption.

1.6.2d Interferons

IFNγ is a type II inflammatory cytokine belonging to a diverse family of inflammatory cytokines with pleiotropic effects (Tak, 2004). IFNγ is produced by activated T lymphocytes and has multiple effects on many cell types. However, with respect to osteoclast formation, IFNγ has been shown to have an anti-osteoclastic action. IFN-γ also interferes with RANK/RANKL signalling via degradation of TRAF6 resulting in inhibition of RANKL-induced activation of NFκB and JNK (Takayanagi et al., 2000) and then priming monocytes to alternative macrophage lineages. Mice lacking IFN-γ receptor (IFNGR1) display notable increases in osteoclast formation and bone destruction (Takayanagi et al., 2000, Takayanagi et al., 2002b). Moreover, IFN-γ inhibits TNF-α and RANKL-induced osteoclast formation and activation (Fox et al., 2000).

Similarly, several observations indicate that IFN-β has an important autocrine inhibitory role in osteoclast formation, limiting the number of osteoclasts that form and preventing excessive resorption. Moreover, IFNβ has been shown to inhibit osteoclastogenesis by

suppressing *c-fos* levels which is an essential transcription factor mediating the effect of RANKL (Takayanagi et al., 2002b).

1.6.2e Interleukins

The interleukin family includes several pleiotropic cytokines that have multiple cellular effects such as IL-1 and IL-6. IL-1 is secreted by bone marrow stromal cells and osteoclast and plays an important role in multiple altered health states such as rheumatoid arthritis, post-menopausal osteoporosis and certain cancers (Kitazawa et al., 1994). It has been shown to promote bone resorption by activation of osteoclast formation via different mechanisms (Lee et al., 2010, Trebec-Reynolds et al., 2010). It is produced by monocyte, bone marrow stromal cells and osteoblasts and is known as one of the most potent inducers of bone resorption.

IL-1 also promotes osteoclast survival by inhibiting apoptosis (Jimi *et al*., 1999). IL-1 also acts as co-stimulator for TNF-α-induced pit resorption, and anti-IL-1α antibodies inhibit resorption (Kudo et al., 2002). Therefore, TNF-α and IL-1 are considered to be critical cytokines in the development of inflammatory joint diseases and are known to promote the formation of osteoclast-like cells and increase osteoclast activity indirectly in bone marrow culture (Pfeilschifter *et al*., 1989). In addition, blocking either IL-1 or TNF alone was not as effective in inhibiting inflammatory bone loss as blocking both cytokines (Dayer, 2002, Zwerina *et al*., 2004). However, IL1α-induced osteoclastogenesis may be RANKL-dependent (Ma et al., 2004), as IL-1 can only induce osteoclast fusion but not differentiation in the absence of RANKL. Additionally, IL-1α can also upregulate the expression of several other cytokines which can stimulate osteoclastogenesis such as M-CSF, IL-6 and RANKL.

IL-6 is a multipotent cytokine produced by many cells including monocyte/macrophages and osteoblasts and has been found to induce osteoclast

differentiation. IL-6 belongs to a family of cytokine which share gp130 as a common signal transducer including leukaemia inhibitory factor (LIF) and IL-11. The role of IL-6 production in bone resorption has been clearly defined, it is produced in response to bone resorptive agents such as TNF-α and IL-1 (Ohsaki *et al*., 1992). Moreover, IL-6 has been suggested to promote bone loss in oestrogen deficiency. Ishimi (1990) showed that IL-6 significantly stimulated osteoclast formation in foetal mouse calvarial cultures. IL-6-null mice display no bone loss following oestrogen deficiency (Poli et al., 1994). It has been reported that IL-6 acts as co-stimulator of PGE2-induced osteoclastogenesis via a mechanism involving an effect on RANKL and OPG expression (Liu et al., 2005). In addition to the cytokines mentioned above there are many other cytokines belonging to the interleukins family involved in the regulation of osteoclast differentiation and activity such as IL-7, IL-8, IL-10, IL-12, IL-18 and IL-17 (Dai et al., 2004, Yamada et al., 2002, Roato et al., 2006).

1.6.2f Tumour necrosis factor-α (TNF-α)

Tumour necrosis factor-α (TNF-α) is a multifunctional pro-inflammatory cytokine belonging to the tumour necrosis factor ligand superfamily, with a wide variety of activities such as regulation of differentiation, proliferation and apoptosis. TNF has two forms (α & β) which have similar biological effects. TNFα is a central modulator of the acute inflammatory response to injury or infection but excessive or prolonged TNF-α production contributes to the development of chronic diseases including bone resorption (Azuma et al., 2000). TNF-α promotes inflammatory gene expression via multiple signal pathways including NFκB (Beutler, 1999, Screaton and Xu, 2000, Kobayashi et al., 2000). TNF-α induces chemokine production, recruiting monocyte and lymphocyte to the site of infection and inducing apoptosis in different types of cells. The biological effect of TNF-α is mediated by transmembrane receptor TNFR1 (p55r) and TNFR2

(p75r). P55 contains a death domain and a third domain that can trigger apoptosis, while p75 lacks a death domain (Kobayashi et al., 2000). TNFR1 (p55r) is the major receptor involved in osteoclast differentiation and bone resorption, and treatment with anti-p55r antibody completely inhibits TNF-α-induced osteoclastogenesis (Kobayashi et al., 2000, Azuma et al., 2000). Furthermore, bone marrow derived from transgenic mice expressing $p55^{+}/^{+}$ had an increased capacity to form osteoclast, while $p55^{-}/^{-}$ $p75^{+}/^{+}$ cells induced fewer osteoclast, suggesting the crucial role of p55 but not p75 in TNF-α's effect (Abu-Amer et al., 2000). Binding of TNF-α to its receptor causes activation of a TNF receptor associated death domain which in turn stimulates two pathways, JNK, protein kinase C and IkB leading to activation of NFκB which subsequently translocates to the nucleus and induces the transcription of TNFα-responsive genes (Kruppa et al., 1992). TNF-α can also activate the Fas activated death domain which triggers the apoptotic signalling cascades (Nanes, 2003).

The mechanism by which TNF-α can affect bone remodelling is complex, and it has both direct and indirect actions as it is able to cooperate with other signals pathways via TARF and NFκB. Injection of TNF-α induced the formation of TRAP positive osteoclast on the surface of bone in the absence of RANKL suggesting that TNF-α may act independently of the RANKL/RANK axis (Kim et al., 2005b). Similarly, TNF-α induced osteoclast formation in cultures of M-CSF dependent precursors by a TNFR1 dependent but RANKL-independent mechanism (Kitaura et al., 2005). In addition, Kudo (2002) showed that TNF-α directly induced human osteoclast formation by a RANKL-independent mechanism, and Zou (2001) showed that TNF-α was sufficient to induce osteoclast formation in the presence of M-CSF (but was less potent than RANKL), while RANKL increased TNF-α expression in RAW264.7 and Balb/c cell lines.

TNF-α can also in-directly affect osteoclast differentiation via an action on several cell types This indirect action is mediated through up regulation of RANKL expression in several cell types such as synoviocytes, osteoblasts, endothelial cells, human microvascular endothelial cells, T cells, and B cells (Cenci et al., 2000, Kanematsu et al., 2000, Page and Miossec, 2005). This in conjunction with TNF-α's direct stimulatory action is thought to be one of the mechanisms leading to the augmentation of osteoclast formation after oestrogen withdrawal (Hofbauer, 1999, Zhang et al., 2001). TNF-α also effects OPG expression in osteoblasts (Nakashima *et al*., 2000), endothelial cells (Collin-Osdoby *et al*., 2001) and in RA synoviocytes (Marotte *et al*., 2005). Moreover, TNF-α enhanced RANKL-induced osteoclast differentiation by stimulating MAP kinase, p38 and JNK pathways. TNF-α also elevated *c-fms* expression, the receptor of M-CSF in osteoclast precursors (Yao et al., 2006).

In addition to its ability to directly induce osteoclast formation or have a synergistic effect with RANKL, TNF-α may also increase bone resorption by stimulating the expression of other pro-inflammatory cytokines (Kurokouchi *et al*., 1998). TNF-α increases the expression of IL-1 from synovial mononuclear cells which could underpin some of the resorption associated with rheumatoid arthritis (Brennan *et al*., 1989). IL-1 is incapable alone of inducing osteoclastogenesis directly, however it is able to increase the resorptive activity of TNF-α or RANKL-induced osteoclast formation. Similarly, TNF-α has also been shown to induce the expression of osteoblastsic IL-6 (Tokuda *et al*., 2004) which is able to directly stimulate osteoclast formation (Kudo *et al*., 2003). It is also reported that TNF-α augments the stimulatory effect of PGE2 on osteoclastogenesis in bone marrow systems.

In addition to its effect on osteoclast, TNF-α can also influence osteoblasts activity and bone formation, reducing bone anabolism. TNF-α decreases *osteocalcin* mRNA

expression in osteoblastsic cell lines due to the suppression of *NFκB* (Li and Stashenko, 1992, Kuno *et al*., 1994). TNF-α also inhibits ALP production which is required for bone mineralisation (Kuroki *et al*., 1994, Nakase *et al*., 1997). Furthermore, TNF-α can promote osteoblasts apoptosis and increase the expression of genes associated with matrix degradation such as proteolytic enzymes that degrade the bone surface such as matrix metalloproteinase (Panagakos and Kumar, 1994).

The role of TNF-α in pathological process is a key area of research. Dysregulation of TNF-α level contributes to chronic autoimmune diseases such as rheumatoid arthritis and psoriatic diseases. TNF-α is also implicated in the development and progression of various cancers and is suggested to act as an autocrine and paracrine tumour promoter in ovarian cancers (Fujiki *et al*., 2002, Wu *et al*., 1993). It has also been reported that TNF-α plays a critical role in ovariectomy-induced bone loss via a stimulatory effect on osteoclast formation and through regulation of various pro-inflammatory cytokines implicated in bone resorption (Kimble *et al*., 1997). Evidence would suggest that TNF-α plays a critical role in the pathogenesis of bone resorption and osteolytic disorders (Nanes, 2003, Kwak et al., 2005, Weitzmann and Pacifici, 2005). In bone, TNF-α can be released from many cells including stroma and osteoblasts (Chaudhary *et al*., 1992, Ralston 1994). Mononuclear cells derived from post-menopausal women also produce increased amounts of TNF-α in comparison to pre-menopausal subjects (Pacifici et al., 1991, Ralston et al., 1990). Similarly, production of TNF-α, IL-1, IL-4, IL-6, and IFN-γ by blood cells are inversely correlated with oestrogen levels in post-menopausal women (Zheng *et al*., 1997). T cells and B cells appear to be a major source of TNF-α in post-menopausal women as elevated numbers of activated T cells produce increased amounts of TNF-α in comparison to oestrogen replete women (Nanes, 2003). This would appear at least in animal models to be a critical event in the bone loss associated with oestrogen

deficiency as nude mice, which are T-lymphocyte deficient, are resistant to bone loss after ovariectomy and mice lacking p55 TNF receptor do not display oestrogen deficiency bone loss (Cenci et al., 2000). Similarly, transgenic mice expressing soluble TNF-α receptor are protected against oestrogen deficiency bone loss (Ammann *et al*., 1997). TNF has also been implicated in other skeletal disorders including rheumatoid arthritis where it is a key therapeutic target. Treatment of RA patient with monoclonal anti-TNF-α antibodies (infliximab) caused a significant improvement in symptoms (Elliot et al., 2008, Maini et al., 1999, St. Clair et al., 2004). The positive effect of infliximab is thought to be due to a reduction in inflammation and a suppression of osteoclastogenesis which may arise in part due to a reduction in synovial NFκB signalling and RANKL expression.

1.6.3 Intracellular regulators of osteoclast differentiation

Osteoclast differentiation, survival and function are tightly controlled by a network of signalling pathways and transcription factors that are required for osteoclast differentiation and maturation. The next section will discuss key elements relating to these factors.

1.6.3a TNFR-associated factors (TRAFs)

RANK is a member of the TNF superfamily and it does not have intrinsic enzyme activity, thus it associates with TNFR-associated factor (TRAF) adaptor proteins to transduce intracellular signals after ligand binding. The TRAF family includes TRAF 1, 2, 3, 4, 5, 6, and 7 that mediate signals induced by a variety of TNF receptors (Inoue *et al*., 2000). TRAFs have the ability to bind to various regions on the cytoplasmic tail of TNF receptors (Inoue *et al*., 2000). The cytoplasmic tail of RANK contains multiple sites for TRAF binding, the region spanning between 235-258- primarily interacts with

TRAF6, while TRAF 2 and 5 associate with amino acids 532-625 (Darnay *et al*., 1998, Wong *et al*., 1998).

TRAF6 is the major adaptor molecule mediating RANKL-induced osteoclast formation and transduces signals to several downstream pathways including NFκB and c-Jun NH_2-terminal kinase (JNK). The essential role of TRAF6 in RANKL-induced osteoclast formation is highlighted by the development of a severe osteopetrosis in TRAF6-knockout-mice due to the absence of osteoclast similar to that seen in $RANK^{-/-}$ or $RANKL^{-/-}$ mice (Naito et al., 1999, Lomaga et al., 1999). However, there is a degree of redundancy in this system as disruption of TRAF6 activity completely abolishes activation of NFκB but not JNK pathway (Wong *et al*., 1998, Lee *et al*., 2000). However the role of TRAF 2 and 5 in osteoclastogenesis is minor in comparison to TRAF6. TRAF5-deficient mice have a mild inhibition of osteoclastogenesis and these animals display a near normal level of NFκB and JNK activation RANKL activation (Kanazawa *et al*., 2003, Kanazawa and Kudo, 2005).

1.6.3b NFκB

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) are a family of dimeric transcription factors that recognize a common sequence of DNA called the κB site (Asagiri and Takayanagi, 2007). *NFκB* is a transcription factor that regulates expression of many genes involved in inflammatory responses (Baldwin, 1996). There are five NFκB proteins in mammals: Rel (cRel), RelA (p65), RelB, NFκB1 (p50) and NFκB2 (p52) (which are processed from their precursors p105 and 100 respectively) (Takayanagi, 2007). NFκB is a vital transcription factor that is important for immune cell development and activation, inflammation and osteoclastogenesis. Following RANK/RANKL binding a signalling cascade mediated through TRAF6 is initiated that results in the activation of *NFκB* (Franzoso et al., 1997, Xing et al., 2003). In particular,

p50 and p52 have a major role in osteoclast differentiation as $p50^{-/-}$ and $p52^{-/-}$ mice develop osteopetrosis due to defective osteoclast formation (Franzoso et al., 1997, Iotsova et al., 1997).

NFκB proteins reside in the cytoplasm of non-stimulated cell but enter the nucleus upon stimulation with different agonists including RANKL (Boyle *et al*., 2003). Activation of *NFκB* is controlled by sequential phosphorylation and ubiqutin mediated degradation of its inhibitory subunits (Miyazaki *et al*., 2000). NFκB drives expression of many important osteoclastic genes and is critical but not sufficient for osteoclast formation. The activation of *NFκB* occurs through classical and alternative pathways. The classical pathway involves the activation of inhibitor of κB kinase (IkB) complex which phosphorylates and enables the ubiquitin mediated destruction of inhibitory kB which masks nuclear localisation sequences on NFκB. IKK consists of two subunits: IKKα and IKKβ and a regulatory subunit IKKα *NFκB* essential modulator (NEMO) (Leibbrandt and Penninge, 2008). The classical pathway is responsible for the activation of p50:RelA which is dependent on IKBβ while the alternative pathway activates p52:RelB which is dependent on IKBα (Novack *et al*., 2003, Hayden and Ghosh, 2004, Hayden and Ghosh, 2008), both pathways are important for osteoclastogenesis. In contrast, IKKα and IKKβ are essential for osteoclastogenesis, although IKKβ but not IKKα may be essential for osteoclast formation *in-vivo* (Ruocco *et al*., 2005). Thus, it seems that *NFκB* classical activation pathways may be the major pathway through which RANK/RANKL signalling can affect osteoclastogenesis.

1.6.3c Activator protein (AP-1)

Activator protein (*AP-1*) is a transcriptional factor that is formed from a range of homo and hetro dimers which has a key role in osteoclast differentiation (Wagner and Eferl, 2005, Takayanagi et al., 2002a). *AP-1* dimers are composed of a range of transcription

factors including *c-fos* (*c-fos*, *c-fos*B, *Fra*-1, *Fra*-2), *Jun*, (*c-Jun*, *JunB*, *JunD*), and ATF (ATFa, ATF2, ATF3, ATF4, B- ATF) proteins (Wagner and Eferl, 2005). AP-1 dimers bind to a range of osteoclastic gene promoters and this interaction is modulated by interaction with other transcription factors and kinases that generates a complex level of regulation (Eferl *et al*., 2004). For instance evidence suggests that *c-fos* may cooperate with *NFATc1* to induce osteoclastogenesis (Macián *et al*., 2000).

c-fos is a cellular proto-oncogene and is upregulated during a range of cellular activities including osteoblast and osteoclast differentiation. c-fos-deficient mice develop osteopetrosis due to the blockade of osteoclastogenesis (Wang *et al*., 1992, Grigoriadis *et al*., 1994). This defect in osteoclast differentiation can be reduced by expression of *c-fos*-related protein Fra-1, suggesting the critical function of *c-fos* and *Fra-1* in osteoclast formation (Fleischmann *et al*., 2000). The Jun family also plays a role in addition to *c-fos*, mice lacking Jun proteins such as *c-Jun* and *JunB* display a minor decrease in osteoclast differentiation, suggesting that members of Jun can substitute for each other during osteoclastogenesis (Kenner *et al*., 2004, Wagner and Eferl, 2005).

1.6.3d NFAT and Ca^{2+} calmodulin-calcineurin

Nuclear factor of activated T cells (NFAT) is a transcription factor family that consists of several members including *NFATc1* (*NFAT*2), *NFAT1* 3, 4, and 5 which are involved in regulating cellular differentiation in several different biological systems such as the cardiovascular, muscular and immune system (Crabtree and Olson, 2002). NFATc1 is considered to be a key regulator of the expression of osteoclastic genes (Takayanagi, 2007) as *NFATc1*$^{-/-}$ embryonic stem cells do not differentiate into osteoclast and loss of *NFATc1* function decreases the capacity to form osteoclast after RANKL stimulation (Takayanagi et al., 2002a). NFATc1 levels display a biphasic profile during osteoclastogenesis, this is characterised by an initial small increase followed by a more

pronounced level of expression after NFATc1 activation, nuclear translocation and auto-regulation of its own gene.

The initial expression of RANKL-induced NFATc1 is dependent on *NFkB* and c-fos (Asagiri et al., 2005). In contrast NFAT activation and secondary expression is dependent on intracellular Ca^{2+} levels which modify the activity of the serine/threonine phosphatase calcineurin. RANKL binding to RANK and co-stimulatory inputs provided by DAP12 and FcRγ stimulate the release of Ca^{2+} from intracellular stores which stimulate Ca^{+2}/calmodulin-dependent calcineurin which promotes *NFATc1* nuclear translocation (Figure 5). Unsurprisingly, calcineurin inhibitors such as FK506 and cyclosporine A therefore prevent osteoclastogenesis (Takayanagi et al., 2002a, Kim et al., 2005c).

In addition to promoting the expression of its own gene NFATc1 regulates a range of other transcriptional targets such as TRAP (Kim et al., 2005c, Matsuo et al., 2004), cathepsin K (Matsumoto et al., 2004, Kim et al., 2005c), calcitonin receptor (CTR) (Anusaksathien et al., 2001, Takayanagi et al., 2002a), and β3 integrin (Crotti *et al*., 2006). NFATc1 in many cases also co-operates with other transcription factors such as PU.1, MITF, and *AP-1* to augment the transcription of genes (Takayanagi et al., 2002a). Moreover, osteoclast-specific co-stimulatory immunoreceptor expression (OSCAR) is one of the first targets for *NFATc1* (Kim et al., 2005a, Kim et al., 2005c). It is not completely clear how the coordinated expression of *NFATc1* target genes promotes the differentiation of osteoclast, but it has been reported that NFATc1-induced *DC-STAMP* expression is essential for cell-cell fusion of osteoclast (Kukita et al., 2004, Yagi et al., 2005). Osteoclast precursors lacking DC-STAMP are unable to fuse and only mononuclear TRAP positive cells are noted in these mice (Yagi et al., 2005).

1.7 Osteocytes

Osteocytes are mature osteoblasts that have become embedded during matrix deposition. They share some morphological and functional characteristics with osteoblasts. Osteocytes are not isolated and form complex connections via canniculi with adjacent osteocytes and other cells especially on the bone surface; in addition to enabling communication this also facilitates the supply of nutrients. Osteocyte formation and maturation takes about three days during which the production of extracellular bone matrix and the formation of dendritic processes occurs (Knothe Tate et al., 2004, Noble, 2008). The precise function of osteocytes is unclear but they are thought to have a role in the adaptive response to mechanical loading, sensing changes in mechanically induced strain and modifying the activity of cells on the bone surface. Osteocytes may also be important for the diffusion of oxygen and nutrients through bone tissue by preventing mineralisation in its environment. Osteocytes have also been demonstrated to produce matrix proteins osteocalcin, osteopontin and osteonection in culture (Aarden et al., 1996), although the *in-vivo* relevance of this is uncertain.

Chapter Two: Nutrition and Bone

Chapter 2

2.1 Introduction

Menopause is a natural process that occurs due to reduced ovarian function, leading to decreased oestrogen (E2) and progesterone levels, which lead to reduced fertility. Hormonal changes during the menopause result in a decline in E2 levels, and play a pivotal role in the generation of chronic disease. One of these is post-menopausal osteoporosis in which reduced E2 levels lead to increased rates of bone remodelling (Schot and Schuurs, 1990, Seeman, 2004). Osteoporosis is a systemic skeletal disease characterized by low bone mineral density, with the World Health Organisation (WHO) defining osteoporosis as a bone mineral density 2.5 S.D below normal T score. Osteoporosis is a serious problem for post-menopausal women and is associated with an increased fracture risk at the distal radius and ulna, vertebral bodies and hip. Multiple treatments are available for post-menopausal bone loss such as calcitonin, bisphosphonates, hormone replacement therapy (HRT) and selective E2 receptor modulators (SERMs) such as rolaxifene (Scharbo-Dehaan, 1996). For a long time hormone replacement therapy (HRT) was the first choice treatment (Gallagher, 2001). However HRT is now not normally prescribed in light of evidence suggesting that long term HRT is associated with a greater risk of developing breast and endometrial cancer, cardiovascular disease and stroke (Hulley *et al*., 1998, Rossouw *et al*., 2002). There are also drawbacks with many of the other forms of therapy for instance bisphosphonates have been suggested to cause kidney damage, osteonecrosis of the jaw and atypical fractures. Therefore there is a need to find alternative treatments to control aberrant remodelling or restore oestrogenic activity (Ruggiero et al., 2004, Migliorati et al., 2005, Cole et al., 2008).

Research from nutritional studies has shown that several dietary compounds may represent a strategy for the treatment of osteoporosis. These include a wide range of vitamins, minerals and other compounds including phytoestrogens (PEs). This chapter

aims to detail the biology relating to E2 and remodelling and the literature examining the effect of phytoestrogens and other selected nutritional factors on bone.

2.2 Oestrogen and bone remodelling

Steroid hormones such as E2 play a pivotal role in skeletal growth and bone homeostasis and a lack of circulating E2 after the menopause leads to the development of several diseases including post-menopausal osteoporosis (Riggs et al., 1998, Weitzmann and Pacifici, 2006a). Thus it is important to understand the cellular and molecular mechanisms through which E2 deficiency causes bone loss. Multiple studies indicate that E2 has the ability to regulate bone homeostasis through an interaction with the immune system, oxidative stress and direct actions on both osteoclast and osteoblast formation and activity. However, the precise mechanism through which E2 deficiency increases fracture risk is still a subject to debate. However, one thing that is clear is that reduced E2 levels lead to an increase in the number of resorptive osteoclasts which then remove excessive amounts of bone and E2 replacement prevents this. For instance E2 administration in post-menopausal women blunted levels of the bone turnover markers, carboxyl-telopepetide of type 1 collagen (NTX) and telopepetide of type 1 collagen, while bone formation marker amino-terminal propeptide of type 1 collagen (P1NPP) was increased (Charatcharoenwitthaya et al., 2007). The mechanism underpinning these changes in cellular activity are not so clear and several hypotheses have been put forward to explain the changes in remodelling activity. These include changes in immune profile, inflammatory and anti-inflammatory cytokine production, disruption of RANKL/RANK/OPG axis, changes in reactive oxygen species (ROS) and direct effects on osteoclast function and lifespan. However, there is little consensus on the precise mechanism through which E2 deficiency exerts it effect on bone tissue and it is likely that multiple actions mediate this effect.

2.2.1 Inflammatory mediators and immune cells

Recently, the interactions between bone and immune cells has been suggested to play a role in the bone loss associated with ovarian dysfunction (Clowes et al., 2005). Many bone and immune cells such as T-cells and B-cells express ERs and are therefore targets for E2 (Cenci et al., 2003, Weitzmann and Pacifici, 2005) and increased levels of pro-inflammatory cytokine expression is as an early change associated with E2 deficiency (Pfeilschifter et al., 2002). Elevated levels of IL-1, IL-7, IL-6, and TNF-α levels have been described in E2 deficiency (Weitzmann *et al*., 2002, D'Amelio *et al*., 2008), and these cytokines could potentially mediate changes in osteoclast formation (Zallone, 2006, Weitzmann and Pacifici, 2007). E2 has been shown to reduce IL-6 expression through an ERα-dependent mechanism that prevents *NFκB* mediated IL-6 promoter activation (Stein and Yang, 1995). E2 also decreases TNF-α levels in cultures of osteoblastsic-like cells (Pfeilschifter *et al*., 2002), which may relate to the E2 dependent production of the repressor of the TNF-α promoter GRIP1 (Cvoro *et al*., 2006).

In contrast to an increase in inflammatory mediator production E2 deficiency has been shown to suppress anti-inflammatory cytokine synthesis. E2 promotes osteoblastsic TGF-β production which in turn stimulates osteoclast apoptosis, suggesting that E2 deficiency may prolong osteoclast life span via a loss of TGFβ-induced apoptosis (Hughes et al., 1996). This is supported by the ability of E2 to inhibit osteoclast formation in cultures of osteoclast precursors and osteocyte-like MLO-Y4 cells in the presence of TGFβ antibodies (Heino et al., 2002).

One important mechanism through which E2 deficiency may stimulate bone loss is through increased T cell number. E2 deficiency increases the proliferation and function of $CD4^+$ and $CD8^+$ cells producing TNF-α in post-menopausal women and ovariectomised mice (Cenci et al., 2000, Roggia et al., 2001, Roggia et al., 2004) and

ovariectomized mice lacking the TNF p55 receptor fail to display bone loss. Moreover, transgenic mice over expressing soluble TNF or mice treated with TNF inhibitory binding protein show decreases in bone resorption (Ammann et al., 1997, Kimble et al., 1997). In addition, E2 dependent changes in TNF-α production in human peripheral blood cells have been noted (Ito *et al*., 2001). TNF-α production is increased in post-menopausal or oophorectomised individuals (Pacifici et al., 1991, D'Amelio et al., 2004), and HRT suppresses TNF-α production *in-vivo* (Bernard-Poenaru et al., 2001). These changes in TNF-α will directly stimulate osteoclast formation and may also have additional in-direct actions to increase RANKL expression or augment the sensitivity of osteoclast precursors to RANKL (Pacifici, 2008).

Expansion of the T cell pool after E2 deficiency could be the main mechanism increasing proinflammatory cytokine levels after loss of ovarian function. How this increase in T cell number occurs is unclear but it has been suggested to potentially relate to changes in follicle stimulating hormone and inflammatory cytokine production or the redox state within bone (Iqbal *et al*., 2006, Lean *et al*., 2005). E2 deficiency is negatively associated with thiol antioxidant enzyme levels in bone including glutathione peroxidase, the main intracellular antioxidant, and glutathione peroxidise expression is augmented following E2 treatment (Lean et al., 2005). Furthermore, E2 increases the expression of antioxidants such as glutathione and thioredoxin in various cells including osteoclast (Lean et al., 2003). In addition, ROS stimulate antigen presentation by dendritic cells, while antioxidants prevent dendritic and T cell activation by repression of MHCII in response to antigen (Maemura et al., 2005). These data are consistent with the concept that E2 deficiency is associated with lower antioxidant levels and increased TNF secretion via stimulation of antigen presenting cell (APC)-induced expansion of T cell pools (Figure 2.1).

Other evidence suggests that the increase in T cell number occurs due to increased antigen presentation by macrophage and dendritic cells (Cenci et al., 2003). This mechanism involves up-regulation of MHC II (CIITA) and costimulatory CD80 expression on macrophage and dendritic cells as a consequence of modified changes in IL-7, TGF-β and IFN-γ levels (Figure 2.1). This leads to presentation of self-antigens to T cell subsets leading to activation and clonal expansion within the T cell pool. CIITA is an immune modulator, which induces MHC II expression (Boss and Jensen, 2003). The increase in CIITA expression itself is dependent on changes in the cytokine profile following decreases in circulating E2 levels.

E2 deficiency up-regulates TNF-α-producing T cells by a complex pathway. In brief, E2 deficiency enhances IL-7 production which suppresses TGF-β secretion followed by activation of IFN-γ production. The high levels of IFN-γ elevates MHCII expression by enhancing the amplification of transcription factor CIITA and then increasing self-antigen presentation to T cells. This promotes the release of TNF-α which directly promotes osteoclast formation and augments the effect of RANKL- (Pacifici, 2008) (Figure 2.1).

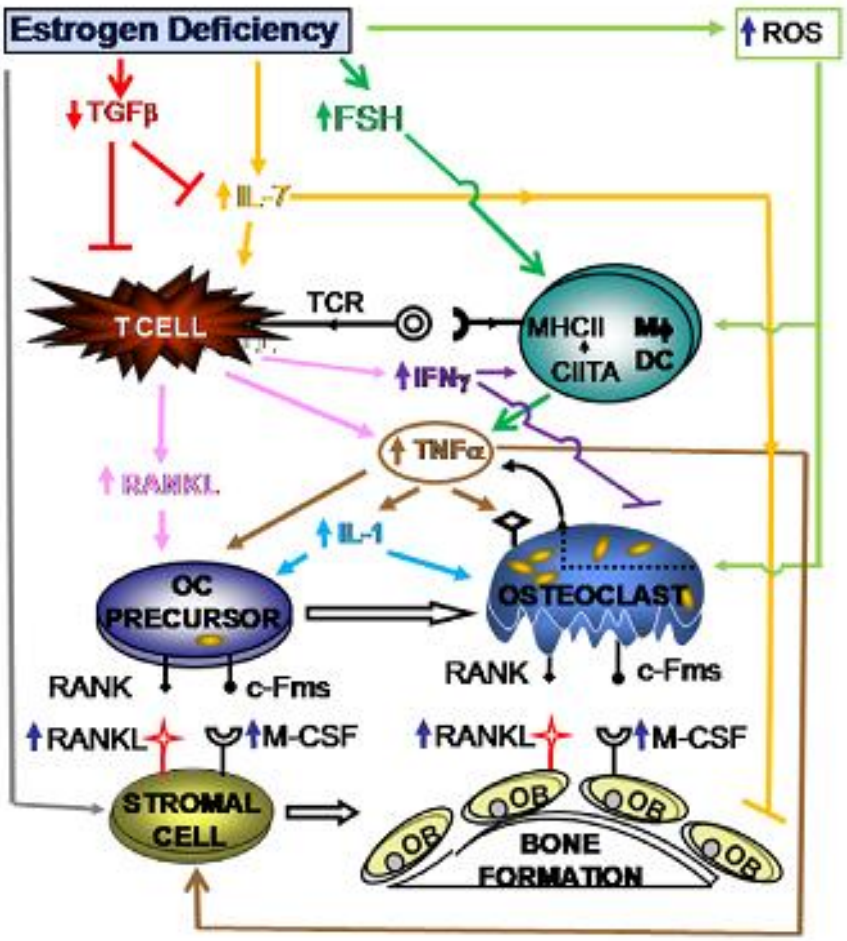

Figure 2.1. Schematic of potential mechanisms by which E2 deficiency causes bone turnover. Follicle-stimulating hormone (FSH) stimulates the production of TNF-α by monocyte. E2 deficiency increases IL-7 production in target lymphoid organs such as bone, thymus, and spleen by at least in part inhibition of TGF-β. This leads to the activation of T cells and the production of proinflammtory cytokines such as IFN-γ which increases antigen presentation by up-regulation of MHCII expression. E2 deficiency also stimulates bone loss by down regulation of antioxidant pathway and thus increasing of reactive oxygen species (ROS) and resulting antigen presenting activation and TNF production. Also, RANKL and TNF production increases osteoclast formation. From (Pacifici, 2008).

2.2.2 Regulation of osteoclast lifespan

E2 deficiency has also been suggested to increase the lifespan of osteoclast. This hypothesis suggests that E2 attenuates resorption by inducing osteoclast apoptosis through the ERα dependent induction of osteoblastsic Fas ligand expression. Interaction of osteoblastsic FasL with Fas on the surface of osteoclast will activate executioner caspase leading to DNA fragmentation and the formation of apoptotic bodies. Moreover, SERMs such as tamoxifen and rolaxifene also stimulate FasL expression in osteoblasts via recruitment of ERα to the FasL promoter, and decreases in trabecular osteoclast and apoptotic profiles was noted in WT but not osteoclast-ERα knockout mice (Krum et al., 2008, Nakamura et al., 2007). This suggests that E2 regulates osteoclast lifespan through modulation of Fas/FasL.

Recent studies investigated the role of Fas expression in bone resorption. These revealed that Fas expression was significantly increased in osteoblasts and decreased in osteoclast of wild type ovariectomised mice. Furthermore, following ovariectomy the number of osteoclast was elevated in wild type but was unchanged in mice lacking Fas (Kovacic et al., 2010).

2.2.3 Modification of the RANKL/RANK/OPG axis

In-vivo and *in-vitro* studies have demonstrated that E2 modifies the ratio of RANKL to OPG in osteoblasts, T and B cells (Shevde et al., 2000, Srivastava et al., 2001, Taxel et al., 2008). E2 can modulate the expression of both RANKL and OPG in osteoblasts. E2 suppresses osteoblastsic RANKL expression supporting the inhibitory effect of E2 on osteoclast formation (Bord et al., 2003). E2 also increases osteoblastsic OPG production (Hofbauer et al., 1999), OPG levels increased in mouse ST-2 stromal cells treated with E2 (Saika et al., 2001). In addition, bone marrow precursors incubated with RANKL and E2 formed fewer TRAP-positive osteoclasts than cells without E2 (Garcia Palacios

et al., 2005). The mechanism through which these changes in RANKL expression occurs is debatable and may occur as a direct effect of E2 on bone cells or as an indirect response to changes in other cytokine levels. Whatever the answer it is clear that the interaction of RANKL with RANK is central to menopausal bone loss. This is highlighted by the effect of the anti-RANKL monoclonal antibody therapy denosumab. Denosumab is a new option for the treatment of post-menopausal osteoporosis in patients with high risk of bone fractures (Moen and Keam, 2011). It binds to RANKL and inhibits osteoclast formation and survival. Long term denosumab treatment reduces bone resorption significantly increasing bone mineral density (BMD) at different skeletal sites and reduces fractures in post-menopausal women (Bone et al., 2008, Lewiecki et al., 2007, Miller et al., 2008). Recent studies found that denosumab promotes a greater increase in BMD than alederonate in post-menopausal women (Kendler et al., 2010). All these data suggested that disruption of RANKL signalling has a key role in post-menopausal bone loss.

In conclusion, E2 regulates bone remodelling through several mechanisms including repression of osteoclastogenic cytokines secreted from bone marrow stroma, osteoblasts, and T-cells. However an overarching mechanism of action has yet to be established. Further studies are needed to fully elucidate the underlying mechanisms.

2.3 Phytoestrogens (PEs)

Phytoestrogens (PEs) are polyphenolic non-steroidal plant-derived compounds that possess oestrogenic agonist and antagonist-like activity. PEs have been the focus of much attention as they are postulated to have protective properties on human health. Publications refer to their potential effect against hormone-dependent breast and prostate cancer, bone resorption, cardiovascular disease and menopausal symptoms (Setchell and Cassidy, 1999, Messina, 2003). It has been noticed that populations

consuming high levels of PE-rich foods have a lower incidence of post-menopausal osteoporosis, certain cancers and cardiovascular disease (Adlercreutz, 1995, Somekawa et al., 2001, Kris-Etherton et al., 2002). Asian populations consume 10-20 times the amount of PEs in comparison to Western populations. This high intake of PEs has been suggested to lower the risk of developing several altered health states, including post-menopausal osteporosis, atherosclerosis, and certain cancers (Messina, 1999, Messina and Bennink, 1998, Clarkson and Anthony, 1998). PEs exhibit weak oestrogenic activity about 10-100 fold less than that of E2 requiring a higher concentration to produce an equivalent biological effect (Zava and Duwe, 1997, Santell *et al*., 1997). On the other hand they may be present in much greater quantities in the body than E2 depending on an individual's diet (100 to 1000 times) (Adlercreutz *et al*., 1986).

PEs are a diverse group of compounds that belong to a family of substituted phenolic compounds known as flavonoids. PEs oestrogenic effect is dependent on two main properties. First the presence of a phenolic ring A, that mimics E2's receptor binding A ring, and second, a similar distance between the 7- and 4'-hydroxyl groups in isoflavones and the C3 and C17 hydroxyl groups of oestradiol (Dixon, 2004) as shown in (Figure 2.2). The presence of a phenolic ring enables PEs to bind to oestrogen receptors (ERs) and provides the major basis of their oestrogenic action (Wuttke *et al*., 2003). In addition to their oestrogenic effect, PEs also function as antioxidants and many such as genistein can modify tyrosine kinase activity (Anderson and Garner, 1998).

PEs are structurally classified as isoflavones or non-isoflavones (Table 2.1). There are also several sub-groups, including coumestans which possess high oestrogenic activity, whilst non-flavones including lignans have been also identified as PEs as they exert oestrogenic actions but they lack a flavone backbone so are not classed as flavonoids

(Cornwell *et al*., 2004). Isoflavones included genistein (5,7,4' trihydroxyisoflavone) and daidzein (7,4' dihydroxyisoflavone); coumestans include coumestrol (3,9-dihydroxy-6-benzofurano [3,2-c] chromenone, while lignans include entradiol and enterolactone as well as secoisolariciresinol and mataresinol. Plant-based foods including fruits and vegetables are thought to be the main source of PEs in most Westerns diets. PEs are also found in leguminous plants such as soy bean (*Glycine max*), which contain isoflavones, while the major source of coumestans are alfalfa sprouts (*Medicago sativa*) and flaxseed (*linum usitatissimum*) is rich in lignans (Table 2.1). Isoflavones are described as most important type of PEs and their usage has been suggested to be beneficial in a wide range of human health states (Heim *et al*., 2002).

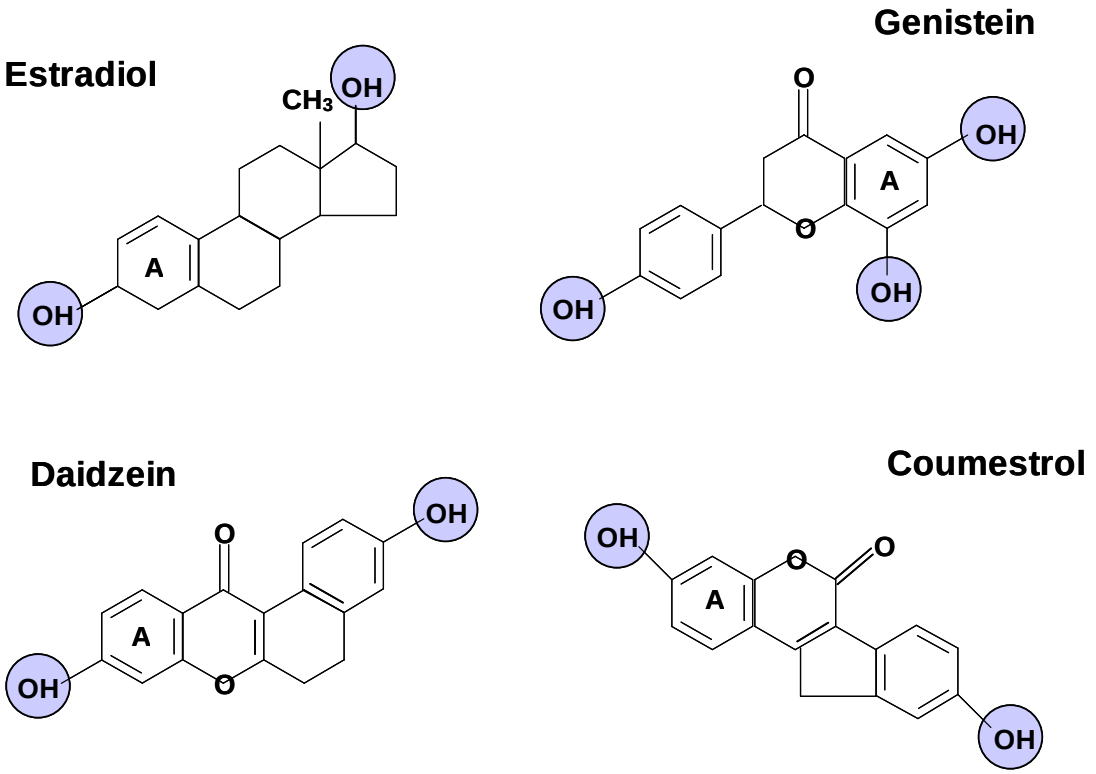

Figure 2.2. Chemical structures of E2, genistein, daidzein, coumestrol and glycitein. The phenolic ring A in isoflavones is responsible for selective high-affinity binding to oestrogen receptors (ERs), and through this interaction with ERs, isoflavones exert their agonist or antagonist action on target cells and tissues. Purple circles highlight the hydroxyl groups (Chen and Anderson, 2002).

Table 2.1. Phytoestrogens of human interest: classes, subtype, and sources (Chiechi and Micheli, 2005).

Class	Subtypes	Source
Isoflavones	Genistein	Legumes
	Daidzein	Soy beans
	Glycein	Peanut
	Fomononetein	Clover
	Biochanin A	Sunflower seed
		Walnut
Coumestans	Coumestrol	Mung beans
	Plicadin	Soy spouts
	Repensol	Alfalfa spout
		Clover
Lignans	Enterolactone	Flaxseeds
	Enterodiol	Rye bread
	Lignan A	Cereals
		Grains
		Fruits
		Vegetables

2.3.1 Phytoestrogen Metabolism

The efficacy of PEs is affected by many factors such as age, diet, gender, dose and the mode of administration. PEs also differ in their rates of absorption, metabolism and excretion (Rowland *et al*., 2003). Many PEs within food are biologically inactive and many PEs require metabolism to active and bio-available forms before absorption occurs and they can influence cell function (Puupponen-Pimia *et al*., 2004). For instance, genistein and daidzein are found in the food as glucosides (conjugated forms) which are converted to active forms by β-glycosidase hydrolysis (Figure 2.3) (Setchell *et al*., 2002b). β-glycosidase is derived from intestinal bacteria (*Lactobacillus, Bacteroides*) and plays an important role in the hydrolysis of PEs and in turn may affect health status as a consequence (Hawksworth et al., 1971). On consumption, intestinal bacteria may further metabolise PEs to their metabolites, for example genistein is hydrolyzed into dihydrogenistein (4',5,7 trihydroxyisoflavones) and further metabolised to 6'-hydroxyl-O desmethylangolesin (6'-hydroxy-O-DMA) (2',4',6', 4" tetrahydroxy-α-methylhydroxybenzoin), whilst daidzein is hydrolyzed into dihydrodaidzein (4', 7 dihydroxyisoflavones) which is further metabolised to O-desmethylangolsin (O-DMA) (4', 6', 4" trihydroxy-α-methyldeoxybenzoin) and equol which both have oestrogenic activity (Heinonen *et al*., 1999, Heinonen *et al*., 2003). Equol is more structurally similar to 17β-oestradiol than daidzein and is therefore more potent. Several studies report that equol and O-DMA bind to ERα and ERβ with a higher affinity than daidzein which only has a low affinity for ERβ (Kinjo *et al*., 2004, Morito *et al*., 2001). In keeping with this, equol has been shown to have more oestrogenic activity than daidzein (Setchell *et al*., 2002a). *In-vitro*, equol and O-DMA were also found to be 100 fold more potent than daidzein at modifying growth and E2 responsive protein expression in breast cancer cells (Kinjo *et al*., 2004) (Figure 3.3). The absorption of PEs is also metabolically dependent, as unconjugated forms of PE are more rapidly absorbed and

more bio-available than polar conjugated forms due to their lipophilic properties (Cassidy, 1996). PEs are absorbed from the small intestine and in the case of genistein this is typically via passive diffusion from the intestine (Crespy *et al*., 2003).

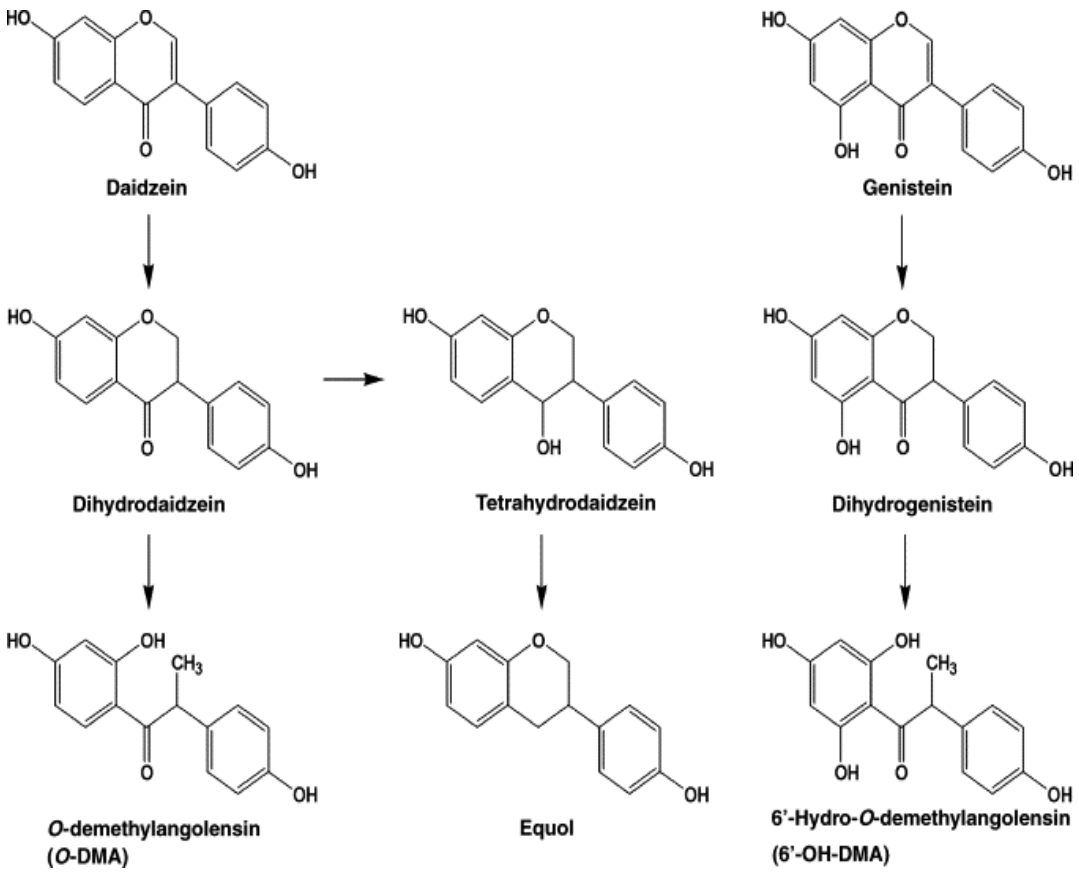

Figure 2.3. Intestinal metabolism of daidzein and genistein. From (Hwang *et al*., 2006).

2.3.2 Mechanism of action

PEs have multiple mechanisms of action including oestrogen agonist and antagonist activity. In addition they are able to influence the production of sex steroid binding proteins, the presence of hydroxyl groups in the 4', 5 position of the aromatic ring gives them antioxidant activity (Adlercreutz *et al*., 1993) and they are able to alter cell function through modification of tyrosine kinase activity (Kurzer and Xu, 1997). However, the mechanism and potency of action of individual PEs is differs (Kuiper et al., 1998, Hwang et al., 2006, Gallo et al., 2005). For instance genistein has been suggested to suppress osteoclast formation *in-vitro* by inhibiting tyrosine kinase activity whereas other PEs shown to inhibit osteoclast formation lack this effect (Gao and Yamaguchi, 2000).

2.3.2.1 Oestrogenic activity

The presence and position of the hydroxyl groups and phenolic ring in PEs and E2 is considered important for their oestrogenic activity (Martucci and Fishman, 1993). The effect of PEs on human health is partly explained through ER-mediated actions (genomic action) due to the capacity of PEs to bind with enzymes and receptors (Adlerceutz, 1998). ERs are expressed in several tissues including bone, but usually at lower levels than that expressed in the reproductive system (Zallone, 2006). ERα and ERβ receptors differ in their C-terminal ligand-binding domain and N-terminal transactivation domain but both interact with specific DNA promoter sequences called oestrogen response elements (EREs). This alters the expression of a raft of genes and proteins modifying cell proliferation and differentiation. The expression profile of ERα or ERβ in a tissue can differ and is also modified in many altered health states. For example prostatic epithelial cells typically express ERβ while prostatic stroma cell express ERα (Ho, 2004). In addition it would appear that ERs may regulate the

expression of each other, ERβ down regulates ERα expression as ERα is elevated in ERβ-knockout mice (Lindberg et al., 2003).

The cellular response elicited by ERα or ERβ also typically differs. However the precise response is contextual depending on the E2 concentration and receptor expression profile. For instance ERβ is expressed in many breast cancers (Fuqua et al., 2003), and it's expression is associated with a proliferative response in breast epithelium in ERβ-knockout mice but not in ERα$^{-/-}$ mice (Gustafsson and Warner, 2000). In contrast, ERα is associated with more differentiated tumours and MCF-7 human breast cancer cells seem to be sensitive to ERα and genistein increases the growth of MCF-7 cells in an ERα dependent manner (Maggiolini et al., 2001). The administration 750ppm of genistein also increased mammary tumour growth in ovariectomised rats via an ERα-mediated effect (Allred et al., 2004). Moreover, genistein increased malignant adenocarsinoma proliferation, while no cancer progression was found in ERα-knockout mice (Day et al., 2001).

In bone ERα and ERβ are expressed by osteoblasts and osteoclasts, and both receptors modulate gene expression in these cells, although evidence suggests that ERα has the major role in protecting bone mass. For instance, cortical bone density still decreased in the absence of ERβ in ovariectomised animal models (Windahl et al., 2001). Moreover E2 had no effect in ERα-knockout mice (Sims et al., 2003). Whereas bone density increased in the absence of ERα in female mice (Sims et al., 2002). Similarly in human MG63 osteoblasts cells E2 increased ALP and bone matrix collagen expression via ERβ (Cao et al., 2003).

PEs bind ERs in a different manner to that of E2 as PE such as genistein and daidzein lack the lipophylic binding region present within E2 (Poulsen and Kruger, 2008). In addition, PEs show a spectrum of affinities for ER, some PEs such as genistein and

coumestrol are good ligands while others bind with a lower affinity (Kuiper *et al*., 1996). Coumestrol however may have a stronger affinity for ER than genistein due to the presence of a carbonyl group at position 2 and the distance between position 2' and 4 being more similar to E2 which may make coumestrol more potent than genistein in some instances (Morito *et al*., 2002). PEs have differential affinities for ERα and ERβ and this plays a crucial role in the agonist and antagonist effect of these compounds. However, the precise role of ERα and ERβ in the response to PE is not fully understood and may depend on the concentration and relative level of receptor expression. Genistein and many other PE's oestrogenic response are primarily mediated via ERβ (Bemis et al., 2004, Kuiper et al., 1997), although this does not preclude actions via ERα. Several studies suggesting that PEs exert agonist effects via ERβ and antagonist effects via ERα. For instance while genistein, apigenin, naringenin, and kaempferol have a stronger affinity for ERβ than ERα, E2, coumestrol and equol only have a partial affinity for ERβ rather than ERα (Kostelac *et al*., 2003). In contrast daidzein has a low affinity for ERβ (Kinjo *et al*., 2004, Morito *et al*., 2001). While the precise role of ERα and ERβ in PE's action is unclear there is certainly evidence for an agonist oestrogenic component to PE's bone effect. For example E2 inhibitors prevented the suppressive effect of genistein on IL-6, RANKL and OPG expression in human and mouse osteoblasts cell lines (Chen et al., 2003a, Viereck et al., 2002). In addition to agonist actions, PEs can also act as antagonists opposing the action of 17-β oestradiol and other sex hormones. PEs can stimulate sex hormone binding globulin synthesis, which would cause a decrease in 17-β oestradiol levels in peripheral blood (Ibarreta *et al*., 2001). Furthermore, the antagonist activity of PEs may also include competitive binding to ER, down regulation of ER, reduced E2 synthesis and inhibition of ER phosphorylation.

2.3.2.2 Non oestrogenic actions

In addition to oestrogenic activity several PEs have been reported to exert various non ER-related actions including inhibition of tyrosine kinase activity, inhibition of protein kinase C, inhibition of DNA topoisomerases I and II, inhibition of angiogenesis, antioxidant effects and modification of prostaglandin synthase activity (Kurzer and Xu, 1997, Arora *et al*., 1998, Markovits *et al*., 1995). Most of these actions require a PEs concentration over 10 μM for an effect. The inhibition of tyrosine kinase by genistein may explain the mechanism by which it impedes cancer cell growth (Ibarreta *et al*., 2001). PEs can also affect steroid bioavailability by interacting with key enzymes involved in the steroid synthesis such as 5α reductase, 17β-OH-steroid-dehydrogenase and aromatase (Makela *et al*., 1995, Kao *et al*., 1998, Rice *et al*., 2006, Lacey *et al*., 2005). In addition, PEs stimulate the production of sex hormone binding globulin (Pino et al., 2000), suggesting that may alter steroid hormone bioavailability.

The antioxidant activity of PEs is related to the presence of free hydroxyl groups in the aromatic ring and ability to bind between aromatic rings (Burda and Oleszek, 2001). The hydroxyl group in the B-ring is important to enable the scavenging of reactive nitrogen or oxygen species (Sekher et al., 2001). The mechanism by which PEs are able to scavenge free radicals is dependent on the ability of these compounds to donate a hydrogen atom or transfer an electron from C-3-OH in the B-ring to hydroxyl to peroxy and peroxynitrite radicals. This stabilizes the reactive species and gives rise to a relatively stable PE radical (Heim *et al*., 2002). Studies suggest that the antioxidant capacity of flavonoids strongly correlates with the number of hydroxyl groups in the B-ring, whereas hydroxyl groups in the A-ring may have little correlation with antioxidant activity (Heim *et al*., 2002). Interestingly, isoflavones such as genistein have also been known to donate hydrogen atoms from the phenolic group (Heim *et al*., 2002). Thus, the

capacity of PEs to scavenge free radicals is primarily attributed to the reactivity of hydroxyl substitutes that participate in the following reaction:-

$F\text{-}OH + R^{\cdot} \longrightarrow F\text{-}O^{\cdot} + RH$ (Heim *et al*., 2002).

(F= phytoestrogens)

Additionally, genistein may also prevent oxidative stress by increasing the production of antioxidant enzymes such as glutathione peroxidase, which maintains the reduction-oxidation reaction (redox) state by reducing oxygen peroxide, thus protecting against oxidative DNA damage (Suzuk *et al*., 2002, Raschke *et al*., 2006).

2.4 Phytoestrogens and human health

E2 deficiency during menopause can lead to an increased risk of developing several altered health states such as osteoporosis, cardiovascular disease and cancer. These disorders were commonly treated with hormone replacement therapy (HRT) or other compounds such as calcitonin, raloxifene or bisphosphonates (Alekel *et al*., 2000). HRT was used for many years as the first line treatment for menopausal symptoms (Gallagher, 2001), however, observations from large scale clinical trials indicate that long term HRT may increase the risk of breast and endometrial cancer and cardiovascular disease in elderly women (Beral, 2007, Rossouw *et al*., 2002). Therefore, it is important to find alternative therapies that exert beneficial effects on bone health without unwanted side effects. PEs represents one group of compounds that could be employed due to their structural similarity with E2 and data suggesting a protective action on human health. Several epidemiological studies suggest that PEs can alleviate diseases associated with E2 deficiency such as cardiovascular disease and menopausal symptoms (Tikkanen *et al*., 1998, Nestel *et al*., 1997). Moreover, multiple investigations indicate a lower

incidence of cardiovascular disease, breast and prostate cancer and osteoporosis in Asian populations with a PEs-rich diet (Adlercreutz, 1998, Clarkson and Anthony, 1998). However, other confounding aspects of the Asian lifestyle could contribute to the beneficial effect of soy-rich diets confusing the precise benefit of these compounds in clinical settings (This *et al*., 2001). Furthermore, due to the complexity of their biological effect it is unclear what aspect of PE's action mediate any change in skeletal or cardiovascular health (Kronenberg and Hughes, 1999). The situation is further confused by wide differences in the ability to metabolise the conjugated inactive PEs form to active metabolites. It is likely that many negative results in clinical trials could at least in part be attributed to not accounting for these non-responders. Collectively, whether PEs can be effective, reliable and safe form of HRT, whether they have additional benefits, and the mechanism of action need to be confirmed with controlled trials to explain the action of PEs on human health.

2.4.1 Cardiovascular Diseases

Following the menopause the risk of coronary heart disease (CHD) is much higher due to the fall in E2 levels (Reckelhoff and Fortepiani, 2004, Reckelhoff, 2006). Multiple studies have examined the association between PEs consumption and improvement of lipid profile, vascular reactivity, and cell proliferation in CHD (Anderson *et al*., 1999). Dietary soy and PEs have been well documented to reduce the risk of CHD, suppress circulating cholesterol and triglyceride levels and increase high density lipoprotein (HDL) (Anderson *et al*., 1995). Several epidemiological and clinical investigations examined the mechanism by which PEs may reduce the progression of atherosclerosis showing that PEs improve the profile of plasma low density lipoprotein LDL-cholesterol, triglyceride lipids, reduce thrombus formation (including a reduction in platelet action) and enhance antioxidant activity (van der Schouw *et al*., 2000). In

addition, other mechanisms are reported to explain the effect of PEs on hypercholestremia including reducing cholesterol synthesis and enhanced cholesterol receptor activity (Glazier and Bowman, 2001). PEs may also improve lipid profiles by modifying LDL levels which are associated with cardiovascular disease. This stems from the ability of PEs to increase bile production and hepatic metabolism both of which lower LDL (Wroblewski Lissin and Cooke, 2000).

In addition, PEs can inhibit LDL oxidation which decreases the likelihood that LDL will be retained in the endothelia where it can promote atherosclerotic lesion development (Steinberg, 1997, Ruiz-Larrea *et al*., 1997, Mitchell *et al*., 1998). Healthy adults administrated genistein, daidzein or equol showed inhibition of LDL oxidation due to the antioxidant properties of PEs (Wiseman *et al*., 2000). Similarly, administration of soy protein to post-menopausal monkeys decreased the risk of coronary artery constriction (Honoré *et al*., 1997). Also *in-vivo* and *in-vitro* observations report that PEs act as antioxidant compounds by increasing levels of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione dismutase (Kurzer and Xu, 1997). Genistein has been shown to decrease platelet action due to its tyrosine kinase inhibitory action (Tham *et al*., 1998). Clinical studies have suggested that isoflavones act as antioxidant agents and can reduce the oxidation of LDL (Ruiz-Larrea *et al*., 1997). However, the relationship between PEs and the lower incidence of cardiovascular disease and the mechanism of action still remain to be determined.

2.4.2 Breast cancer

Breast cancer is the most common cancer in women in Western countries. Epidemiological studies of dietary intake and cancer risk have been recently reviewed concluding that there is some evidence of a protective role of PEs on breast cancer

although the picture is more complex than first appreciated (Velentzis *et al*., 2008). The data from *in-vitro* studies, animal experiments, and human trials revealed that PEs are oestrogenic agonists in breast epithelium (Petrakis et al., 1996). It is found that enterolactone is associated with a lower risk of breast cancer (Sonestedt *et al*., 2009). Recent analyses also shown a protective role of isoflavone intake an breast cancer incidence (Trock et al., 2006, Qin et al., 2006, Wu et al., 2008), pre- and post-menopausal Asian women eating the highest amount of dietary isoflavones had a 29% reduction in breast cancer risk when compared to low-level isoflavone consumers. Similar results have been noted in other studies examining dietary intake (Fink *et al*., 2007, McCann *et al*., 2004, Silva *et al*., 2004). Furthermore, the administration of flaxseed has been shown to reduce tumour proliferation and stimulate apoptosis (Thompson *et al*., 2005).

Several *in-vitro* studies with breast cancer cells have shown a positive correlation between PEs and inhibition of tumour growth at high concentrations (Peeters *et al*., 2003, Ziegler, 2004). Genistein in the presence of E2 competes to bind with ERs and slightly decreased cellular proliferation. Moreover, genistein at doses more than 10 μM inhibited cell proliferation via inhibition of tyrosine kinase activity. (This *et al*., 2001). Similarly, low concentrations of quecertin and genistein reduced proliferation or had no stimulatory effect on ER-negative MDA-MB-231 cells (Balabhadrapathruni *et al*., 2000).

Tumour development in animal models is also influenced by PEs, however the response is not necessarily always a positive one. The growth of E2 responsive tumours in mice is promoted by genistein (Allred *et al*., 2001, Hsieh *et al*., 1998), and administration of a genistein-rich diet to ovariectomized nude mice stimulated MCF-7 tumour number and burden (Hsieh *et al*., 1998). Whereas other studies report that treatment of new born

female rats with genistein reduces the incidence and number of mammary tumours induced by carcinogenic agents (Lamartiniere *et al*., 1995, Brown *et al*., 1998).

There are several potential mechanisms through which the beneficial effect and anti-proliferative action of PEs on breast cancer risk could be mediated. PEs may reduce the risk of breast cancer by effecting endogenous sex-hormone levels or the length of menstrual cycle. Numerous studies *in-vivo* and *in-vitro* suggest that PEs stimulate the production of sex-hormone binding globulin (SHBG) by liver cells. The increase in SHBG would be expected to reduce free circulating sex-hormone concentrations and prolong the menstrual cycle and thus lower the risk of breast cancer (Lu et al., 1996, Wu et al., 2000, Pino et al., 2000).

Another mechanism involves ERα and ERβ mediated actions by binding with ERs and eliciting a weak-oestrogenic effect. PEs tend to bind to ERβ with a higher affinity than ERα whereas E2 typically does not show a selective action (Kostelac *et al*., 2003, Margeat *et al*., 2003, Brzezinski *et al*., 1997), and for this reason PEs may function more like selective E2 receptor modulators (SERMs). Other possible mechanisms included inhibition of tyrosine kinase activity, inhibition of angiogenesis, inhibition of several enzymes such as s13β- hydroxysteroid, 17β-hydroxysteroid, 5α-reductase, topoisomerase I and II, and reduction of oxidative stress (Kurzer and Xu, 1997, Makela *et al*., 1995, Kao *et al*., 1998). In addition, genistein has the ability to stimulate apoptosis of cancer cells *in-vivo* and *in-vitro* (Pettersson and Gustafsson, 2001, Adlercreutz and Mazur, 1997). Collectively epidemiological studies suggest that PEs rich diets may reduce the risk of developing breast cancer. The role of PEs in reducing established tumours is less clear and may be dependent on dosage, E2 status and if the tumour expresses ERα or ERβ.

2.4.2.1 Safety of phyotoestrogens and breast cancer

Evidence in recent years has increased attention on PEs as an alternative to HRT. HRT is associated with an increased risk of breast cancer, however to date there is no recorded adverse effect associated with short or long time of use PEs in humans and in some cases beneficial actions have been noted. The action of PEs and E2 depends on many factors including target tissue, endogenous E2 and ER status.

The anti-cancer effect of PEs can be explained by a dual mechanism suppressing steroid hormone biosynthesis and promoting of E2 metabolism. Many breast cancers are E2-dependent and a large proportion express ERs ER-dependent mechanism could modulate tumour progression or inhibition. E2 in breast tumour present 20 fold than in circulating while it is with 5 levels in pre-menopausal women (Nakata et al., 2003, Pasqualini and Chetrite, 2005). The loss of ovarian function stimulates local E2 production through the action of key enzymes such as 17β-hydroxysteroid dehydrogenase (17β-HSD) and aromatase that catalyses E2 formation from circulating androgens (Labrie, 2003). PEs have been shown to inhibit aromatase and 17β-hydroxysteroid dehydrogenase activity, genistein has been shown to inhibit 17β-HSD 2, while coumestrol inhibit 17β-HSD 1 (Brooks and Thompson, 2005). This would reduce E2 levels and reduce the tumour promoting effects of E2. However, high concentration of PEs are required to inhibit local E2 synthesis and the relative contribution of this to their action is uncertain.

ERα and ERβ are expressed in a cell and tissue specific manner in distinct tissue so E2 has different effects in different tissues, and many complications are involved to explain the diverse action of ERs. The relative binding affinity of PEs and E2 to ERs differs and this may effect their agonist and antagonist activity; it was found that E2 has the same affinity to both ERs, while PEs typically have a higher affinity for ERβ (Kuiper et al.,

1998), with genistein, daidzein and biochanin A having a 16-fold higher affinity for ERβ compared with ERα (Liu et al., 2001a). Interestingly tumour development is associated with ERα activation and tumour inhibition ERβ activation. Furthermore, the ERα:ERβ ratio is increased in tumours (Skliris et al., 2003). This suggests that ERβ could silence ERα by forming ERα/β heterodimers (Lindberg et al., 2003). The affinity of PEs for ERβ may be a possible protective mechanism antagonising the promoting effects of ERα. Through modulation of ERβ isoforms genistein may inhibit E2-induced cell growth, silencing ERα-dependent promoting effects. However, further studies are needed to determine the interaction of PEs with ERβ isoforms and determine the importance of this interaction on breast tumour progression. Moreover, the biological activity of PEs may depend on the formation of dimers between both ERα and ERβ because these receptors may form homo- or heterodimers. Moreover, genistein at high concentration can inhibit breast tumour growth while at low concentration induced tumour growth (de Lemos, 2001). Thus, the effect of PEs depends on the level expression of ERs and physiological dose.

In addition to the direct effect of PEs on ER they also reduce levels of available hormones by increasing the expression of human sex hormone binding globulin (HSBG) which reduces active hormone levels (Adlercreutz et al., 1998). PEs through anti-oxidant activity also reduces reactive oxygen species that play a crucial role in the development and growth of breast cancer (Wei et al., 1995, Wilson et al., 2002). Genistein can also effect on cell growth via inhibition of protein tyrosine kinase.

Therefore most evidence suggests a protective effect of PEs on breast cancer through an action on ERβ, reduction of free active hormone, anti-oxidant activity and inhibition of cell signalling. In conclusion, at the present time is not possible to decide if PEs are

completely safe since further experiments are needed to determine the long-term action of PEs at therapeutically relevant concentrations.

2.4.3 Prostate cancer

Prostate cancer is the one of the most common cancers in men in Western countries with a much higher incidence in comparison with Asian populations (Severson *et al*., 1989). The protective effects of PE-rich diets and lower risk of prostate cancer has been demonstrated (Alonso *et al*., 2009, Chen *et al*., 2009, Zhang *et al*., 2008, Syed *et al*., 2007). Many different environmental and genetic factors are associated with the development of prostate cancer including age, race, diet and infection (Tominaga and Kuroishi, 1997). Higher urinary concentrations of daidzein and genistein have been noted in healthy individuals in comparison to prostate cancer patients, suggesting a inverse link with disease development (Park *et al*., 2009). High plasma concentrations of genistein have also been associated with a lower subsequent risk of prostate cancer progression (Travis *et al*., 2009). Furthermore, additional studies have evaluated the effect of isoflavones on prostate specific antigen (PSA) levels, which is a diagnostic marker of prostate cancer. Some of these studies revealed that a low PSA levels was associated with consuming 83 mg isoflavones/day in men (Adams *et al*., 2004). A further study in which 28 men diagnosed with prostate cancer were given 50g of bread supplemented with heat-treated soy grits found a significant decrease in PSA in the supplemented group supporting the protective hypothesis that PEs reduce prostate cancer progression (Dalais *et al*., 2004). Another study using 450mg/day of genistein and aglycan showed variable results, seven patients had a 50% reduction in PSA levels while eight patients showed no effect (deVere White *et al*., 2004). *In-vitro* studies have shown that genistein and biochanin A inhibit androgen sensitive LNCaP and androgen insensitive PC-3 prostate cancer cell proliferation at least in part by stimulating

apoptosis (Davis *et al*., 1999, Raffoul *et al*., 2006, Peterson and Barnes, 1993). Similar effects have been noted in LNCaP treated with enterolactone (Chen *et al*., 2007), and genistein inhibits tumour progression in animal cancer models (Landström *et al*., 1998, Mentor-Marcel *et al*., 2005) and metastasis of tumours to distant sites (Lakshman *et al*., 2008) potentially by inhibiting matrix metalloproteinase production (Xu and Bergan, 2006). However a minority of studies indicate that PEs may also promote prostate cancer growth. Similar actions of genistein have been noted in animal models where it has been shown to increase tumour burden and augment metastatic potential (El Touny and Banerjee, 2009).

Several mechanisms may mediate the effect of PEs on prostate cancer growth. PEs can exert oestrogenic effects on prostate cancer cells through binding to ERs in particular ERβ, as activation of ERβ has been suggested to suppress cell proliferation and induce differentiation in prostate cells *in-vitro* and *in-vivo* (Morrissey and Watson, 2003, Usui, 2006). Isoflavones have also been shown to modify sex steroid receptor expression, feeding male rats 25 and 250 mg genistein/Kg decreased the expression of androgen receptors (AR), ERα and ERβ. Thus, the protective action of dietary PEs in Asian populations may be attributable to the down-regulation of sex steroid receptors (Fritz *et al*., 2002). Furthermore, genistein at a high concentration can inhibit protein-tyrosine kinase (PTK) activity which plays an important role in cell proliferation and apoptosis, and this may be one of several mechanism by which PEs effect cell growth (Kyle *et al*., 1997, Sarkar and Li, 2002). Decreases in prostaglandin synthesis have also been noted after PE treatment and this has been proposed as a potential mechanism mediating their suppressive action on mitosis (Swami *et al*., 2009). The antioxidant capacity of PE has also been shown to protect prostate cells from DNA damage caused by a range of factors through the ability to modify glutathione transferase and reductase levels (Raschke *et al*., 2006, Steiner *et al*., 2007).

Thus the majority of *in-vitro* and *in-vivo* experiments suggest a chemoprotective role of PEs on prostate cancer progression. Whether PEs have antagonist or adverse effects in human is not clear yet. However further long term intervention studies need to be performed to confirm these results in the clinical setting.

2.4.4 Phytoestrogens and osteoporosis

There is much evidence that osteoblast and osteoclast activity is dependent on a range of signalling inputs to maintain bone mass. Interestingly, the expression of ERs in bone and the biological properties of PEs provide evidence that they may play an important role in modifying bone remodelling. E2 deficiency after the menopause can lead to the development of an osteoporotic skeleton that is prone to fracture. There is much evidence to support the use of HRT and SERMs to attenuate and prevent bone loss in post-menopausal women. However, observations from large scale clinical trials indicate that long term HRT may increase the risk of breast and endometrial cancer and cardiovascular disease in elderly women (Beral, 2007, Rossouw *et al*., 2002). HRT may also increase the risk of stroke through elevation of venous thrombosis formation (Rank et al., 2012). Therefore, PEs have been suggested as an alternative therapy to protect bone mineral density in post-menopausal women (Rod, 2010).

A number of studies have been published investigating the beneficial effect of several PEs in post-menopausal women and most of these suggest that PEs protect or increase BMD (Dalais et al., 1998, Ho and Liao, 2002, Morabito et al., 2002). Epidemiological and clinical studies show a positive correlation between PEs consumption and bone mineral density (Yuebin et al., 2006, Kanno et al., 2004, Lee and Choi, 2005, Uchiyama and Yamaguchi, 2007). A positive association between PEs intake and skeletal health has been reported in Japanese and Chinese post-menopausal women and some studies using Western populations (Ho et al., 2003, Horiuchi et al., 2000). Additionally, in

Asian populations isoflavone consumption is associated with a higher BMD (Sebastian, 2005, Gallagher *et al*., 2004, Uesugi *et al*., 2002, Yamori *et al*., 2002). Epidemiological studies on post-menopausal Chinese women showed a positive association between PEs intake and BMD in lumbar spine and femur (Mei *et al*., 2001). Similarly, genistein administration in post-menopausal Japanese women positively correlates with lumbar BMD (Greendale *et al*., 2002). Another study revealed a significant increase in lumbar BMD in early post-menopausal women supplemented with 54 mg/day of genistein for 12 months (Morabito et al., 2002), markers of bone resorption were significantly reduced while markers of bone formation were increased in these subjects suggesting an anti-resorptive and anabolic action (Morabito et al., 2002). BMD in post-menopausal women was also increased after six months of isoflavone administration (Potter et al., 1998).

Animal models of bone loss show a similar bone sparing and anti-osteoclastic action of PEs. Soy protein rich diets prevented bone loss in ovariectomised rats but had no effect on ALP, a marker of bone formation, suggesting that this may have occurred due to decreased resorption (Arjmandi *et al*., 1998). In addition, subcutaneous injection of isoflavones at 0.4 mg/day increased BMD but not with 0.7 mg/day (Ishimi *et al*., 2000). A low dose of genistein (0.5 mg/day) was also shown to decrease femoral bone loss in ovariectomised rats (Anderson *et al*., 1998). Genistein (10^{-7} to 10^{-5} M) inhibited PTH-induced bone resorption which could be related to increased osteoblast formation or increased osteoclast apoptosis (Gao and Yamaguchi, 2000). Picherti *et al*. (2000) reported similar effects with genistein and daidzein, which both prevented ovariectomy-induced bone loss and an even great suppressive action of daidzein was noted on bone resorption. This may reflect the strong oestrogenic action of equol a metabolite of daidzein (Setchell *et al*., 2002a), which has been shown to directly inhibit osteoclast formation in RAW264.7 cells (Hwang *et al*., 2006). Ishida *et al*. (1998) also found that

genistein and daidzein 50 mg/Kg/day increased BMD and calcium/phosphorous content in ovariectomised rats, although no direct comparison was made between potency. The administration of genistein for five weeks reduced ovariectomy-induced increases in body weight in mice and also decreased *RANKL* and *cathepsin K* mRNA expression and enhanced OPG expression in the tibial head, which is suggestive of a positive effect of genistein on bone mass (Zhang et al., 2009). In addition to genistein and daidzein, other PEs exhibit beneficial effects on bone mass and inhibit osteoclast differentiation such as ferutinin (Palumbo *et al*., 2009), stilbene, matairesnol, and 8-prenylnaringenin (Cornwell *et al*., 2004). Other studies suggest that PEs such as quercetin and kaempferol may also influence osteoclast differentiation (Wattel et al., 2003, Wattel et al., 2004), resveratol, a phenolic compound found in grape skin, and red wine has also been shown to increase osetoblastic differentiation and proliferation (Mizutani *et al*., 1998). Moreover, it has been observed that quercetin, myricetin, kaemferol, isohamnetin, curcumin, hesperdin, and astaxanthin decreased PTH-induced osteoclast formation, while only quercetin significantly increased diaphyseal calcium contents *in-vitro* (Yamaguchi *et al*., 2007). In addition to suppression of bone resorption PE have also been suggested to promote osteoblasts activity. PEs consumption increased BMD and bone formation markers, administration of genistein to post-menopausal women increased bone-specific ALP and osteocalcin (Morabito et al., 2002, Atkinson et al., 2004). .

However, not all data shows a positive effect of PEs on the skeleton. For instance, supplementation of post-menopausal Chinese women with 40 mg/day of daidzein had no effect on BMD (Chen et al., 2003b). Similarly, studies using isoflavones at doses 40-150 mg/day for up to nine months observed no effect on BMD (Chiechi *et al*., 2002, Uesugi *et al*., 2004, Potter *et al*., 1998). These discrepancies are likely to reflect differences in the nature and concentration of the PE delivered, there metabolism and

the stage of menopause. For instance not all individuals are able to metabolise daidzein to its more potent metabolite equol. Further human studies are required before a clear relationship between PEs and bone health is established.

The cellular mechanism mediating the effect of PE on bone resorption is not clear but is likely to involve a suppressive effect on osteoclast differentiation and cytokine production. A plethora of *in-vitro* studies demonstrate several potential mechanisms through which PEs regulate osteoclast activity. Genistein and daidzein have been shown to suppress osteoclast activity by inducing apoptosis, modifying protein tyrosine kinase activity, altering intracellular [Ca^{2+}], membrane depolarization and changes in pro-osteoclastic cytokines (Blair et al., 1996, Williams et al., 1998, Okamoto et al., 2001, Gao and Yamaguchi, 1999c, Gao and Yamaguchi, 2000). In osteoblastsic cells isolated from young piglets daidzein increased the production of OPG and decreased soluble RANKL by an ER-mediated mechanism (de Wilde *et al*., 2004). Genistein has also been shown to reduce osteoclast formation in healthy pre or post-menopausal women by increasing OPG/RANKL ratio in bone marrow stromal cells, suggesting that changes in these important regulators of osteoclast differentiation may mediate the effect of genistein on bone resorption (Heim et al., 2004, Crisafulli et al., 2004). Interestingly, genistein has been shown to modulate the action of PTH on osteoblastsic SaoS-2 cells. It was found that the stimulatory effect of PTH on RANKL expression and suppressive action on OPG expression was abolished by pre-treatment with genistein, suggesting that genistein may block the stimulatory action of PTH on osteoclast formation (Chen and Wong, 2006). Inflammatory cytokines can also stimulate osteoclast or osteoblasts apoptosis and in addition many also directly influence bone cell differentiation. Genistein and daidzein suppress the synthesis of the pro-inflammatory cytokine IL-6 and prostaglandin E2 (PGE2) which are able to directly stimulate osteoclast formation (Suh et al., 2003, Chen and Anderson, 2002) . With genistein inducing ER binding to

specific IL-6 promoters to repress IL-6 transcription and synthesis (Chen et al., 2002). Genistein also significantly inhibited the production of IL-1 and TNF-α in post-menopausal women supplemented with soy-rich diet (Huang *et al*., 2005) as well in ovariectomised rats (Li and Yu, 2003).

In addition it has been reported that genistein decreases osteoclast formation by inhibition of DNA topoisomerase I and II, 5-α reductase, aromatase and MAPKs in murine cell culture (Makela *et al*., 1995, Kao *et al*., 1998, Yamagishi *et al*., 2001). Williams *et al*. (1998) demonstrated an inhibitory effect of genistein on hydrochloric acid transport via suppression of Cl^- channel activity and inhibition of tyrosine kinase activity. Daidzein has similar actions inhibiting bone resorption and osteoclast formation via ER-dependant apoptosis of osteoclast progenitors (Rassi et al., 2002). Daidzein as well as E2 reduce the life span of osteoclast progenitors by inducing apoptosis via a selective caspase-8 mediated mechanism (Earnshaw *et al*., 1999). Genistein acts at high doses as a tyrosine kinase inhibitor and also induces apoptosis as a consequence (Sandy *et al*., 1998), while at lower concentrations it appears to act as a weak oestrogenic agonist which may explain why cells display a biphasic response to it (Anderson *et al*., 1998).

Several *in-vitro* studies using different osteoblastsic cell lines have investigated the cellular and molecular mechanism mediating the effect of PEs on bone formation. While genistein and daidzein increased osteoblastsic proliferation, coumestrol only slightly increased proliferation (Kanno *et al*., 2004). In addition, enterolactone, enterodiol and coumestrol at various concentrations increased ALP activity, bone mineralisation, and extracellular Ca^{2+} and phosphorous content in cultured osteoblastsic cells (Kanno et al., 2004, Feng et al., 2008). The culture of osteoblastsic MC3T3-E1 cells with daidzein stimulated osteoblast differentiation and activity by elevating DNA and protein content and ALP activity which was prevented in the presence of

cyclohexamide, a protein synthesis inhibitor. Similarly, the isoflavone biochanin A stimulated MC3T3-E1 cell growth, ALP activity, osteocalcin production and type I collagen expression. Moreover, the effect of biochanin A was blocked by cyclohexamide and tamoxifen indicating that this effect on protein synthesis was mediated by an ER dependent. These findings indicate that PEs may have a stimulatory effect on cell proliferation by increasing DNA and protein content (Sugimoto and Yamaguchi, 2000b, Sugimoto and Yamaguchi, 2000a).

The anabolic effect of genistein on osteoblastsic proliferation and maturation has also been examined in primary culture using mouse bone marrow-derived cells (Okumura et al., 2006, Liao et al., 2007, Pan et al., 2005). Genistein increased NO synthase and cGMP activity which was associated with increasing of osteoblast differentiation and *Runx2* expression, which was prevented by ICI 182,780, an ER antagonist (Pan et al., 2005). Other PEs have also been shown to promote osteoblasts activity. Resveratrol increased ALP activity, elevated DNA synthesis and inhibited prostaglandin E2 production in MC3T3-E1 cells (Mizutani *et al*., 1998). In addition, stimulatory effects on the differentiation and proliferation of MC3T3-E1 cells have also been noted with kaempferol, glabridin, apigenin, and luteolin (Miyake *et al*., 2003, Choi, 2005, Choi, 2007a, Choi, 2007b). Collectively, much of the data points towards a positive effect of PEs on osteoblast differentiation and activity, although the primary mechanism through which this occur is still to be fully ascertained.

2.5 Zinc and bone

Zinc (Zn^{2+}) plays an important physiological role in cell function (Yamaguchi et al., 1992, Yamaguchi, 1995). It is widely found in different sources: meat, milk, bread and cereal products. It is also known as an essential factor for growth in humans and animals (Chan et al., 1998, Ryz et al., 2009, Shinde et al., 2006). Zn^{2+} is found within

the bone matrix, being concentrated in the osteoid layer before calcification. Zn^{2+} also occurs in the mineralised component of bone tissue and is released during resorption to affect bone cell activity or to be subsequently reincorporated back into mineral during bone formation (Drzazga et al., 2007). Studies suggest that urinary Zn^{2+} excretion can be used as a marker for bone resorption in post-menopausal women (Herzberg et al., 1990, Herzberg et al., 1996, Colpan et al., 2005). Thus, in addition to fluctuations in the level of dietary sources, the local concentration of Zn^{2+} is at least in part dependent on the resorption rate.

Zn^{2+} has an important role in bone development and remodelling and the effect of Zn^{2+} on bone mass is summarised in (Figure 2.4). Multiple studies have investigated the association between Zn^{2+} and skeletal health (Ryz et al., 2009, Hosea et al., 2004). These studies showed that Zn^{2+} deficiency was associated with a reduction in BMD and impaired skeleton maturation. Furthermore, Zn^{2+} blunted osteocalcin and type I collagen levels, and also decreased bone development and recovery in rats feed a Zn^{2+}-deficient diet. Zn^{2+} deficient diets have also been shown to modulate the expression of genes involved in bone formation and induce a significant reduction in femur weight, ALP expression, femoral calcium and phosphorus content (Sun et al., 2011). Furthermore, Zn^{2+} deficiency reduces Runx2 expression in osteoblastsic MC3T3-E1 cells and also decreases ALP production in these cultures (Kwun et al., 2010). Zn^{2+} accumulates in bone tissues and starts to induce ALP production, collagen synthesis and calcification. Zn^{2+} sulphate has also been shown to stimulate a significant increase in osteoblasts proliferation, ALP activity, calcium and collagen expression in MC3T3-E1 cells (Yamaguchi and Matsui, 1996, Seo et al., 2010). This is supported by reports showing that Zn^{2+} increases osteoblasts proliferation and differentiation as inferred from increased ALP activity, osteocalcin, and TGF-β (Yamaguchi and Ohtaki, 1991, Yamaguchi and Hashizume, 1994, Hashizume and Yamaguchi, 1993). Similar effects of

Zn^{2+} sulphate on protein synthesis in bone of new-born rats has been reported, which was abolished by cyclohexamide (Ma and Yamaguchi, 2001). More recent studies found that osteoblasts mineralisation was augmented after Zn^{2+} treatment which may occur as a consequence of increased ALP production (Kawakubo et al., 2011). Similarly, culturing of human osteoblastsic SaoS-2 cells with Zn^{2+} significantly increased osteoblast differentiation and enhanced ALP activity and mineralised nodule formation (Cerovic et al., 2007). Collectively these studies suggest a positive effect of Zn^{2+} in skeleton growth primarily but not exclusively via an action on osteoblast differentiation.

The stimulatory effect of Zn^{2+} on osteoblast differentiation appears to be mediated via several potential responses. For instance Zn^{2+} has been shown to promote bone growth via a mechanism involving enhanced expression of IGF-I, TGF-β1 or osteocalcin expression as well as protein tyrosine phosphate activity *in-vitro* (Yamaguchi and Fukagawa, 2005). Additionally, Zn^{2+} has been shown to stimulate *Runx2* mRNA expression which induces pre-osteoblast differentiation (Yamaguchi et al., 2008). Zn^{2+} also impacts on osteoclast differentiation and activity. In the presence of M-CSF, Zn^{2+} has a suppressive effect on RANKL-induced osteoclastogenesis in mouse marrow culture (Yamaguchi and Uchiyama, 2004). Zn^{2+} decreased TRAP-positive osteoclast formation from mouse bone marrow cells *in-vitro* induced by PTH, IL-1α or PGE2 (Kishi and Yamaguchi, 1994). Furthermore, Zn^{2+} reduced mRNA expression of RANK, c-fos, cathepsin K and c-Jun, and through an extracellular signal-regulated kinase (ERK) dependent mechanism (Hie and Tsukamoto, 2011). Yamaguchi *et al*. (2008) showed a stimulatory effect of Zn^{2+} on *OPG* mRNA expression in osteoblastsic lineages. A recent study also demonstrated a suppressive effect of Zn^{2+} on NFκB activation in osteoblast and osteoclast, and Zn^{2+} also antagonises the inhibitory effect of TNF-α on Smad activation induced osteoblast differentiation through TGFβ/BMP-dependent mechanism

suggestion that Zn^{2+} can act as skeletal protective agent (Yamaguchi and Weitzmann, 2011).

There is many diet sources can include both PEs and zinc with different amounts depending on the sources; these sources including soybeans, peanuts, cheese, sesame products, cereals, green beans, chick peas, vegetables and meat (Institute of Medicine, 2001, U.S. Department of Agriculture, 2011). The concentration of zinc ingested depends on the nature and amount of food consumed. In meat product such as beef it is 7 mg per 85 g and chicken breast 1.33 mg per 140 g, cereals 3.7 mg per 30 g, chickpeas 1.66 mg per 240 g, beans 15.79 mg per 254 g, green peas 1.12 mg per 170 g, peanut 1.08 mg per 28.35 g, walnut 0.88 mg per 28.35 g and 0.88 mg per 28.35 g in cheese (cheddar).

Several studies investigated the serum zinc concentration in relationship to its consumption by individuals. The supplementation of 7.7 µmol of zinc sulphate to 28 hemodialysis patients for 90 days was associated with $6.1x10^{-6}$ M of serum zinc on day 90 after treatment (Jern et al., 2000). Similar result were found in another study, zinc sulphate supplementation increased serum zinc concentration from $4.89x10^{-6}$ M at to $6x10^{-6}$ M (Chevalier et al., 2002). Moreover, the supplementation of hemodialysis patients with 220 mg of zinc sulphate capsule for 42 days increased serum zinc concentration from $3.55x10^{-6}$ M to $5.47x10^{-6}$ M on 42 day (Rashidi et al., 2009).

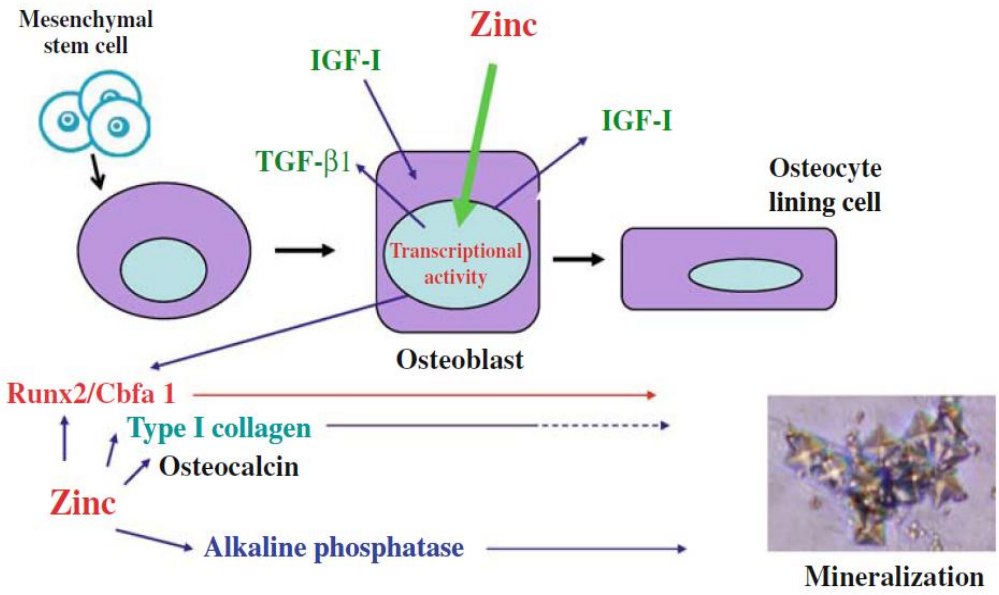

Figure. 2.4. The effect of zinc on osteoblast differentiation and mineralisation. Zinc stimulates key regulators which are controlled this differentiation and bone formation such as RUNX2, ALP, type-I collagen and osteocalcin expression. From (Yamaguchi, 2010).

2.6 Aim of study

Consumption of PEs and other nutritional factors by post-menopausal women may prevent bone loss and other age-related diseases. Several human and animal studies suggest a beneficial effect of supplementation on bone mass. However, the effect of PEs on bone metabolism needs to be established to generate an accurate therapy recommendation for prevention and treatment of post-menopausal osteoporosis.

The aim of the following studies is to examine the effect of physiologically relevant concentration of key PEs such as genistein, coumestrol and daidzein on bone resorption and formation. These studies will be divided into three strands, first their effect on TNFα-induced osteoclast formation; second the effect on osteoblasts formation and bone matrix generation and finally the effect of PEs on Th-lymphocyte number and cytokines production. The studies will also look at potential interactions between PEs and Zn^{2+}.

Chapter Three: General Material and Methods

3. Materials and Methods

3.1 Media and reagents

Dulbecco's minimum essential medium, RPMI 1640 phenol red free was obtained from Invitrogen (GIBCO, UK). The non-selective oestrogen antagonist ICI 182,780 was obtained from Tocris Biosciences (Bristol, UK). Recombinant murine TNF-α and anti-human TNF-α antibodies were purchased from Insight Biotechnology (Wembley, UK). Genistein, daidzein and coumestrol were purchased from Sigma (Poole, Dorset, UK). Cell Titer 96 AQeous non-reactive cell proliferation assay kit was purchased from Promega (Promega, Madison, WI, USA). Antibiotics and other chemicals, reagents and assay kits were obtained from Sigma Aldrich (Poole, Dorset, UK) unless stated. All plastic-wear flasks or tubes were purchased from Fisher scientific (Nunc, UK).

3.2 Cell culture

Murine RAW264.7 TIB.71 monocytic cell line was obtained from American Type Culture Collection (ATCC, UK), human osteogenic sarcoma cells (SaoS-2) and human leukemic T cell lymphoblasts (Jurkat E6.1) were purchased from European Collection of Cell Culture (ECCAC, UK). All experiments were performed in medium without phenol red supplemented with 10% charcoal stripped foetal calf serum (Autogen Bioclear, UK), 2mmol/l glutamine, 100IU/ml benzylpenicillin and 100mg/ml streptomycin (all from Sigma, Poole, Dorset, UK). Incubations were performed at 37^{o}C in a humidified 5% CO_2 incubator. Cultures were fed every 2-3 days by replacing half of the culture medium with fresh medium and cytokines.

3.3 Cell cryopreservation and reanimation

Cells of passage 3-7 were cryopreserved and then used for experiments as required. In brief, adherent cells were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA) solution for no longer than 5 minutes at 37^{o}C. After detachment, cells were

washed with the culture medium to deactivate trypsin and centrifuged at 1000rpm for 5 minutes at room temperature. After centrifugation, cells were resuspended in sterile 90% FCS and 10% dimethyl sulphoxide (DMSO) solution, placed in cryopreservation tube and frozen at -80°C for 24 hour before transfer to liquid nitrogen for long term storage. To reconstitute frozen cells, vials were removed from liquid nitrogen and rapidly thawed and then immediately transferred into 5ml of cell culture media, centrifuged to remove DMSO and then cultured in $25cm^2$ flasks until 80% confluence was reached.

3.4 Phytoestrogens concentrations

PEs concentrations between (10^{-5}-10^{-10} M) have been shown to be effective at modifying bone cell and tissue function and my concentrations were chosen to reflect this range (Crisafulli et al., 2004, Chen and Wong, 2006, Kanno et al., 2004, Sugimoto and Yamaguchi, 2000b). Genistein (10^{-7}-10^{-9} M) inhibited osteoclast formation in murine bone marrow cultures and reduced RANKL/OPG ratios in osteoblastic cells (Sliwiński et al., 2005). PTH-induced osteoclast formation was also prevented by genistein (10^{-5}-10^{-7} M) (Gao and Yamaguchi, 1999b, Gao and Yamaguchi, 1999c) and genistein (10^{-5}-10^{-8} M) decreased pit formation on bone slices (Li and Yu, 2003). This concentration range also appears to be effective at modifying bone cell function in animal models of bone remodelling and loss. Subcutaneous injection of genistein ($2.5x10^{-6}$ M) which generated serum concentrations of $1x10^{-9}$ M partially prevented ovariectomy-induced trabecular bone loss with no uterine effects noted (Ishimi et al., 2000). Furthermore, TRAP+ cell number was reduced in the presence of genistein (10^{-6}-10^{-9} M) and serum ALP and osteocalcin levels significantly increased in ovariectomised rats while TNF-α and IL-1β levels were reduced after OVX rats were administered genistein 45 mg/kg/day. Genistein and daidzein at (10^{-5} M) induced ALP expression and calcium content in bone tissue of female rats (Gao and Yamaguchi, 1999a); and

osteoblast PGE2 and IL-6 production was blunted by genistein (10^{-5} M) (Suh et al., 2003). The range of concentrations used in these studies also reflect those measured in the serum of Asian and Western populations (Morton et al., 2002). In Japanese individuals plasma concentrations of genistein were $4.9x10^{-7}$ M whereas those in Western Europeans $3.3x10^{-8}$ M (Morton et al., 2002). Similarly Valentin-Blasini recorded genistein and daidzein serum concentrations of $1.7x10^{-8}$ and $1.5x10^{-8}$ M respectively (Valentin-Blasini et al., 2003) and genistein and daidzein serum concentrations of $1.4x10^{-8}$ and $1.1x10^{-8}$ M were associated with a reduced incidence of breast cancer in post-menopausal women (Verheus et al., 2007).

Several studies have assessed the relationship between dietary PE intake and serum concentration in Asian and Western population. For instance an investigation on Japanese males and females found that the serum concentration of daidzein and genistein was $1.2x10^{-7}$ M and $4.7x10^{-7}$ M in those receiving either 18.3 mg/day daidzein or 31.4 mg/day genistein (Yamamoto et al., 2001). A further study showed that increasing soy milk intake in pre-menopausal British women was associated with higher plasma concentration of genistein and daidzein; consuming 149 ml or 248 ml of soy milk resulted in serum concentrations of $3.9x10^{-8}$ M and $1.3x10^{-7}$ M for daidzein and 1.1 $x10^{-7}$ M and $3.7x10^{-7}$ M for genistein (Verkasalo et al., 2001). Similar results was found in Frankenfeld's (2003) study, which revealed that intakes ofof 8.1 mg/wk or 6.9 mg/wk of genistein and daidzein by post-menopausal women was associated with plasma concentration of $1.2x10^{-8}$ M and $6.9x10^{-8}$ M of genistein and daidzein respectively. Therefore, data provides evidence for a positive relationship between the intake of phytoestrogens and subsequent serum or plasma levels. These concentrations are similar to those in the mid-range of my dose response and are similar to the genistein

and coumestrol concentrations that had the most pronounced effect on osteoclast differentiation.

3.5 Assessment of Tartrate-Resistant Acid Phosphatase (TRAP) activity

Osteoclast formation was evaluated by staining for the specific osteoclastic marker tartrate resistant acid phosphatase-positive (TRAP) using a modification of the method of Burstone (Burstone, 1958) using a acid phosphatase, leukocyte (TRAP) kit (Sigma, Poole, Dorset, UK) and naphthol AS-BI phosphate as a substrate. The assay was performed following manufacturer's instructions. Briefly, after treatment, cells were fixed onto the bottom of well plates with 10% formaldehyde/PBS for 10 minutes at room temperature, then washed twice with distilled water (dH_2O) and stained with AS-BI phosphate and fast garnet (GBS) base as a chromogen for 1 hour at 37^{o}C in a humidified incubator. Cultures were then rinsed with dH_2O and osteoclast differentiation scored using the following method. The number of TRAP-positive mononuclear and multinuclear cells containing three or more nuclei was measured using an inverted light microscope fitted with an eyepiece graticule using an objective of x20 (Olympus, Japan). Five defined areas for each well were counted for TRAP-positive mono- and multi-nucleated osteoclast and the results expressed as the mean number of cells per cm^2 ± S.E.M. All experiments were performed in triplicate.

3.6 Bone resorption

Bone resorption was measured using slices of bovine cortical bone (kind gift of Dr Karen Fuller). Bovine cortical bone slices were cut using diamond saw (Buehler, Coventry, UK) according to Klein et al. (Klein et al., 1994) method. Bovine bone was cut into slices 30 μm in thickness followed by cleaning in distilled water by ultrasonication for 15 minutes. Then, bone slices were washed with acetone for 10 minutes and immersed in Hank's balanced salt solution (HBSS). Slices were then

sterilised in 70% ethanol at room temperature and cut into small squares 20mm^2 in area. Slices were stored at -20^{o}C until used. RAW264.7 cells were seeded onto the slices in 24 well plates at 10^5 cells per well. After incubation, cells were removed from the surface of the slices by immersion in 10% (vol/vol) sodium hypochlorite for 10 minutes, 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 to enable visualization of resorption pits. The percentage of bone surface resorbed was quantified by reflected light microscopy using an eyepiece gratitude and magnification of x100 on an Olympus BHB microscope with a Schott KL1500 light source.

3.7 Measurement of cell proliferation and apoptosis

Cell proliferation was measured using the Cell Titer 96 AQeous non-reactive cell proliferation assay kit (Promega, Madison, WI, USA). This assay measures proliferation using mitochondrial NADH/NADPH-dependent dehydrogenase activity as a marker of cell proliferation, this enzyme converts a substrate (3-(4,5-dimethylthiazol-2-gl)-5-(3-carboxymethoxylphenyl)-2-(4-sulphophenyl-2H) tetrazolium compound (MTS) into an formazan dye which can then be quantified at A490. The absorbance of the formazan is linearly proportional to the number of viable cells.

At the end of an experiment 20µl of MTS solution was added to each well and incubated for 4hr at 37^{o}C. After incubation, the absorbance in each well was measured at 490nm using a 96 well plate reader (Molecular Devices, USA). The viable cell number in each well was then calculated using the equation of the line $y = mx + c$ and a standard curve plotted from absorbance of wells containing know cell numbers. All experiments were performed in triplicate and data expressed as the mean ± S.E.M.

Apoptosis was determined using an Apo-One Homogeneous caspase 3/7 kit (Promega, Madison, WI, USA) that measured caspase 3/7 activity in osteoclast following the

manufacture's protocol. After incubation 100µl of apo-one caspase-3/7 reagent was added to each well and the plate incubated for 18 hour with shaking at 37°C. Fluorescence was then measured using a fluorescence multi-well plate reader (Perseptive Biosystem, USA) at excitation wave length of 490 nm and emission wave length 521 nm on a. Experiments were performed in triplicate.

3.8 NFATc1 immunofluorescent staining

RAW 264.7 cells at ($1x10^4$ cell/well) density were seeded overnight onto glass coverslips and then incubated with appropriate treatments. The distribution of NFATc1 protein 24 and 48 hours after stimulation was assessed according to previously published protocols (Evans and Fox, 2007). Coverslips were removed, washed in PBS, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with 1% goat serum and incubated with a specific anti-mouse NFATc1 monoclonal antibody (1:50, Santa Cruz, USA) for 1 hour. Cells were washed in PBS, incubated for 2 hours with biotinylated goat anti-mouse secondary (Vector Labs, USA) and then incubated for 15 minutes with fluorescein conjugated streptavidin (Vector Labs, USA). Fluorescence was visualized using a Leica HC microscope. The percentage of cells displaying nuclear staining was then quantified and 100 cells per group were measured from 3 separate coverslips per group. Photographs were taken with a JVC digital camera linked to image pro-plus at a magnification of x400. Results are displayed as mean ± S.E.M from three separate experiments.

3.9 Measurement of mineralisation and alkaline phosphatase (ALP) activity

ALP activity was measured by staining cultures with *p*-nitrophenyl phosphate (1 mg/ml) in 0.2 M tris buffer at 37ºC for 30 min (Sabokbar et al., 1994). 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 zinc sulphate (10^{-5} M) on ALP activity was assessed as follows. SaoS-2

cells ($1x10^4$ per well) were incubated in 96 well plates for 24 hr to enable cells to adhere. Cultures were then incubated in relevant treatments in presence of β-glycerophosphate (β-GP) (10 mM) and L-ascorbic acid (L-AA) (50 mg/l) for four days. Absorbance was measured at 405 nm and results were then normalised to total cell number and expressed as the amount of ALP required to liberate 1 mmol of *p*-nitrophenol / min / 10^4 cells.

Mineralisation was assessed using a modification of Hale's methodology (Hale et al., 2000). 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}-10^{-9} M) with or without zinc sulphate (10^{-5} M). After 18 days of incubation culture medium was aspirated, the monolayer washed with PBS and incubated in culture medium containing 1 µg/ml calcein for four hours at 37ºC. Cultures were then washed three times in PBS and fluorescence measured by a cytofluor II fluorescence multiwell plate reader (Perseptive Biosystem, USA) at 485 nm excitation and 530 nm emission.

3.10 Molecular biology

3.10.1 RNA extraction and reverse transcription

Total RNA was isolated using a commercially available GenElute™ mammalian total RNA miniprep kit (Sigma, Poole, Dorset, UK), which utilizes a column based technique to isolate and purify RNA. After incubation, cells were washed with DPBS and total RNA extracted according to the manufacture's protocol. All consumables and reagents used were free of contaminating DNAase and RNAase. Genomic DNA was removed using an on-column DNase-I treatment step. RNA and DNA quantity and purity was measured using a nandrop spectrophotometer (ND-1000) (Labtech, UK). Absorbance

was measured with A260 for nucleic acid and the ratio A260:A280 was used to assess sample purity. RNA concentrations were then adjusted to 250µg/µl in molecular biology grade H_2O, RNA (1µg) was reversed transcribed to cDNA using M-MLV reverse transcriptase reaction in thin walled PCR tubes (Sigma, Poole, Dorset, UK) using a GeneAmp PCR System 9700 machine. RNA was denatured at 70°C for 10 min in the presence of dNTPs (0.5mM) (dATP, dCTP, dGTP, TTP) and random nonamers (1µM). Reactions were cooled on ice for 5 minutes and then 1 unit of MMLV-reverse transcriptase was added to each reaction. Reactions were then incubated at room temperature for 10 min, 37°C for 50 min and 94°C for 5 min. RTs were stored at 4º C until used. Negative controls consisting of reactions lacking RT were run in all experiments.

3.10.2 Verification of PCR primers and RT

Primer sequences were designed using the NCBI website and according to the mRNA sequence of each gene published in the same website using primer blast software. Designed primers were purchased from Eurofins MWG Operon (Germany). 2 µl of cDNA was used for each PCR reaction. Each reaction contained 10µM of forward and reverse primers, dNTPs (0.5mM), Taq DNA polymerase (5 unit/µl) and PCR buffer (10x) in a final volume of 25µl. Reaction conditions were 94^{o}C for 2 minutes, followed by 40 cycles at 94^{o}C for 30 seconds, 60^{o}C for 30 seconds and 72^{o}C for 30 seconds.

Product size and primer specificity was then confirmed using agarose gel electrophoresis. PCR samples had 2.5 µl of loading buffer (orange G dye) added and was then loaded onto a 2% (w/v) TAE agarose gel. Gels were made by dissolving agarose (electrophoresis grade, Fisher scientific) in an appropriate volume of TAE buffer (40mM Tris-base, l0mM EDTA and 0.1% acetic acid) which was heated in a microwave for 2 minutes and then cooled to 50°C. 1µl of ethidium bromide (10mg/ml)

was then added to the gel to enable visualization of DNA under UV light. Gels were run at 60-100V according to the size of the product for an appropriate time. Bands were checked for presence and size using UV gel documentation system (UVi Tech, Japan) linked to a PC (Toshiba, Japan).

3.10.3 Real-time quantitative polymerase chain reaction (RT-PCR)

Quantitative PCR is a highly sensitive technique that enables the absolute quantification of gene expression in biological samples. RT-PCR was used in my studies to establish the effect of PEs on the expression of genes involved in osteoblast, osteoclast and T cells formation and activity. Quantitative RT-PCR was performed using a 48 well Step One PCR system linked to Stepone v.2 analytical software (Applied Biosystems Warrington, Cheshire, UK) and using the DNA-binding dye SYBR green for detection of PCR products. β-actin was used as a house keeping gene to normalise mRNA levels. Reactions containing the following, 2µl of external plasmid standard or cDNA added to a final reaction volume of 25µl which contained 0.05U/µl Taq, SYBR green, PCR buffer (300 nM) reference dye (Rox) and specific sense and antisense primers (0.2µM).

For the generation of standard curves, the corresponding cDNA was cloned into pGEM-T Easy (Promega, UK) (see section 3.8.3.6). The concentration of DNA plasmid stock was determined by measurement of optical density at 260 nm. Copy number for each plasmid was calculated from these measurements. A 100 ng/ml of target insert solution was prepared and used as a stock to prepare serial dilutions to generate a standard curve with copy numbers from 500 to $5x10^6$ copies. 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 minutes, followed by 40 cycles of 94°C for 30 seconds,

60°C for 30 seconds and 72°C for 30 seconds. 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 normalized against 10^6 β-actin copy numbers. The mRNA copy number was calculated for each sample from the standard curves by the instrument's software. Samples were analysed in triplicate.

3.10.4 Preparation of internal standards for quantitative PCR

To enable the quantification of absolute copy number of target genes the coding sequences of target genes were sub-cloned into a plasmid vector (pGEM-T Easy, Promega, UK). This vector is pre-linearized by EcoRI digestion and insertion of 3'terminal thymidine to both ends prevents recircularisation and creates compatible overhanging bases for ligation of PCR products. Retroviral PBabe puromycin resistant vector was linearized using one restriction enzyme chosen according to the suitability with the DNA insert. Known copy numbers of these plasmids were then used to generate standard curves to enable the measurement of copy number in samples.

The target cDNA was amplified by RT-PCR from cultures of osteoblasts or osteoclasts using specific primer pairs. Products were electrophoresed on 1% TAE agarose gel and bands detected by ultraviolet light (UV) using ethidium bromide staining. DNA fragments of correct size were excised from the gel using a sterile scalpel, placed into 1.5 ml microcentrifuge tubes and DNA extracted using GenElute gel extraction kit (Sigma, Pool, Dorset, UK) according to the manufacturer's instructions. Solubilisation solution was added to the gel slices which were then incubated in a water bath at 50-60°C with vortexing every 2 minutes, until the gel slices were completely dissolved. Samples were then applied to DNA binding column and centrifuged at 13,000xg for 1 minute. The column was then washed to remove remaining agarose and centrifuged at 13,000xg for 1 minute. The DNA insert was eluted in 50µl of elution buffer and the

concentration and purity quantified using a nanodrop spectrophotometer and then stored at -20°C until the ligation reaction was performed.

The DNA insert was then ligated into pGEM-T easy vector (Promega, UK) using T4 DNA ligase. For maximal ligation efficiency, an insert : vector ratio of 3:1 was used. The amount of DNA fragment was calculated according to the following equation:

$$\frac{\textbf{ng vector x size of insert (kb)}}{\textbf{size of vector (kb)}} \textbf{ x insert: vector ratio} = \textbf{amount of DNA required (ng)}$$

Ligation reactions also contained 2x T4 DNA ligase buffer, 1µl of T4 DNA ligase (3U/µl) and water up to 10µl final volume. Ligations were incubated overnight at 4°C. Control ligations were setup without insert DNA. Following ligation plasmids were transformed into competent JM109 bacteria cells (*Escherichia coli* DH5α, Promega, UK) which is capable of high efficacy transformation at $1x10^8$ CFU/µg. In brief, 50µl of thawed bacteria was added to 2µl of ligation reaction and incubated for 20 minutes in an ice bath. Bacteria were then heat-shocked in a water bath at 42°C for 50 seconds and incubated again on ice for 2 minutes. 950µl of SOC medium was added to the bacteria and incubated for 1.5 hours in a shaking water bath at 37°C. 100µl of transformed bacteria were then plated out on LB agar/ampicillin (100µg/ml)/x-gal (80µg/ml)/IPTG (0.5 mM) plate and incubated overnight at 37°C. On these plates transformed colonies appear white while colonies that don't contain plasmid with an insert are blue. Several colonies were selected from the agar plate and used to inoculate 5 ml of LB/ampicillin broth and the culture incubated in water bath at 37°C overnight with shaking (150 rpm) to enable plasmid amplification prior to extraction.

Following overnight culture a GeneElute HP plasmid miniprep kit (Sigma, UK) was used for extraction of plasmid DNA and to check for the presence of target insert. Bacterial cells were harvested from 3-5ml of culture and then transferred to a 1.5ml

microcentrifuge tube and centrifuged at 13,000xg for 5 minutes. Bacterial pellets were resuspended in 200µl of suspension solution, and then 200µl of lysis solution added to release the plasmid. Cell debris was precipitated by adding 350µl of neutralization/binding buffer and centrifuging the samples at maximum speed in a micro-centrifuge for 10 minutes. Subsequently, the supernatant was transferred onto binding column and centrifuged at maximum speed for 1 minute. The pellet containing plasmid DNA was then washed twice with wash solution and then resuspended in 50µl of elution buffer. An EcoRI restriction enzyme digest was then performed to check for the presence of target insert in the DNA vector. Digests were performed for 1 hour at 37^{o}C and then run on a 1% agarose TAE gel to check for the presence of target insert of the correct size. Successfully ligated plasmids were then streaked on LB/ampicillin agar plate and incubated overnight at 37°C. Colonies were selected from these plates and grown in 250ml of LB medium at 37°C. Plasmids were then purified from these cultures using a GenElute High performance plasmid midiprep kit according to manufacturer's instructions (Sigma, UK). Plasmids were stored at -20°C.

3.10.5 Molecular biology solutions

3.10.5a Luria-Bertani (LB) broth

20 g of LB broth was dissolved in 1 litre of distilled water, autoclaved and ampicillin added to a final concentration of 100 µg/ml.

3.10.5b LB/ampicillin/x-Gal agar plate plates

15g agar and LB broth were dissolved in 1 litre of distilled water, autoclaved then chilled at room temperature to approximately 50°C. Ampicillin (100µg/ml)/x-gal (80µg/ml)/IPTG (0.5 mM) were added to the LB agar media and poured into 94mm petri dish to a depth of 10mm of thickness , left to set, then stored at 4°C.

3.10.5c 10x Tris-acetate-EDTA (TAE) buffer

0.4M Tris-base, 0.5M EDTA and 1M acetic acid was dissolved in 1L of d.H_2O. The pH was adjusted to 8.5 and then diluted to make 1x TAE buffer.

3.11 Statistical analysis

Differences between groups were assessed using Fisher's analysis of variance (Statview; Abacus concepts, USA) or Student's T-test as appropriate. All data are analysed using SPSS program 18 (IBM SPSS Statistics software). A difference of $P<0.05$ was considered statistically significant.

Chapter Four : Effect of Phytoestrogens on TNF-α-Induced Osteoclast Differentiation

Chapter 4

4.1 Introduction

Post-menopausal osteoporosis is characterized by low bone mass and increased fracture risk. Worldwide, osteoporotic fractures are a major health concern especially in countries with aging populations. Prior to menopause osteoblastsic bone formation and osteoclastic bone resorption are balanced such that there is little net bone loss during each remodelling cycle. E2 deficiency disrupts this equilibrium increasing bone turnover and skewing remodelling in favour of resorption. Recent evidence suggest that this is at least in part due to elevated levels of the potent pro-inflammatory cytokine TNF-α (Weitzmann and Pacifici, 2007). Several studies show increased TNF-α production by bone marrow and T cells following E2 deficiency in mice (Cenci et al., 2003, Grassi et al., 2007). Furthermore, mice lacking T cells or TNF-α receptors are resistant to ovariectomy-induced bone loss (Roggia et al., 2001). Human peripheral blood mononuclear cells (PBMC) also display E2-dependent changes in TNF-α production, PBMC from post-menopausal or oophorectomised individuals synthesize elevated levels of TNF-α (D'Amelio et al., 2004), whereas E2 replacement suppresses PBMC TNF-α production *in-vivo* (Bernard-Poenaru et al., 2001). In addition to promoting post-menopausal bone loss TNF-α is also a key regulator of osteolysis associated with chronic inflammatory conditions such as rheumatoid arthritis (Boyce et al., 2005). TNF-α promotes osteoclast differentiation through several actions, it augments RANKL induced osteoclastogenesis and also directly stimulates osteoclast formation from human or mouse monocytes (Komine et al., 2001, Fox et al., 2008) and has been suggested to activate osteoclast formation independent of RANKL signalling (Kudo et al., 2002). Thus, TNF-α has a central role in post-menopausal bone loss directly promoting osteoclast formation and augmenting the response to other resorptive stimuli.

Post-menopausal women are typically prescribed bisphosphonates to prevent bone loss, however complications such as induction of the acute phase response and osteonecrosis of the jaw can occur (Cole et al., 2008). As a consequence, other potential therapeutic interventions have been examined including PEs. PEs are a diverse group of plant derived compounds with a structure and function similar to oestradiol that are used clinically to control tumour progression. Some epidemiological studies suggest that diets with high PEs content may be associated with a more robust skeleton; positive associations have been noted between soy protein intake and spinal and hip bone mineral density in Asian women (Horiuchi et al., 2000, Kim et al., 2002a, Mei et al., 2001) and women with the highest dietary soy levels have the lowest resorption rates (Mei et al., 2001).

Intervention studies have also noted beneficial actions of PEs on post-menopausal women's spinal (Alekel et al., 2000, Atkinson et al., 2004), trochanter (Chen et al., 2003b) and Ward's triangle BMD (Wu et al., 2006). Decreases in resorption markers have also been described following PEs supplementation (Weaver et al., 2009). However, not all studies have observed beneficial actions of PEs on the skeleton (Gallagher et al., 2004), suggesting that the response may be dependent on the nature of the treatment regime or is modified by other lifestyle factors. While PEs may have a beneficial action on skeletal mass, the cellular and molecular mechanism mediating this response is not fully understood and is likely to differ depending on the PEs examined. Previous studies have shown that PEs reduce osteoclast formation by directly suppressing the response of monocytes to osteoclast-inductive stimuli and also indirectly by reducing osteoblastsic RANKL expression (Garcia Palacios et al., 2005, Gao and Yamaguchi, 1999b, Bandyopadhyay et al., 2006, Gao and Yamaguchi, 2000, Rassi et al., 2002, Uchiyama and Yamaguchi, 2007, Li and Yu, 2003). Surprisingly, in

spite of evidence showing that TNF-α has a crucial role in post-menopausal and inflammatory bone loss no study has examined the direct effect of PEs on TNF-α induced osteoclast differentiation. This is of importance as while there is an overlap between the intracellular signals activated by TNF-α and RANKL the early events in the their transduction cascades are dissimilar, each binding to a distinct receptor linked to different groupings of TRAF signalling factors. RANKL activates TRAFs 2, 3 and 6 while TNF-α predominantly signals via TRAFs 2 and 3. Furthermore, RANKL activates a broader range of signal transduction pathways than TNF-α, which requires other co-stimulatory factors to facilitate osteoclast formation (Fox et al., 2008). This raises the possibility that TNF-α and RANKL-induced osteoclast may respond differently to anti-osteoclastic compounds that target initial stages of osteoclast differentiation. In keeping with this the anti-resorptive effect of bisphosphonates is blunted in inflammatory conditions associated with high TNF-α levels such as rheumatoid arthritis (Zhang et al., 2005) and E2 at least partly suppresses RANKL-induced osteoclast formation via a TRAF6 dependent action (Robinson et al., 2009). The mechanism by which TNF-α can affect bone remodelling is complex as it can cooperate with other signalling pathways downstream of TNFR1.

Thus, to determine if PEs are able to directly inhibit TNF-α-induced osteoclastogenesis and to establish the molecular mechanism of the anti-osteoclastic action I examined the effect of genistein, coumestrol and daidzein on TNF-α-induced osteoclast formation from the monocytic cell line RAW264.7.

4.2 Materials and methods

All general materials and methods relating to cell culture and molecular biology experiments are described in chapter three. However, key methods relating to this chapter are described in detail below.

4.2.1 Cell culture

Extensive research proves that RAW264.7 cells are an excellent model to study osteoclast biology in-vitro due to the similar characteristics they have with primary human and animal osteoclastic precursors. Like primary monocytes RAW264.7 cells form osteoclast in response to a range of resorptive stimuli and readily form resorption pits on bone, in addition unlike primary cells they are a homogenous population enabling the direct study of osteoclast regulatory factors without the interference of other cell types (Abe et al., 2012, Collin-Osdoby and Osdoby, 2012, Cuetara et al., 2006). RAW264.7 cells were cultured in phenol-free DMEM medium supplemented with 10% foetal calf serum, 2mmol/l glutamine, 100IU/ml benzylpenicillin and 100mg/ml streptomycin. Cells were maintained by replacing half the medium with fresh medium every 2-3 days.

4.2.2 Osteoclast differentiation

To determine the direct effect of PEs on TNF-α-induced osteoclast differentiation, RAW264.7 cells were transferred to 24-well plates at a density of 10^5 cells per well and cultured with combinations of TNF-α (50ng/ml), genistein (10^{-5} to 10^{-9} M), daidzein (10^{-5} to 10^{-9} M) or coumestrol (10^{-5} to 10^{-9} M) for 4 days with or without the E2 antagonist ICI 182,780 (10^{-6} M). To determine the effect of PEs on mature TNF-α-induced osteoclast, RAW264.7 cells were incubated in the presence of TNF-α (50ng/ml) for four days to generate osteoclast cultures which were then incubated for 24 hours with PE concentrations shown to inhibit osteoclast formation in the initial experiment

with or without the E2 antagonist ICI 182,780 (10^{-5} M). Raw264.7 cells were then fixed and stained for TRAP to determine osteoclast formation as explained in chapter three (3.4). The number of TRAP-positive cells was counted and the results expressed as the number of cells per cm^2. All experiments were performed in triplicate.

4.2.3 Bone resorption and NFATc1 immunofluorescent staining

To assess the effect of PEs on TNF-α-induced bone resorption RAW264.7 cells were seeded onto 20mm^2 slices of devitalised bovine bone in 24 well plates at a density of 10^5 cells per well. Cells were incubated in TNF-α (50ng/ml) with or without PEs for eight days in the presence or absence of ICI 182,780 (10^{-5}M). After incubation cells were removed from the surface of bone slices and stained for assessment of bone resorption area using inverted light microscopy as described in chapter three (3.5). The percentage of bone surface resorbed was measured as a mean from three separate experiments.

For NFATc1 immunofluorescent staining, RAW 264.7 cells were seeded overnight onto glass coverslips and then incubated in TNF-α (50ng/ml) with or without genistein (10^{-7} M), coumestrol (10^{-7} M) or daidzein (10^{-5} M). The distribution of NFATc1 protein 24 & 48 hours after stimulation was assessed according to the procedure detailed in chapter three (3.6).

4.2.4 Cell viability and apoptosis

The effect of PEs on cell viability was assessed using an AQueous one solution cell proliferation assay (Promega UK). Cells were incubated with TNF-α (50ng/ml) or TNF-α plus genistein, coumestrol or daidzein (10^{-4} to 10^{-9} M) for four days, proliferation was then assessed. The effect of anti-osteoclastic concentrations of PEs on apoptosis was assessed by incubating RAW cells with TNF-α (50ng/ml) or TNF-α plus genistein

(10^{-7} M), coumestrol (10^{-7} M) or daidzein (10^{-5} M) for 24 hours. Caspase 3/7 activity was then measured using the apo-One homogeneous caspase assay (Promega, UK).

4.2.5 Real time quantitative PCR analysis

Quantitative PCR was used to determine the expression of key regulators of osteoclast differentiation. All the results are expressed as a copy number normalised to 10^6β-actin mRNA copies. RAW264.7 cells (5 x 10^5) were incubated in 25cm^2 flasks for 24, 48 or 96 hours with combinations of TNF-α (50ng/ml), genistein (10^{-7} M), coumestrol (10^{-7} M) or daidzein (10^{-5} M). Total RNA was extracted from these cultures using a Genelute RNA isolation kit and reversed transcribed with M-MLV reverse transcriptase and QPCR was carried out using SYBR Green DNA binding dye as described in chapter three (3.8.2). The sequences of the primers used are shown in (Table 4.1). The generation of standard curves was described in chapter three using the corresponding cDNA and cloned into pGEM-T Easy (Promega).

Table 4.1. Primer sequence of murine genes examined.

Murine Genes	5´-3´ Forward primer	3´-5´ Reverse primer
β-Actin	GTCATCACTATTGGCAACGAG	CCTGTCAGCAATGCCTGGTACAT
NFATc1	CCGTTGCTTCCAGAAAATAACA	TGTGGGATGTGAACTCGGAA
c-fos	CCATCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
DC-STAMP	AAAACCCTTGGGCTGTTCTT	GTTCCTTGCTTCTCTCCACG
NFκB	GTGGAGGCATGTTCGGTAGT	GTCCAGAAGGCTCAGGTCAG
p38	CGACCACGTTCAGTTTCTCA	AGGTCAGGCTCTTCCACTCA

4.2.6 Generation of c-fos expressing retroviral vector

The role of c-fos in the effect of PEs on TNF-α-induced osteoclastogenesis was examined by infecting RAW264.7 cells with a *c-fos* expressing retroviral vector. Constitutively active *c-fos* (N-core-cfos) was transduced using a retroviral vector, pBabe puro, which expresses cDNA inserts under the control of a retroviral enhancer-promoter (Morgenstern and Land, 1990). The coding region for the N terminal and core regions of *c-fos* were PCR amplified from total mouse RNA and cloned into PGEM T easy (Promega, UK). EcoRI digest fragments were then sub-cloned into pBabe puro. The resulting plasmid pBabe-cfos was sequenced (MWG Eurofins). The pBabe-*cfos* and pBabe-empty (control) vectors were then transfected into the Phoenix retroviral packaging cell line using FuGene (Roche, Indianapolis). After 48 hours stably transfected cells were selected by incubating with 2.5 μg/ml puromycin. Stably transfected clones were then picked 4-7 days later and grown to confluence in 25cm^2 flasks. After incubation in fresh medium for an additional 2 days, stably transfected cells were selected with puromycin for 2 days.

4.2.7 Infection of RAW264.7 cells with c-fos expressing retroviruses

Raw264.7 cells were added to the wells of 96 well plates (2x10^4 cells/well) containing thermonax coverslips or slices of devitalized bovine bone and cultured for 24 hours prior to infection. Medium was then removed and replaced with filtered (0.45μm) supernatant from pBabe-*cfos* or pBabe-empty virus-producing Phoenix cells in the presence of 8μg/ml polybrene. Cultures were then incubated for 16 hours before the addition the treatment. After incubation for a further two days stably infected cells were selected by the addition of puromycin (2.5 μg/ml) for two days. Cells were then stained for TRAP and bone resorption as described earlier. To examine the effect of pBabe-*cfos* on *NFATc1* expression, Raw264.7 cells were seeded into 25cm^2 flasks and then

incubated with supernatant from pBabe-*cfos* or pBabe-empty virus-producing Phoenix cells in the presence of 8μg/ml polybrene. Cultures were then incubated for 16 hours before the addition of TNF-α (50ng/ml) with or without genistein (10^{-7}M), daidzein (10^{-5}M) or coumestrol (10^{-7} M) in the presence of puromycin (2.5 μg/ml) for 48 hours. Total RNA was then isolated and NFATc1 levels determined by quantitative PCR as described above.

4.2.8 Statistical analysis

Differences between groups were assessed using Fisher's one way analysis of variance (Statview; Abacus concepts, USA) or Student's t-test as appropriate. A difference of $p<0.05$ was considered statistically significant.

4.3 Results

4.3.1 Genistein, coumestrol and daidzein suppress TNF-α-induced osteoclast differentiation and bone resorption

All PEs at 10^{-4} M significantly reduced cell viability whereas lower concentrations had no detrimental effect on proliferation (Figure 4.4) and for this reason subsequent experiments on osteoclast formation used PEs concentrations from 10^{-5} to 10^{-9} M. TNF-α directly stimulated the formation of strongly TRAP positive mononuclear and multinuclear osteoclasts within four days, which similar to previous findings readily formed resorption pits on bone slices (Figure 4.1A) (Fuller et al., 2002). Genistein, coumestrol and daidzein all directly suppressed TNF-α-induced osteoclast formation and bone resorption. Genistein at 10^{-7}M significantly reduced TNF-α-induced osteoclast formation (11% of TNF-α treated group, 10^{-7} M; $P<0.001$) (Figure 4.1A) and inhibited TNF-α-induced bone resorption (19% of TNF-α treated group) (Figure 4.1B). Similar suppressive effects on osteoclast formation and resorption were noted with 10^{-7} M coumestrol (TRAP positive osteoclast formation 12% of TNF-α treated group, bone resorption area 22% of TNF-α treated group) (Figures 4.2 A&B). In contrast only the highest concentration of daidzein (10^{-5} M) suppressed osteoclast formation and bone resorption (TRAP positive osteoclast formation 13% of TNF-α treated group, bone resorption area 74% of TNF-α treated group) (Figures 4.3 A&B). In addition, anti-osteoclastic concentrations of genistein, coumestrol and daidzein also significantly reduced TRAP expression in cultures of mature TNF-α-induced osteoclasts (Table 4.2).

To determine if the anti-osteoclastic effect of PEs was mediated through an ER dependent mechanism cells were cultured in the presence of the E2 antagonist ICI 182,780. ICI 182,780 prevented the suppressive action of genistein (10^{-7} M), coumestrol (10^{-7} M) and daidzein (10^{-5} M) on TNF-α-induced bone resorption and TRAP positive

osteoclast formation (Figure 4.1, 4.2, 4.3), suggesting that PEs suppress TNF-α-induced osteoclastogenesis via an ER dependent mechanism. In addition, in the presence of ICI 182,780 genistein and coumestrol (10^{-5}-10^{-6} M) also displayed a pro-osteoclastic effect, significantly augmenting TNF-α-induced osteoclast formation and bone resorption (Figure 4.1, 4.2, 4.3). In contrast, daidzein had no augmentative action on osteoclast differentiation or bone resorption at any concentration in the presence or absence of ER antagonist. Thus, genistein and coumestrol possess multiple antagonistic actions on osteoclast formation; however it is clear that when ER signalling is intact the anti-osteoclastic action counteracts any potential pro-osteoclastic effect. Therefore all PEs studied only displayed a suppressive effect on TNF-α-induced osteoclast differentiation when delivered in isolation.

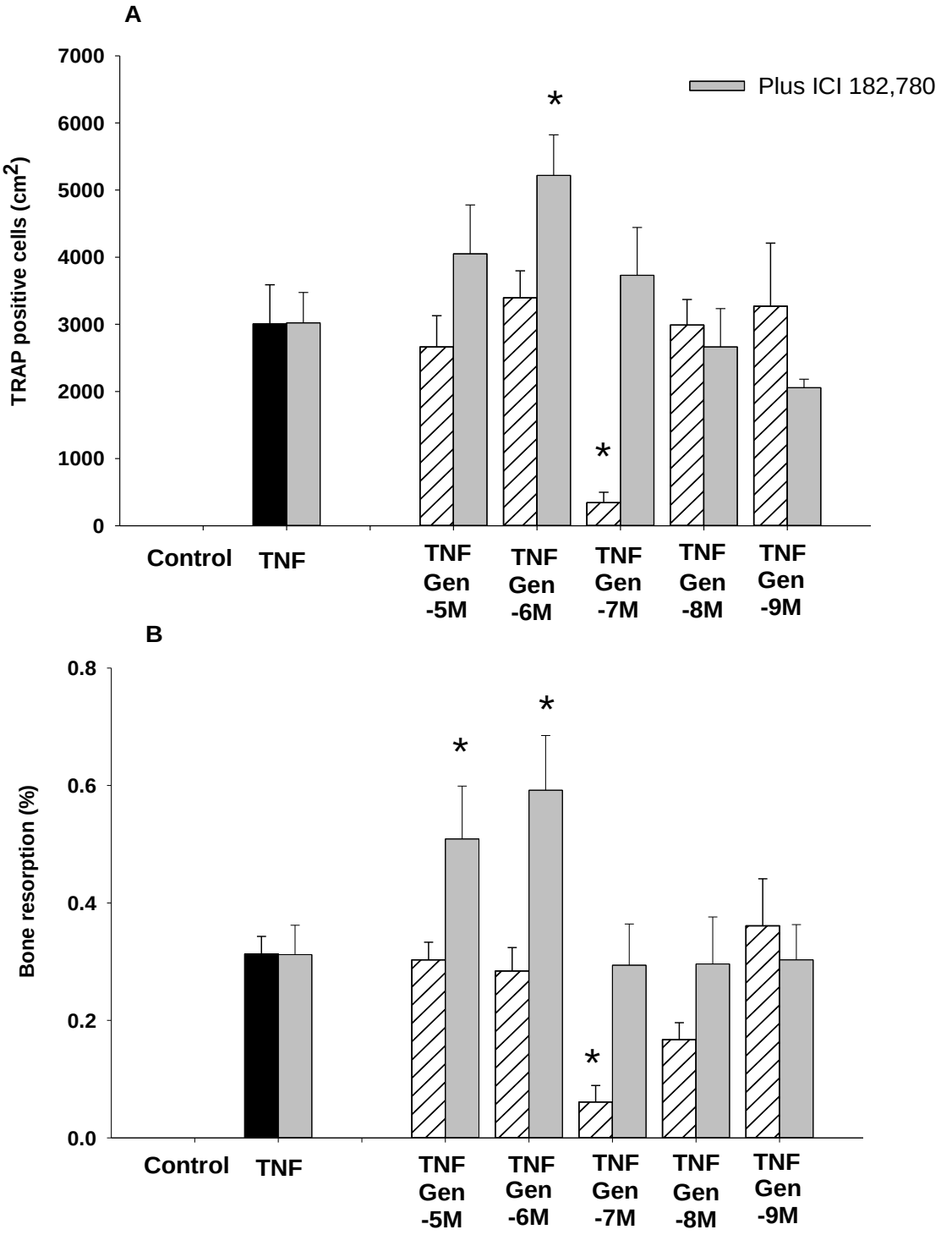

Figure 4.1. Genistein (10^{-7} M) significantly reduced TNF-α-induced TRAP positive osteoclast formation (A) and bone resorption (B), which was prevented by the ER antagonist ICI 182,780 (solid grey bars). In the presence of ICI 182,780 higher concentrations of genistein (10^{-5}, 10^{-6} M) increased osteoclast formation and bone resorption. RAW264.7 cells treated with TNF-α (50ng/ml) and phytoestrogens (10^{-5}-10^{-9} M), and after four days TRAP-positive cells was scored, while the percentage of bone surface displaying resorption pits was analysed after eight days. Values are expressed as the mean ± SEM of three separate experiments n=18, * P< 0.05 versus TNF-α treated group.

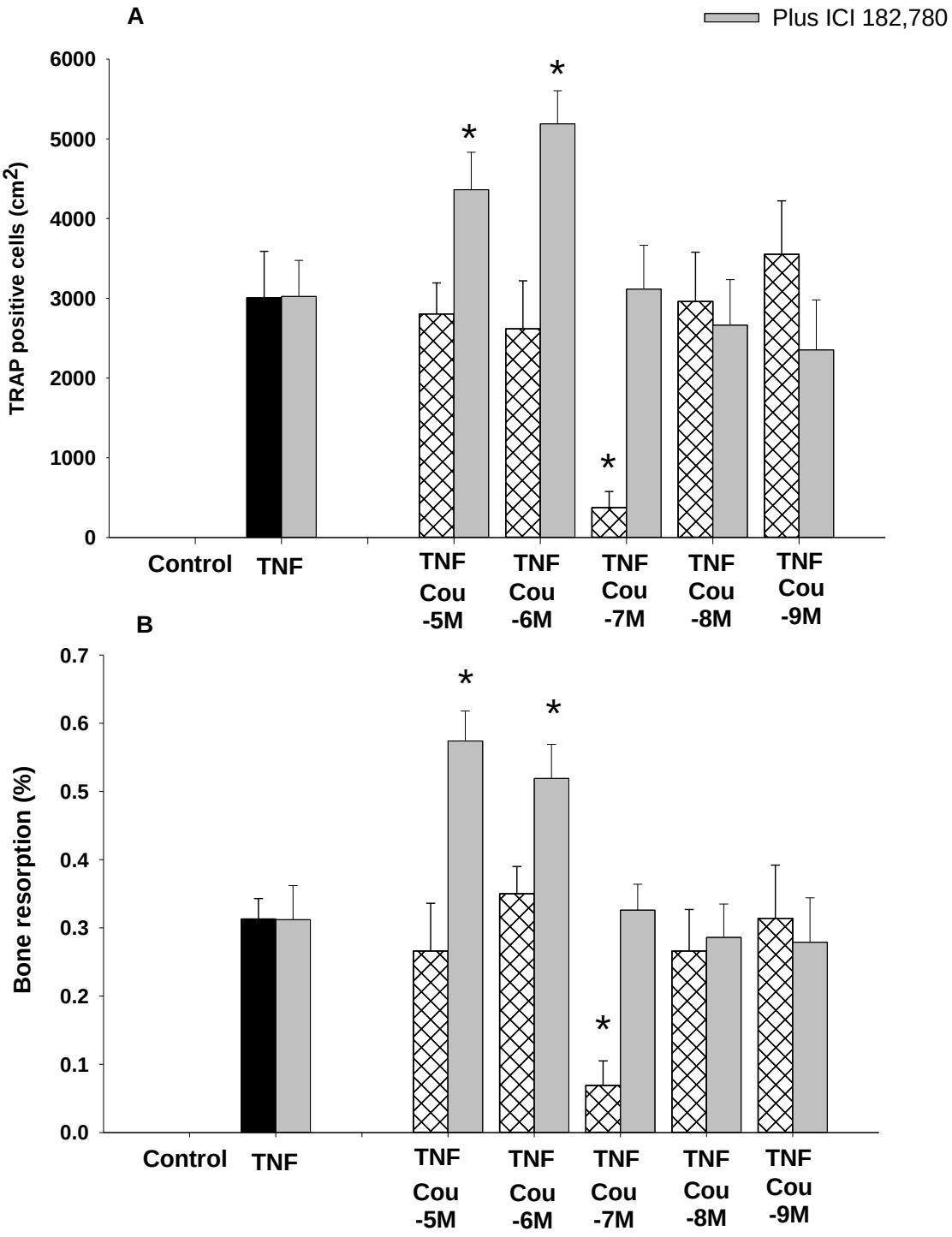

Figure 4.2. Coumestrol (10^{-7}M) significantly reduced TNF-α-induced TRAP positive osteoclast formation (A) and bone resorption (B), which was prevented by the ER antagonist ICI 182,780 (solid grey bars). In the presence of ICI 182,780 higher concentrations of genistein (10^{-5}, 10^{-6} M) increased osteoclast formation and bone resorption. RAW264.7 cells treated with TNF-α (50ng/ml) and phytoestrogens (10^{-5}-10^{-9} M), and after four days TRAP-positive cells was scored, while the percentage of bone surface displaying resorption pits was analysed after eight days. Values are expressed as the mean ± SEM of three separate experiments n=18, * P< 0.05 versus TNF-α treated group.

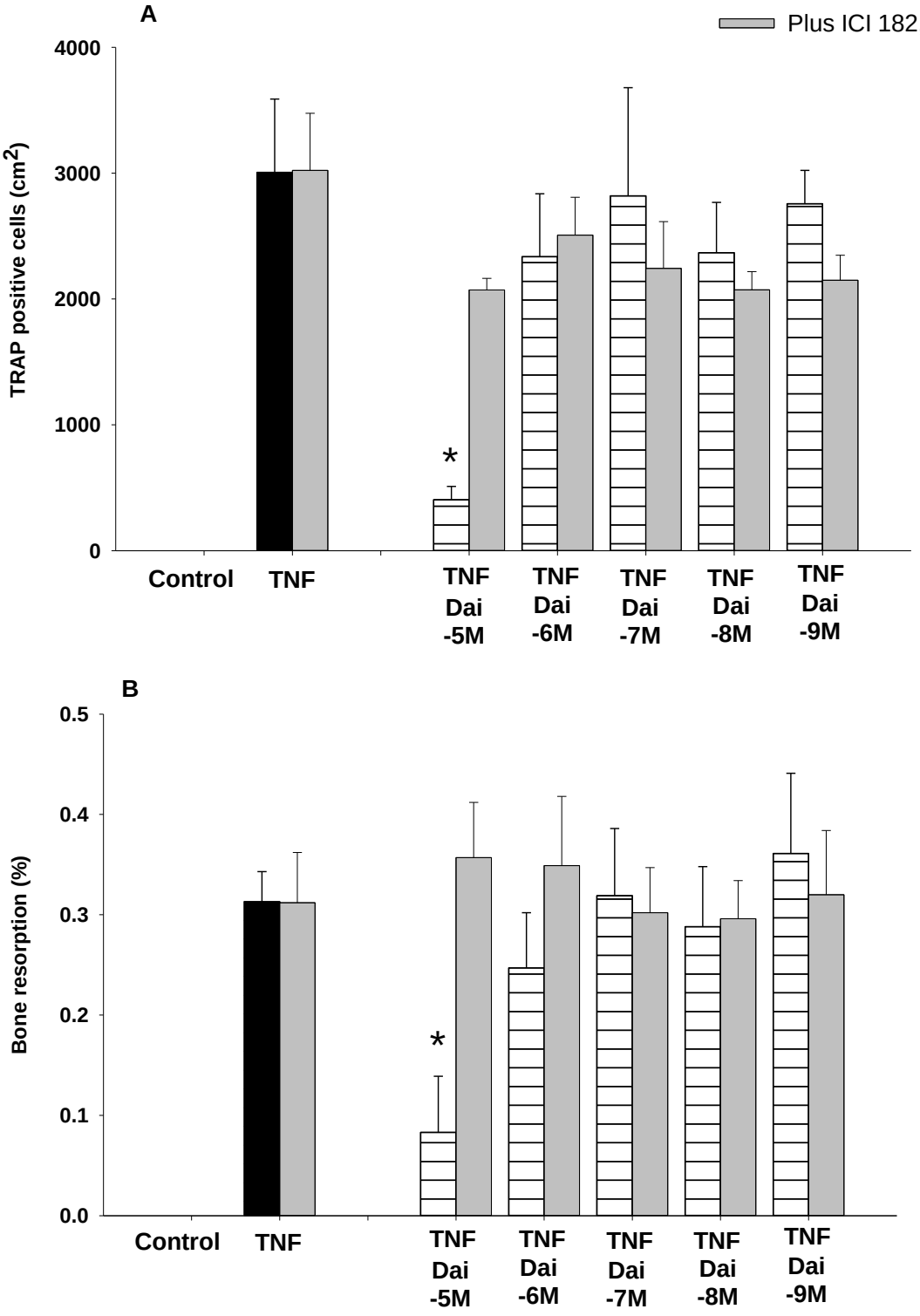

Figure 4.3. Daidzein (10^{-5} M) significantly reduced TNF-α-induced TRAP positive osteoclast formation (A) and bone resorption (B), which was prevented by the ER antagonist ICI 182,780 (solid grey bars). RAW264.7 cells treated with TNF-α (50ng/ml) and phytoestrogens (10^{-5}-10^{-9} M), and after four days TRAP-positive cells was scored, while the percentage of bone surface displaying resorption pits was analysed after eight days. Values are expressed as the mean ± SEM of three separate experiments n=18, * $P< 0.05$ versus TNF-α treated group.

Table 4.2. PEs significantly reduced the number of TRAP positive mature osteoclasts. TNF-α 50 ng/ml, genistein (10^{-7} M), coumestrol (10^{-7} M), daidzein (10^{-5} M) * P<0.05 versus TNF-α.

Groups	TRAP positive cells (cm2)		TRAP positive cells (cm2) + ICI 182,780	
	Mean	SEM	Mean	SEM
Control	0	0	0	0
TNF-α	317	55	296	42
Genistein and TNF-α	14*	8	238	34
Coumestrol and TNF-α	0*	0	250	63
Daidzein and TNF-α	160*	35	307	42

4.3.2 Anti-osteoclastic concentrations of PEs have no effect on cell viability or apoptosis

I then went on to determine the cellular and molecular mechanism through which genistein, coumestrol and daidzein suppressed TNF-α-induced osteoclast formation. As shown in (Figure 4.4) anti-osteoclastic PE concentrations (genistein 10^{-7} M, daidzein 10^{-5} M and coumestrol 10^{-7} M) had no detrimental effect on cell viability and an increase in cell number was noted with genistein (10^{-6} to 10^{-7} M), coumestrol (10^{-5} to 10^{-7}M) and daidzein (10^{-6} to 10^{-7} M). Furthermore, caspase 3/7 activity was also unaffected by these concentrations (Figure 4.5), suggesting that the reduction in osteoclast formation and activity noted was mediated via an action on osteoclast differentiation.

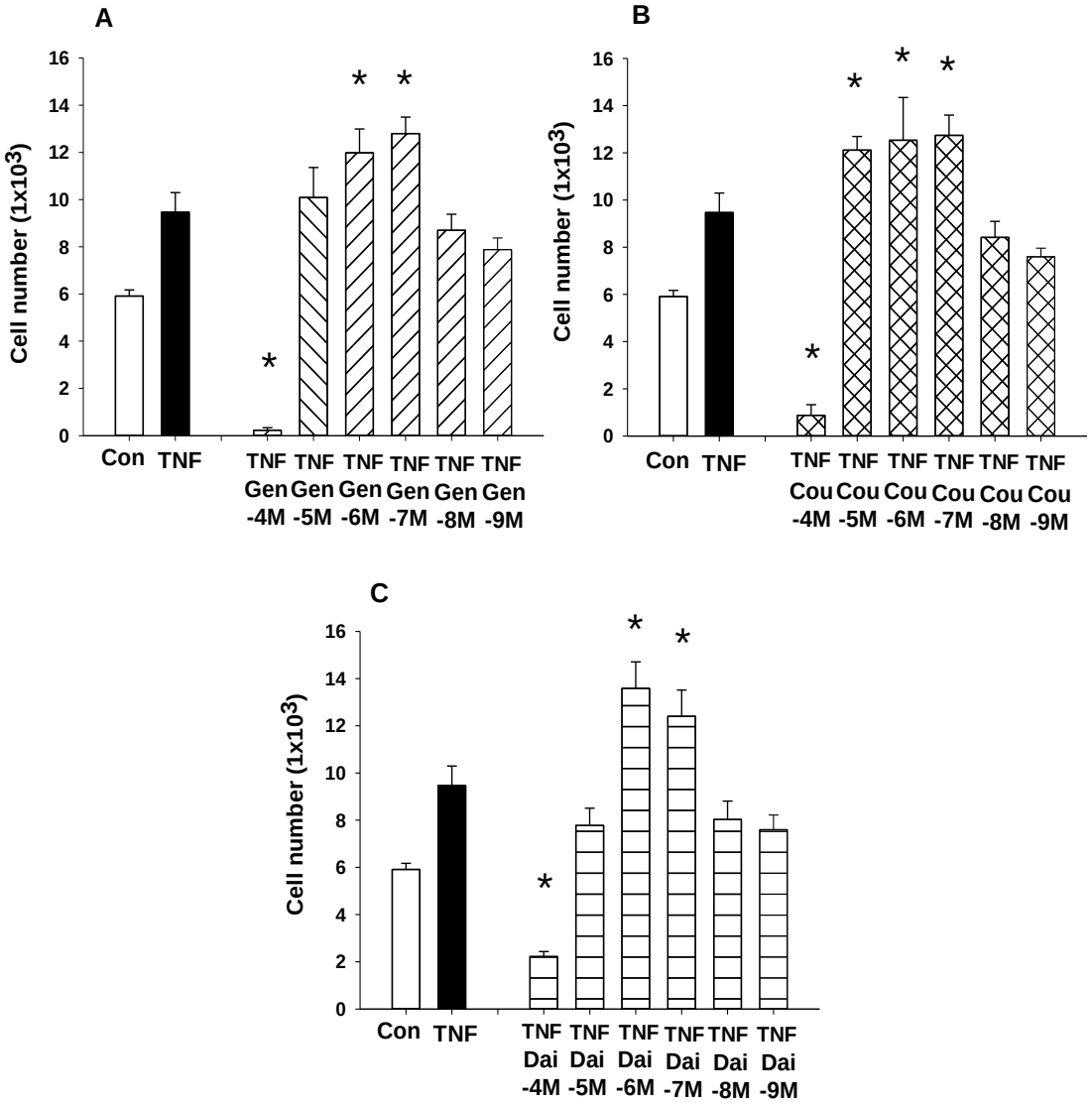

Figure 4.4. Genistein, coumestrol and daidzein (10^{-4}M) significantly decreased RAW264.7 cell viability after four days of incubation. All other concentrations had no detrimental effect on monocyte number. RAW264.7 cells treated with TNF-α (50ng/ml) and phytoestrogens (10^{-4}-10^{-9} M), and after four days, the number of viable cells was quantified using an MTS assay. Values are expressed as the mean ± SEM of 3 separate experiments n=18, * P< 0.05 versus TNF-α treated group.

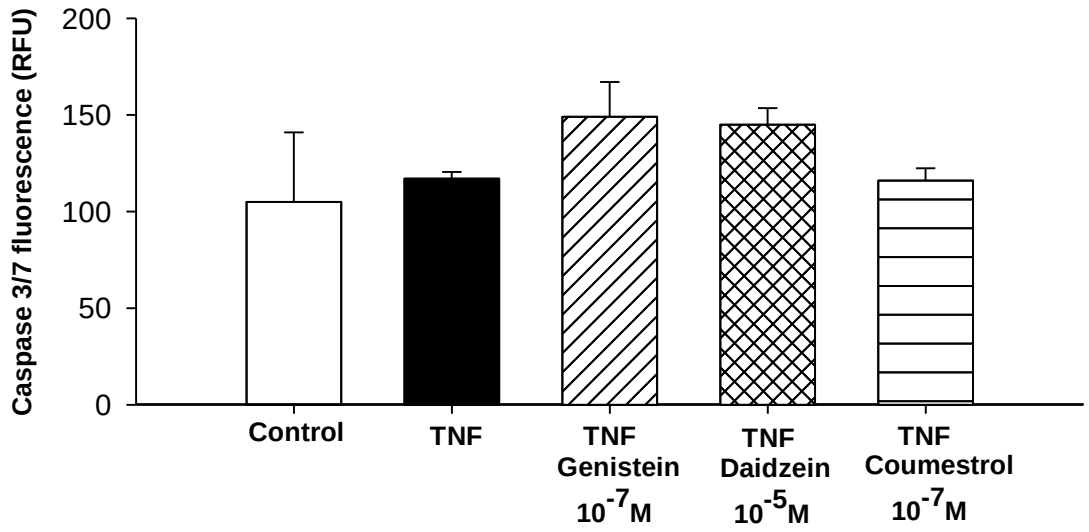

Figure 4.5. Anti-osteoclastic concentrations of PEs had no effect on monocyte caspase 3/7 activity. Values are expressed as the mean ± SEM mean from five replicate experiments.

4.3.3 Genistein, coumestrol and daidzein suppress the expression of regulators of osteoclast differentiation

Osteoclast differentiation is controlled by a network of signalling factors that regulate the expression of genes typical of osteoclasts such as *TRAP* and *DC-STAMP*. Central to this is the c-fos dependent induction of NFATc1 expression. Mice deficient in either NFATc1 or c-fos lack osteoclasts and are severely osteopetrotic as a consequence. Evidence also suggests that NFATc1 is sufficient stimulus on its own to promote osteoclast formation (Matsuo et al., 2004). Therefore to determine the effect of PEs on this master regulatory system I analysed changes in *c-fos* and *NFATc1* expression using real time quantitative PCR. TNF-α induced a significant 3.82-fold increase in *NFATc1* expression and a significant 2.82- fold increase in *c-fos* expression within 48 hours (Figure 4.6A & 4.7A). TNF-α-induced *NFATc1* and *cfos* expression was significantly suppressed in the presence of concentrations of genistein (10^{-7}M) (Figure 4.6 A & B), coumestrol (10^{-7} M) (Figure 4.7 A&B) or daidzein (10^{-5} M) (Figure 4.8 A & B) shown to reduce osteoclast formation and bone resorption in my earlier experiments. These values were not significantly different from non-treated control. No other concentrations had any significant inhibitory effect. The suppressive action of genistein, coumestrol and daidzein was prevented by ICI 182,780 suggesting that the reduction in mRNA expression was ER dependent. In keeping with the decrease in *NFATc1* mRNA expression genistein, coumestrol and daidzein also suppressed TNF-α-induced NFATc1 nuclear translocation as shown by a significant reduction in the number of nuclei displaying TNF-α-induced NFATc1 immunostaining (Figure 4.9).

Interestingly, in the presence of ICI 182,780 genistein and coumestrol (10^{-5} to 10^{-6} M) significantly augmented TNF-α-induced *NFATc1* expression again suggesting that high concentrations of these compounds have additional ER independent actions (Figure 4.6A, 4.7A). However in keeping with the changes in osteoclast differentiation and

bone resorption described earlier no augmentation of *NFATc1* expression was noted in the absence of ICI 182,780, suggesting that genistein's and comestrol's ER-mediated inhibitory action negates any stimulatory effect. Furthermore, no increase in *c-fos* expression was noted with any combination of PEs and E2 antagonist suggesting that the increase in *NFATc1* expression generated by high concentrations of genistein and coumestrol was not mediated via changes in *c-fos* transcription or turnover.

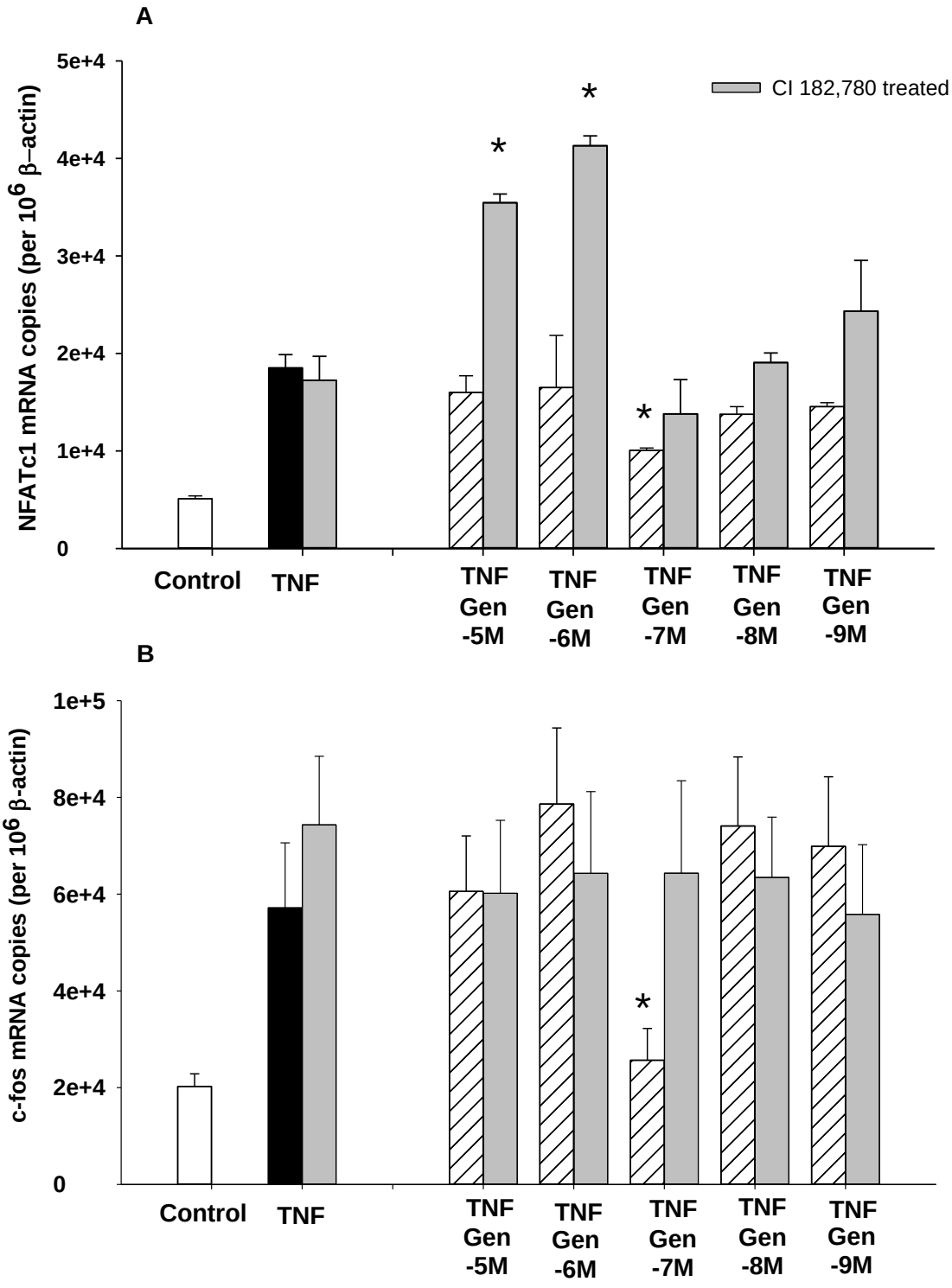

Figure 4.6. TNF-α-induced *NFATc1* (A) and *c-fos* (B) expression is suppressed by genistein (10^{-7} M) in an ER dependent manner. RAW264.7 cells were incubated in combinations of TNF-α (50ng/ml), genistein (10^{-5}-10^{-9} M) in the presence or absence of ICI 182,780 and after four days the expression of *NFATc1* and *c-fos* was then assessed by quantitative real time PCR. Data is expressed normalized to 10^6 copies of β-actin and represent the mean ± SEM from three separate experiments. * $P<0.05$ versus TNF-α treated group.

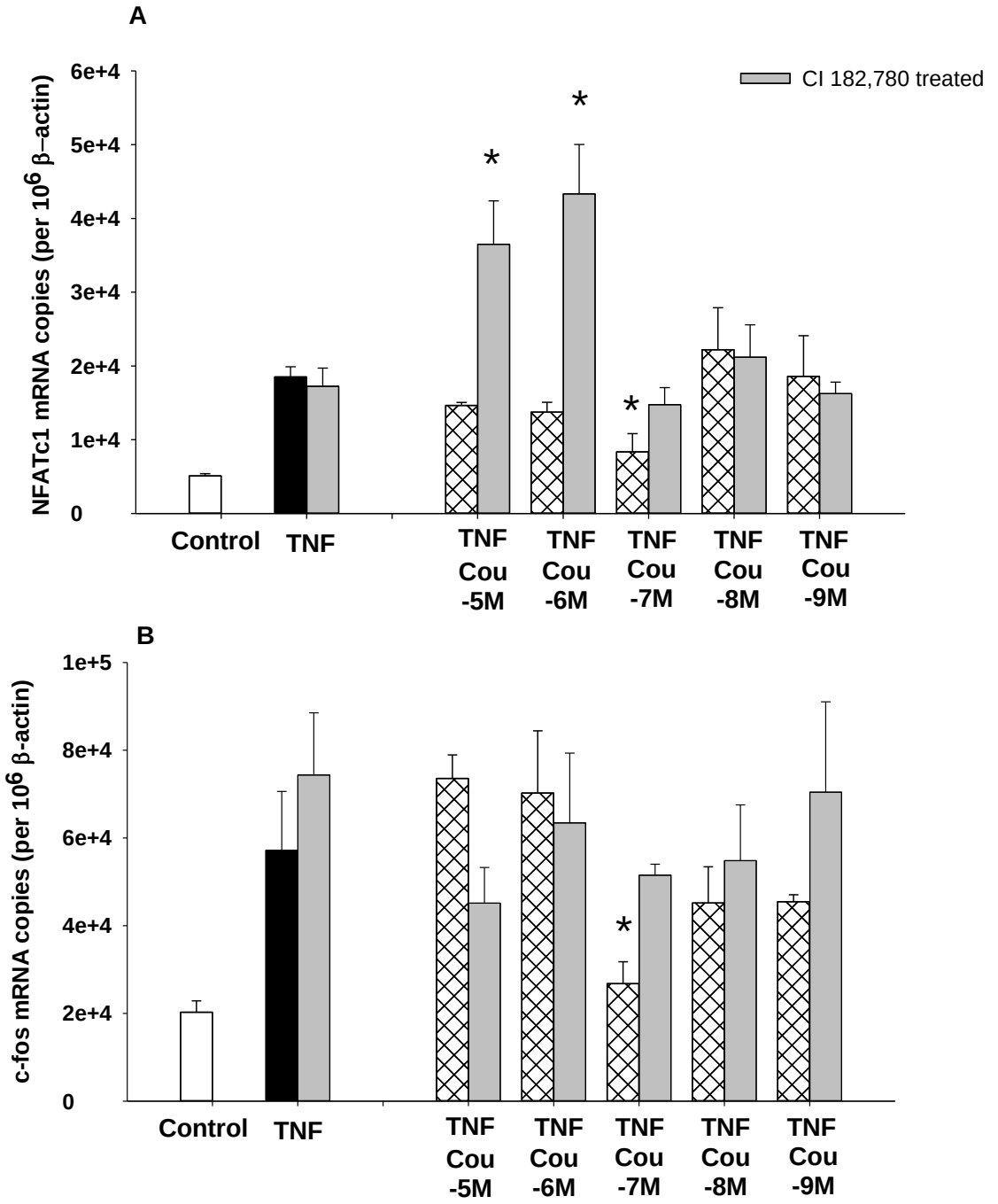

Figure 4.7. TNF-α-induced *NFATc1* (A) and *c-fos* (B) expression is suppressed by coumestrol (10^{-7} M) in an ER dependent manner. RAW264.7 cells were incubated in combinations of TNF-α (50ng/ml), coumestrol (10^{-5}-10^{-9} M) in the presence or absence of ICI 182,780 and after four days the expression of *NFATc1* and *c-fos* was then assessed by quantitative real time PCR. Data is expressed normalized to 10^6 copies of β-actin and represent the mean ± SEM from three separate experiments. * $P<0.05$ versus TNF-α treated group.

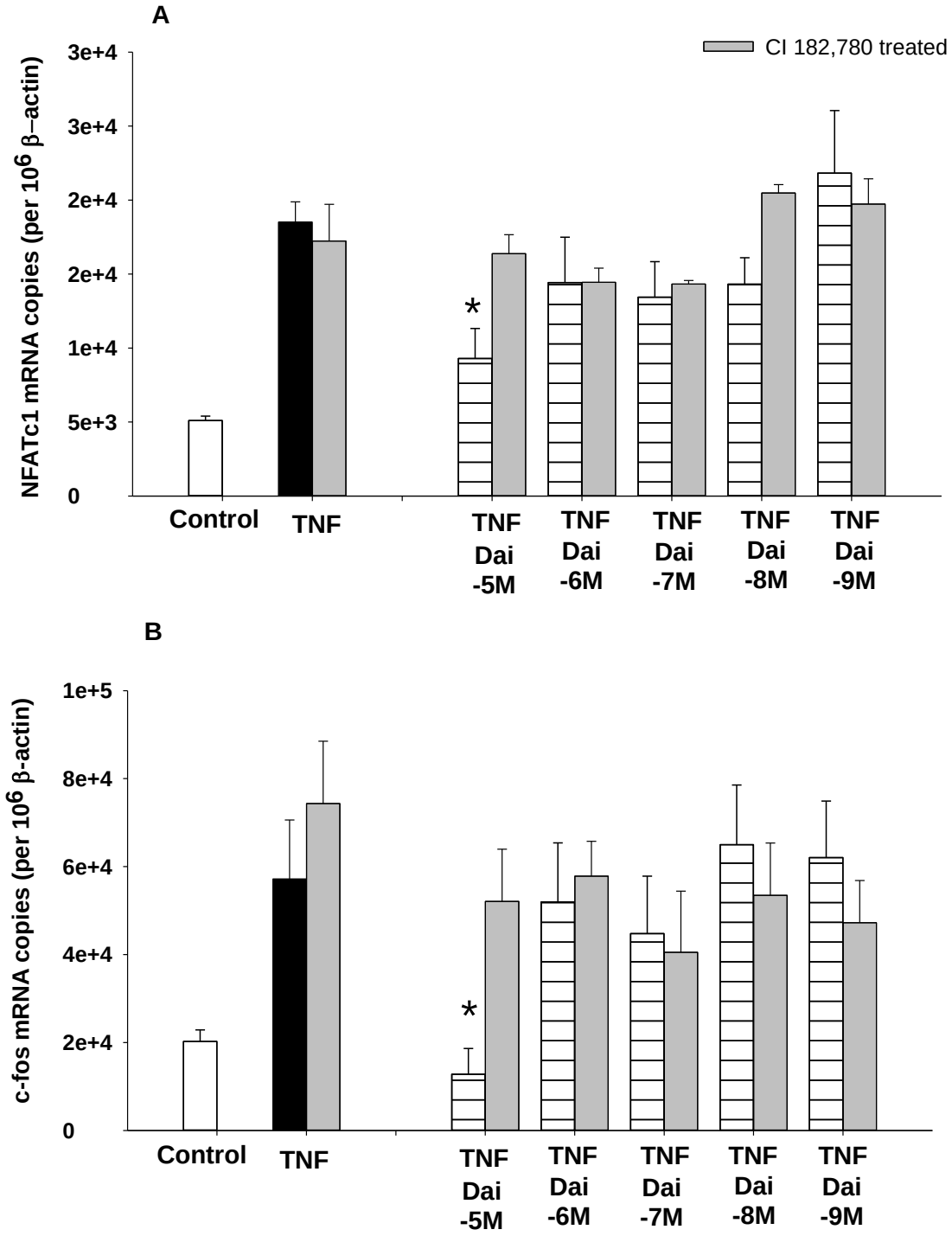

Figure 4.8. TNF-α-induced *NFATc1* (A) and *c-fos* (B) expression is suppressed by daidzein (10^{-5} M) in an ER dependent manner. RAW264.7 cells were incubated in combinations of TNF-α (50ng/ml), daidzein (10^{-5}-10^{-9} M) in the presence or absence of ICI 182,780 and after four days the expression of *NFATc1* and *c-fos* was then assessed by quantitative real time PCR. Data is expressed normalized to 10^6 copies of β-actin and represent the mean ± SEM from three separate experiments. * $P<0.05$ versus TNF-α treated group.

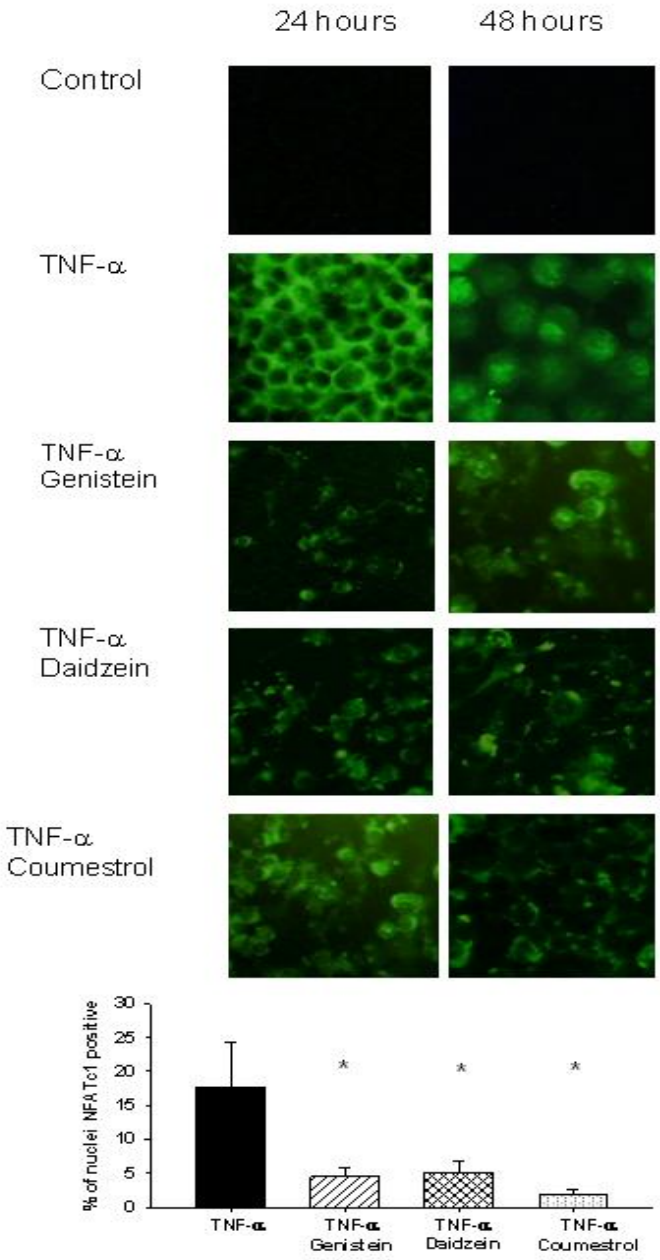

Figure 4.9. NFATc1 immunofluorescent staining in RAW264.7 cells cultured for 24 or 48 hours in the presence of combinations of TNF-α, genistein, coumestrol or daidzein. PEs reduced the intensity of TNF-α-induced NFATc1 cytoplasmic staining and significantly reduced the number of cells displaying NFATc1 nuclear localization. * P<0.05 versus TNF- α-treated group.

While c-fos and NFATc1 are master regulators of osteoclast differentiation, other signalling pathways have been shown to modify osteoclast differentiation. Therefore, to determine the potential role of these pathways, I examined the effect of anti-osteoclastic concentrations of genistein, coumestrol and daidzein on *p38* and *NFκB* expression. TNF-α-induced a significant 3.8 fold increase in *NF-κB* expression within 24 hours (Figure 4.10) which was prevented in the presence of genistein (10^{-7} M), coumestrol (10^{-7} M) or daidzein (10^{-5} M) in an ER dependent manner (Figure 4.10). In contrast anti-osteoclastic concentrations of PEs had no effect on TNF-α-induced *p38* expression (Table 4.3). In addition to changes in transcription factor levels I also noted a significant inhibitory action on the expression of the essential regulator of osteoclast aggregation and fusion *DC-STAMP*. TNF-α induced a significant 4.3 fold increase in *DC-STAMP* expression 48 hours after stimulation, which was prevented in the presence of genistein (10^{-7} M), coumestrol (10^{-7} M) or daidzein (10^{-5} M) (Table 4.3).

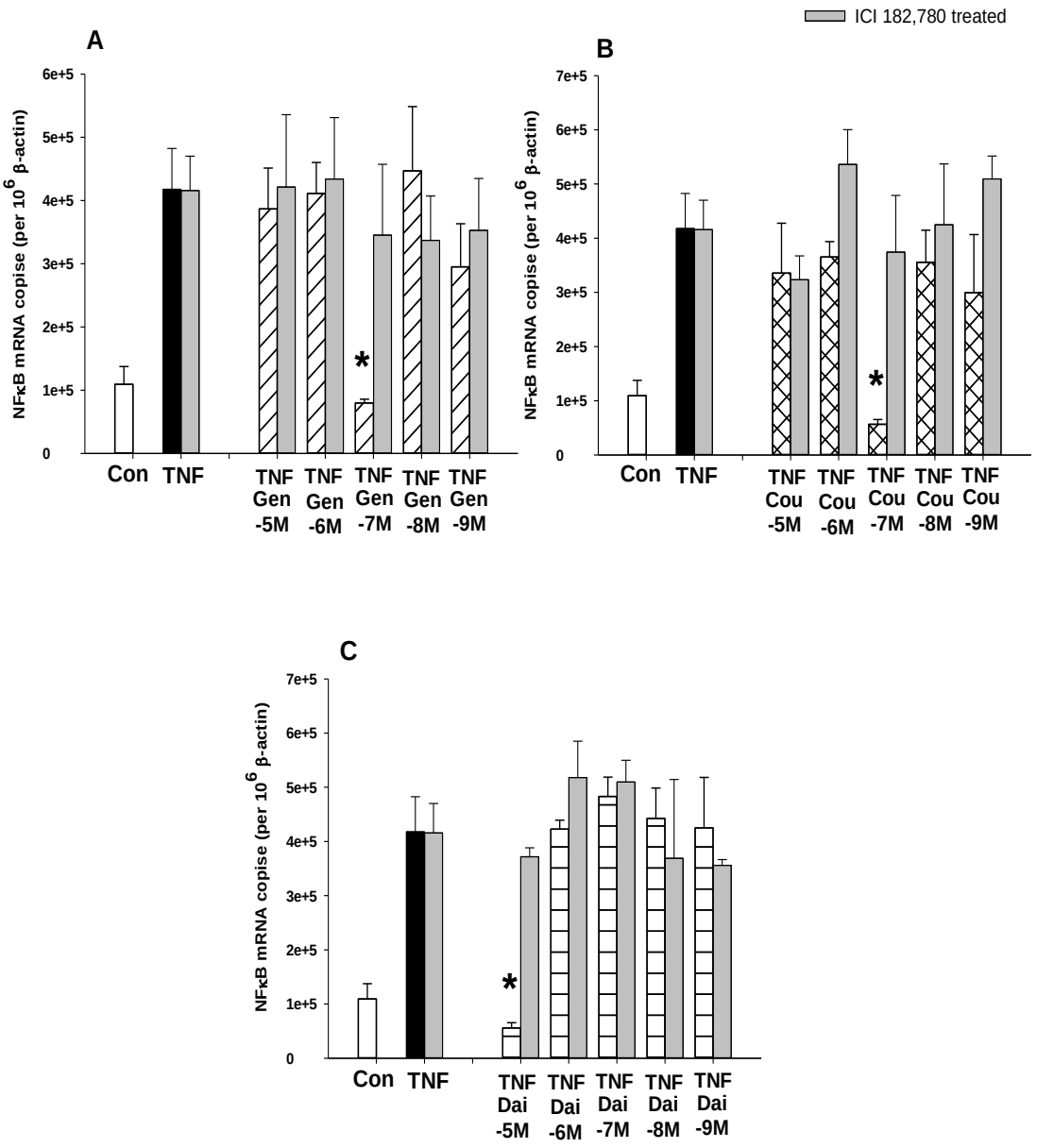

Figure 4.10. Genistein, coumestrol and daidzein inhibit *NFkB* expression. RAW264.7 cells were incubated in combinations of TNF-α (50ng/ml), genistein, coumestrol or daidzein (10^{-5}-10^{-9} M) and then mRNA expression was assessed after four days of treatment by quantitative real time PCR. Data expressed normalized to 10^6 copies of β-actin and represent the mean ± SEM from three separate experiments. * $P<0.05$ versus TNF-α treated group.

Table 4.3. Genistein, coumestrol and daidzein inhibit *DC-STAMP* but not *p38* expression. RAW264.7 cells were incubated in combinations of TNF-α (50ng/ml), genistein (10^{-5} M), coumestrol (10^{-7} M) or daidzein (10^{-5} M) and then mRNA expression was assessed by quantitative real time PCR. Data expressed normalized to 10^6 copies of β-actin and represent the mean ± SEM from three separate experiments. a $P<0.05$ versus TNF-α treated group.

	P38 mRNA copies per 10^6 β-actin copies		**DC-STAMP mRNA copies per 10^6 β-actin copies**	
	Mean	SEM	Mean	SEM
Control	10535	1239	3026	368
TNF-α	18577	2314	12994	3040
Genistein and TNF-α	18258	2629	7064 [a]	1696
Coumestrol and TNF-α	15775	1059	5658 [a]	355
Daidzein and TNF-α	19550	2254	9207 [a]	1642

4.3.4 Retroviral c-fos expression prevents the inhibitory effect of PEs on TNF-α-induced NFATc1 expression, osteoclast formation and resorption

The initial stimulus for the cytoplasmic accumulation of NFATc1 is provided by c-fos. NFATc1 is subsequently activated by Ca^{2+} calmodulin dependent signals leading to nuclear translocation and auto-amplification of its expression. Without these separate stimuli insufficient NFATc1 levels are generated to promote osteoclast differentiation. The ability of PEs to substantially reduce c-fos expression raises the possibility that this represents a key molecular mechanism mediating their action on osteoclast formation. To assess this I generated monocytic precursors (c-fos-pBabe) expressing constitutively active c-fos under the control of a retroviral expression vector and exposed them to concentrations of PEs shown to suppress osteoclast formation in my earlier experiments. This study found that retroviral driven c-fos expression increased basal *NFATc1* mRNA levels 3.25-fold and led to a significant 4-fold increase in TNF-α-induced NFATc1 expression (Figure 4.11).

Moreover, constitutive c-fos expression prevented the inhibitory effect of anti-osteoclastic concentrations of PEs on TNF-α-induced osteoclastogenesis and bone resorption (Figures 4.12), suggesting that PEs suppress TNF-α-induced osteoclast formation through a c-fos dependent action.

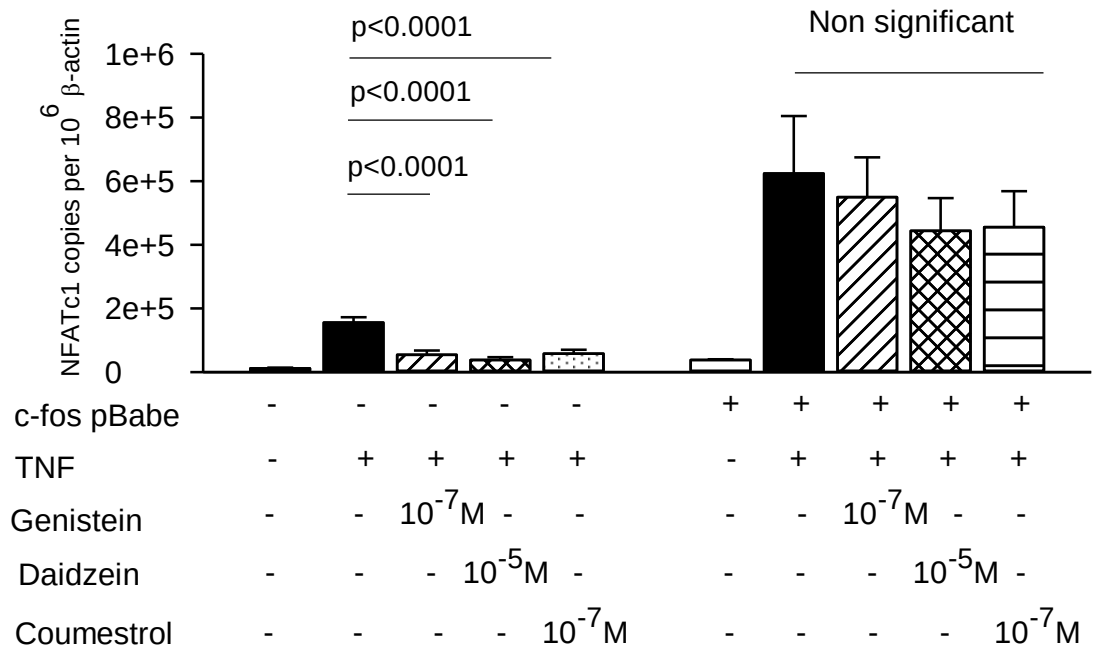

Figure 4.11. Constitutive c-fos expression prevents the anti-osteoclastic effects of PEs on TNF-α-induced NFATc1 expression. RAW 264.7 cells were infected with control or c-fos (c-fos pBabe) expressing retroviruses and the effect of TNF-α (50 ng/ml), genistein (10^{-7} M), coumestrol (10^{-7} M) and daidzein (10^{-5} M) on NFATc1 expression was examined. Experiments were performed in triplicate.

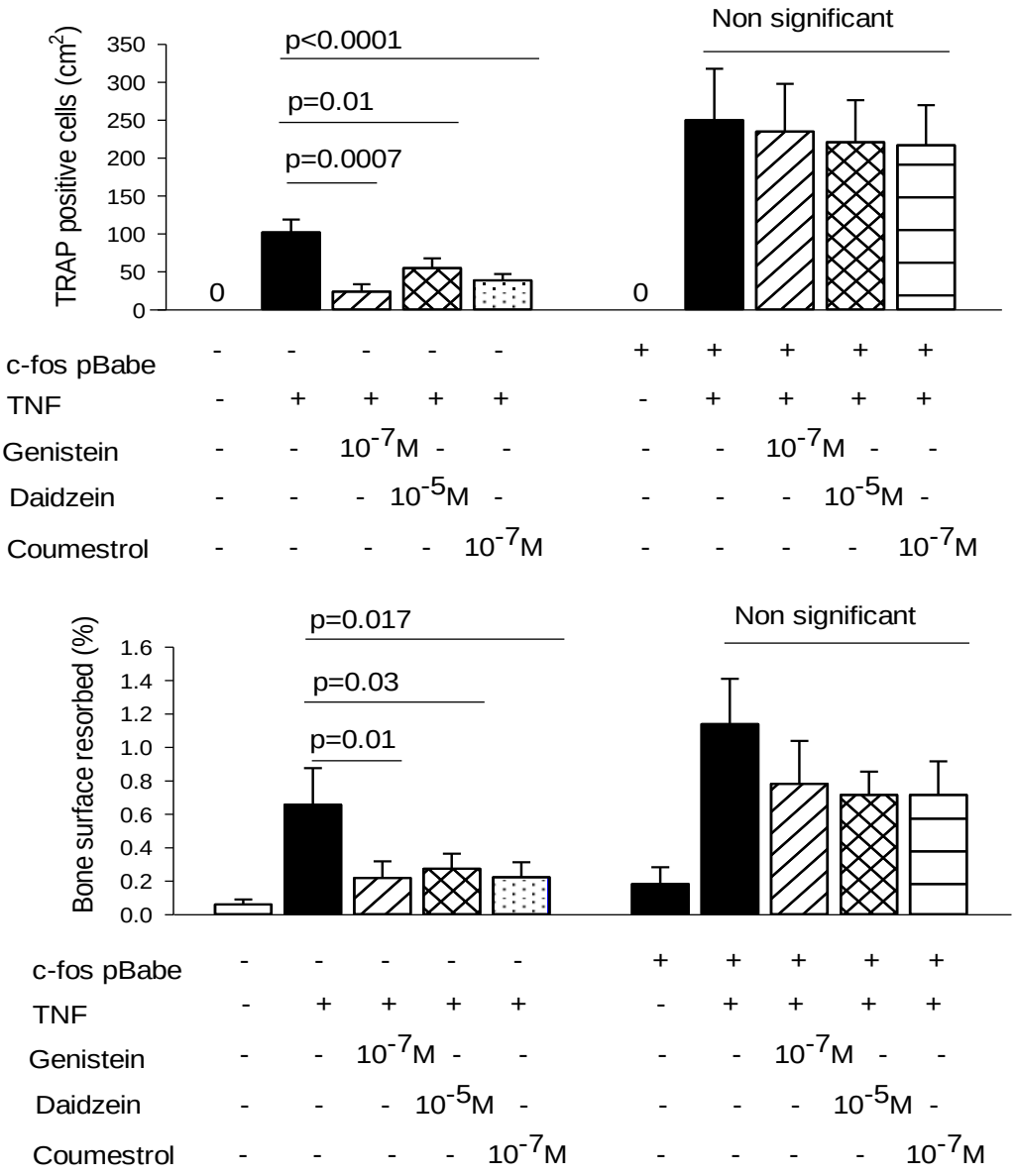

Figure 4.12. Constitutive c-fos expression prevents the anti-osteoclastic effects of PEs on TNF-α -induced osteoclast formation and bone resorption. RAW 264.7 cells were infected with control or c-fos (c-fos pBabe) expressing retroviruses and the effect of TNF-α (50 ng/ml), genistein (10^{-7} M), coumestrol (10^{-7} M) and daidzein (10^{-5} M) on TRAP positive osteoclast formation and bone resorption was examined. Experiments were performed in triplicate.

4.4 Discussion

An increasing body of evidence implicates TNF-α-induced osteoclast formation in post-menopausal and inflammatory bone loss. TNF-α directly stimulates the formation of resorptive osteoclast from human monocytes in the presence of OPG (Kudo et al., 2002), whereas blockade of TNF-α using a range of biological agents reduces osteoclast number and prevents bone destruction *in-vivo* (Barnabe and Hanley, 2009). Therefore, an understanding of how potential antiresorptive therapies such as PEs modify TNF-α-induced osteoclastogenesis is essential. Genistein, coumestrol and daidzein have previously been shown to suppress RANKL-induced osteoclast differentiation (Gao and Yamaguchi, 1999b, Garcia Palacios et al., 2005, Rassi et al., 2002). Comparable concentrations of these PEs also reduced TNF-α-induced osteoclast differentiation and bone resorption in my study, with genistein and coumestrol having a more potent effect than daidzein. This anti-osteoclastic action was mediated via a direct effect on monocyte differentiation and not via in-direct osteoblastsic actions as suggested in previous studies using mixed cell populations (Chen et al., 2003a). Genistein, coumestrol and daidzein also directly reduced TRAP expression in mature TNF-α-induced resorptive osteoclasts. These results strengthen the data for the use of PEs as agents in the treatment of post-menopausal bone loss and inflammatory osteolytic diseases where they may suppress TNF-α-induced osteoclast formation and resorptive activity.

Serum concentrations of PEs vary between populations with Asians having significantly higher levels than Westerners (Morton et al., 2002). This has been suggested to contribute to the lower incidence of osteoporotic fractures in Asian women. Interestingly, the antiresorptive concentration of genistein and coumestrol noted in my study is similar to levels measured in Asian populations but higher than those achieved by Western diets (Morton et al., 2002). In contrast, both Asian and Western diets are

unable to generate serum concentrations of daidzein similar to those shown to suppress TNF-α-induced osteoclastogenesis in my studies. However, these concentrations could be achieved with daidzein supplementation which generates tissue levels several orders of magnitude higher than dietary sources (Gardner et al., 2009). The antiresorptive effect of genistein and coumestrol was noted over a narrow concentration range with doses higher or lower than this having no effect on differentiation or resorption. This response likely reflects antagonistic interactions between genistein's and coumestrol's multiple biological actions. For instance in addition to its classical oestrogenic effect genistein also inhibits tyrosine kinase activity (Aggarwal and Shishodia, 2006) and alters redox states which are known to have a bimodal effect on osteoclast formation (Kim et al., 2006). Similarly, coumestrol activates MAPK activity which promotes osteoclast differentiation (Jeng et al., 2009). Furthermore, the ER-independent actions of genistein and coumestrol tend to have higher EC_{50}, thus it is conceivable that while concentrations of 10^{-7} M stimulate ER dependent anti-osteoclastic actions higher concentrations activate antagonistic non-oestrogenic effects that promote osteoclast formation and bone resorption. This assertion is strengthened by the increase in osteoclast formation and resorption noted when ER signalling was inhibited with ICI 182,780 at higher concentrations of coumestrol and genistein (10^{-5} to 10^{-6} M). These concentrations also promoted NFATc1 expression in the presence of E2 antagonist indicating that increased osteoclast number most likely resulted from enhanced levels of this key osteoclastic transcription factor. In contrast, daidzein which acts exclusively via ER did not augment osteoclast formation, bone resorption or NFATc1 expression in the presence of ICI 182,780 at any concentration examined. The precise nature of the ER independent action which augments NFATc1 transcription is unclear but would not appear to be mediated via an increase in *c-fos* or *NFκB* expression as neither genistein nor coumestrol increased levels of these transcription factors. Furthermore, while ICI

182,780 prevented the suppressive action of all PEs there is a possibility that other ER-independent actions may still have a role in the inhibitory effect.

Importantly, while coumestrol and genistein appear to possess pro and anti-resorptive actions my data suggests that the antiresorptive action predominates with no augmentative effect apparent when ER signalling is intact. Genistein and coumestrol could therefore be used at relevant doses to decrease resorption in post-menopausal osteoporosis or inflammatory osteolysis with little theoretical chance of them enhancing resorption if this dose was exceeded. However, it does indicate caution in the use of genistein and coumestrol as antiresorptive agents in women with ER-positive breast tumours that have preferentially metastasized to the skeleton. ER antagonists are often prescribed to these patients to reduce fracture risk and therefore in this setting high doses of genistein and coumestrol could potentially augment tumour associated osteolysis and increase the occurrence of skeletal related events.

Previous data suggest that PEs suppress RANKL-induced osteoclastogenesis by enhancing precursor apoptosis and disrupting intracellular signals regulating osteoclast differentiation (Gao and Yamaguchi, 1999b, Garcia Palacios et al., 2005, Rassi et al., 2002, Uchiyama and Yamaguchi, 2007). In contrast my data suggest that their suppressive action on TNF-α-induced osteoclast formation is predominantly mediated via an effect on differentiation with no impact on viability or apoptosis. This may relate to differences in the strength of survival signals stimulated by TNF-α and RANKL. Evidence suggests that TNF-α but not RANKL is able to induce levels of anti-apoptotic factors, such as Bcl-xL, sufficient to induce resistance to subsequent apoptotic stimuli (Zhang et al., 2005). In keeping with this antiresorptives such as alendronate and pamidronate are relatively ineffective at preventing focal inflammatory bone loss in rheumatoid arthritis where high TNF-α levels are the predominate resorptive driver (Lodder et al., 2003). Alternatively, the PEs examined in my studies may induce

apoptosis via a TRAF6 mediated action, which would be expected to modify RANKL but not TNF-α-induced survival signals. Whatever the answer it is clear that unlike their dual suppressive action on RANKL-induced osteoclastogenesis the predominant effect of PEs on TNF-α-induced resorption is mediated via suppression of osteoclast differentiation.

TNF-α-induced osteoclastogenesis is dependent on the coordinated expression of transcription factors that drive differentiation along the osteoclast lineage. Pivotal to this is NFATc1 which has been suggested to be sufficient for osteoclast formation due to its ability to promote expression of osteoclastic genes such as *TRAP* and *DC-STAMP* (Matsuo et al., 2004). NFATc1 displays a biphasic expression pattern during osteoclastogenesis; initially there is a small cytoplasmic increase followed by a larger increase in expression after nuclear translocation and autoregulation of its own gene. These processes are controlled by separate signals, cytoplasmic expression is c-fos dependent whereas nuclear accumulation is Ca^{2+} calmodulin dependent (Grigoriadis et al., 1994). In addition auto-amplification of NFATc1 expression is dependent on the presence of c-fos to stabilize NFATc1's interaction with its own promoter (Asagiri et al., 2005). This places c-fos centrally in the regulation of NFATc1 and osteoclast differentiation. My studies suggest that genistein, coumestrol and daidzein may directly inhibit osteoclast differentiation by suppressing *c-fos* levels. The mechanism leading to a reduction in *c-fos* transcription is uncertain, but this could arise as a consequence of a direct ER mediated effect on the *c-fos* gene or indirectly via modification of an upstream regulator. The PEs-induced reduction in *c-fos* would in turn be expected to prevent NFATc1 reaching levels sufficient to enable osteoclast differentiation, maintaining precursors in a non-committed state or allowing them to differentiate towards alternative macrophage lineages. This conclusion is strengthened by the inability of all PEs to inhibit osteoclast formation and bone resorption in cells

constitutively expressing high levels of c-fos. However, while it is clear that c-fos suppression is associated with the inhibitory action of PEs the levels generated by retroviral constructs are likely to be higher than those induced by TNF-α and may not therefore represent a physiological response.

While previous studies noted similar actions of genistein on NFATc1 expression during RANKL-induced osteoclast formation (Uchiyama and Yamaguchi, 2007); this to my knowledge is the first report of an inhibitory effect of coumestrol, genistein or daidzein on monocytic *c-fos* expression. This is also the first report of an action of these compounds on *DC-STAMP*. DC-STAMP has a key role in later stages of osteoclast differentiation, its expression is elevated on mononuclear osteoclasts and it is enables their subsequent aggregation and fusion to form large multinuclear osteoclast. Mice lacking DC-STAMP develop a mild osteopetrosis and have no multinuclear osteoclasts (Kukita et al., 2004, Miyamoto, 2006). The PE-induced reduction in *DC-STAMP* most likely occurs as a secondary consequence of decreased *NFAT* and *c-fos* expression which would be expected to lower *DC-STAMP* promoter activity (Yagi et al., 2007).

In conclusion, genistein, coumestrol and daidzein directly suppressed TNF-α induced osteoclast formation and bone resorption. The concentration of genistein and coumestrol required to suppress resorption are achievable by diets containing high soy content, whereas to achieve effective daidzein levels would require dietary supplementation. The anti-osteoclastic action is at least in part mediated by suppression of *c-fos* expression in osteoclast precursors which would prevent nuclear accumulation of NFATc1 a key regulator of osteoclast formation. These results provide further evidence that PEs are potential therapeutic candidates in the prevention of bone loss associated with aberrant TNF-α levels in post-menopausal osteoporosis and inflammatory osteolysis.

Chapter Five: Effect of Phytoestrogens on Osteoblast Differentiation and Bone Matrix Formation

Chapter 5

5.1 Introduction

The skeleton constantly remodels in response to changes in mechanical load, serum calcium and micro-damage (Martin and Seeman, 2008, Henriksen et al., 2009). 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. Osteoblasts and osteoclasts activity is tightly regulated such that during each remodelling cycle osteoblasts formation is temporally coupled to resorption ensuring there is little net bone loss. However, this balance is disrupted in many skeletal disorders such as post-menopausal osteoporosis and osteomyelitis (Manolagas et al., 2002, Nair et al., 1996). In post-menopausal women the reduction in circulating E2 increases bone turnover and skews remodelling in favour of osteoclastic resorption (Manolagas et al., 2002). The resulting bone loss increases fracture risk at elements with a high trabecular content such as the femoral neck and distal radius and ulna.

HRT has been shown to prevent the increase in osteoclast formation and thereby reduce fracture risk (Writing Group for the Women's Health Initiative Investigators, 2002). HRT also has an anabolic action increasing bone formation and volume in rats and humans (Khastgir et al., 2003, Chow et al., 1992). This contrasts with other antiresorptive drugs, such as bisphosphonates, which typically only suppress osteoclast activity. However, the widespread use of HRT has been re-assessed in light of large scale clinical trials that showed a substantial increase in the risk of breast cancer and coronary heart disease in older women prescribed combination HRT (Writing Group for the Women's Health Initiative Investigators, 2002). Therefore, several alternative compounds with oestrogenic actions have been examined for their antiresorptive and anabolic potential, these include PEs. Some epidemiological studies suggest that diets with high PE content such as soy rich diets may generate a more robust skeleton. A

positive association between soy consumption and BMD has been noted in Asians (Mei et al., 2001, Horiuchi et al., 2000, Kim et al., 2002a) and supplements have also been shown to have beneficial effects on BMD (Alekel et al., 2000, Morabito et al., 2002, Atkinson et al., 2004, Wu et al., 2006). Genistein has also been shown to increase the expression and activity of the osteoblastsic marker ALP, alter the OPG and RANKL expression by osteoblasts (Sugimoto and Yamaguchi, 2000a). Moreover, genistein (10^{-6}-10^{-7} M) increased osteoblast formation in preosteoblastsic KS483 cells via an ER-dependent mechanism (Dang *et al.*, 2002). However, not all studies note a positive effect at all skeletal sites and efficacy varies depending on the PEs and dose studied (Ricci et al., 2010)

The protective effect of PEs is thought to occur through a combination of osteoclast and osteoblast mediated actions. Several studies note decreases in resorption markers following PEs supplementation (Mei et al., 2001, Weaver et al., 2009) and *in-vitro* studies show a direct suppressive effect of PEs on cytokine-induced osteoclast differentiation (Karieb and Fox, 2011, Gao and Yamaguchi, 1999b). In addition to suppressing resorption, PEs have also been shown to increase bone formation markers such as serum ALP and osteocalcin levels in post-menopausal women (Morabito et al., 2002, Roudsari et al., 2005). Genistein has also been shown to increase mineral apposition and bone formation rates in ovariectomised rats (Dai et al., 2008) and PEs stimulate osteoblast differentiation and mineralisation *in-vitro* (Kanno et al., 2004, Yadav et al., 2011, Wu et al., 2009).

In addition, other nutritional factors have been shown to influence remodelling activity. Zn^{2+} promotes osteoblasts activity *in-vitro* (Kwun et al., 2010). Zn^{2+} deficiency is associated with osteopenia in men (Hyun et al., 2004) and Zn^{2+} supplements prevent exercise-induced falls in long bone mass in rats (Seco et al., 1998). Osteoclast activity is

also decreased by Zn^{2+} (Yamaguchi and Uchiyama, 2004, Uchiyama and Yamaguchi, 2007). The ability of dietary factors to not only prevent further bone resorption but 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 *osx* mRNA expression which regulates the formation of mature osteoblasts has not been investigated. Therefore, this study examined the effect of genistein, coumestrol and daidzein in the presence of Zn^{2+} on osteoblast and osteoclast differentiation, function and bone matrix formation *in-vitro*.

5.2 Material and methods

5.2.1 Cell culture

SaoS-2 human osteoblast like cells were obtained from ECACC, Porton Down, UK (ECACC cat. num. 89050205) and cultured in phenol red free RPMI1640 supplemented with 10% charcoal stripped foetal calf serum (Autogen Bioclear, UK), 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin (all from Sigma, UK). RAW264.7 monocytes (ATCC, UK, cat. num. TIB-71) were incubated in phenol red free Dulbecco's minimum essential medium supplemented with 10% charcoal stripped foetal calf serum (Autogen Bioclear, U.K.), 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin (all from Sigma, UK). All incubations were performed at 37^{o}C in 5% CO_2, and cultures fed every 2–3 days by replacing half of the culture volume with fresh medium. Zinc sulfate heptahydrate (zinc) was obtained from Sigma (Poole, Dorset, UK). The non-selective E2 antagonist ICI 182,780 was obtained from Tocris Biosciences (Bristol, UK). Recombinant murine TNF-α was purchased from Insight Biotechnology (Wembley, UK). All other reagents and kits were obtained from Sigma (Poole, Dorset, UK) unless stated.

5.2.2 Measurement of mineralisation and alkaline phosphatase (ALP) activity

Preliminary studies established that concentrations of zinc sulphate less than 10^{-5} M showed no interaction with PEs. All subsequent studies therefore used zinc at a concentration of 10^{-5} M. The effect of coumestrol, daidzein and genistein (10^{-5} to 10^{-9} M) in the presence or absence of zinc sulphate (10^{-5} M) on ALP activity was assessed as described in chapter three (3.8). SaoS-2 cells were incubated in 96 well plates with relevant treatments in the presence of β-glycerophosphate (β-GP) (10 mM) and L-ascorbic acid (L-AA) (50 mg/l) for four days. ALP activity was measured by staining cultures with *p*-nitrophenyl phosphate (1 mg/ml) and the absorbance was measured at

405 nm and results were then normalised to total cell number and expressed as the amount of ALP required to liberate 1 mmol of *p*-nitrophenol / min / 10^4 cells.

Mineralisation was assessed using a modification of Hale's methodology (Hale et al., 2000) by measuring calcein incorporation as described in chapter three (3.8). Briefly, cells were treated with β-GP (10 mM) and L-AA (50 mg/l) in the presence of genistein, daidzein or coumestrol (10^{-5}-10^{-9} M) with or without zinc sulphate (10^{-5}M). After 18 days of incubation the monolayer was incubated in culture medium containing 1 µg/ml calcein for four hours at 37ºC, and fluorescence measured by a cytofluor II fluorescence multiwell plate reader (Perseptive Biosystem, USA) at 485 nm excitation and 530 nm emission.

5.2.3 Cell Proliferation

SaoS-2 cells were cultured in 96 well plates at a density of $1x10^4$ 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 zinc sulphate (10^{-5} M) for 4 days. Proliferation was then assessed using a commercial AQueous one solution cell proliferation assay (Promega, UK) according to manufacturer's instructions described in chapter three (3.6).

5.2.4 Real time quantitative PCR analysis

SaoS-2 cells (5 x 10^5 per well) were incubated in 6 well plates for 24, 48 or 96 hours with coumestrol (10^{-7} M), genistein (10^{-7}M) or daidzein (10^{-5}M) with or without (10^{-5} M) of zinc sulphate. Total RNA was extracted from these cultures as described in chapter three (3.8.1) and reversed transcribed with M-MLV reverse transcriptase using random nonamer primers. Real time-PCR was performed on a StepOne PCR system (Applied Biosytems, UK) using the DNA-binding dye SYBR green. Primers used for each human transcription factor are shown in (Table 5.1). The generation of standard curves was

described in chapter three (3.8.2) using the corresponding cDNA and cloned into pGEM-T Easy (Promega, UK).

Table 5.1. Primer sequence of osteoblastsic transcriptional factors.

Genes	5´-3´ Forward primer	3´-5´ Reverse primer
β-Actin	GCGCGGCTACAGCTTCACCA	TGGCCGTCAGGCAGCTCGTA
Runx2	AGACCCCAGGCAGGCACAGT	GCGCCTAGGCACATCGGTGA
Osterix	GCACCCTGGAGGCAACTGGC	GAGCTGGGTAGGGGGCTGGA
Type I collagen	CCTGGCAGCCCTGGTCCTGA	CTTGCCGGGCTCTCCAGCAG
Osteocalcin	CCCAGCGGTGCAGAGTCCAG	CCTCCCTCCTGGGCTCCAGG
RANKL	ACAGGCCTTTCAAGGAGCT GTGC	ACCAGATGGGATGTCGGTGGC
OPG	AATCGCACCCACAACCGCGT	AGCAGGAGACCAAAGACACTGC A

5.2.5 Osteoclast differentiation and bone resorption assays

To examine the direct effect of zinc on the anti-osteoclastic action of PEs, RAW264.7 cells were transferred to 96-well plates at a density of $1x10^4$ cells per well. The effect of Zn^{2+} on the anti-resorptive action of PEs was assessed by seeding 10^4 RAW264.7 cells onto $20mm^2$ slices of devitalised bovine bone in 96 well plates. Cells and bone slices were then incubated with combinations of TNF-α (50ng/ml), genistein (10^{-5} - 10^{-9}M), daidzein (10^{-5} - 10^{-9} M) or coumestrol (10^{-5} -10^{-9} M) with or without zinc sulphate (10^{-5} M) for four days for assessment of TRAP-positive osteoclast formation or eight days for bone resorption. Cells were stained for TRAP positive cells using naphthol AS-BI phosphate as a substrate as mentioned in chapter three (3.4). Bone resorption area was assessed in bone slices after staining with the protocol described in chapter three (3.5) and the percentage of bone resorbed was quantified by reflected light microscopy. All experiments were performed in triplicate.

5.2.6 Statistical analysis

Differences between groups were assessed using Fisher's one way analysis of variance (Statview; Abacus concepts, USA) or Student's t-test as appropriate. A difference of $P<0.05$ was considered statistically significant.

5.3 Results

5.3.1 Zinc has no effect on the direct anti-osteoclastic action of phytoestrogens

Excessive resorption is central to bone loss in several skeletal disorders including post-menopausal osteoporosis. As has been shown previously, PEs possess antiresorptive actions, directly suppressing cytokine-induced osteoclast differentiation and bone resorption (Karieb and Fox, 2011). However, the effect of combinations of PEs and Zn^{2+} on this direct anti-osteoclastic action has not been examined. Thus to establish if the direct action of PEs is modified in the presence of other nutritional factors, RAW264.7 monocytes were cultured with genistein, coumestrol or daidzein and a concentration of Zn^{2+} shown in preliminary studies to modify the effect of PEs. As shown in the previous chapter, coumestrol, daidzein and genistein all significantly suppressed TRAP positive osteoclast formation and bone resorption. The dose response for the PEs 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^{2+} alone. Furthermore, the anti-osteoclastic action of all PEs was not affected by the addition of Zn^{2+} (Figures 5.1, 5.2, 5.3).

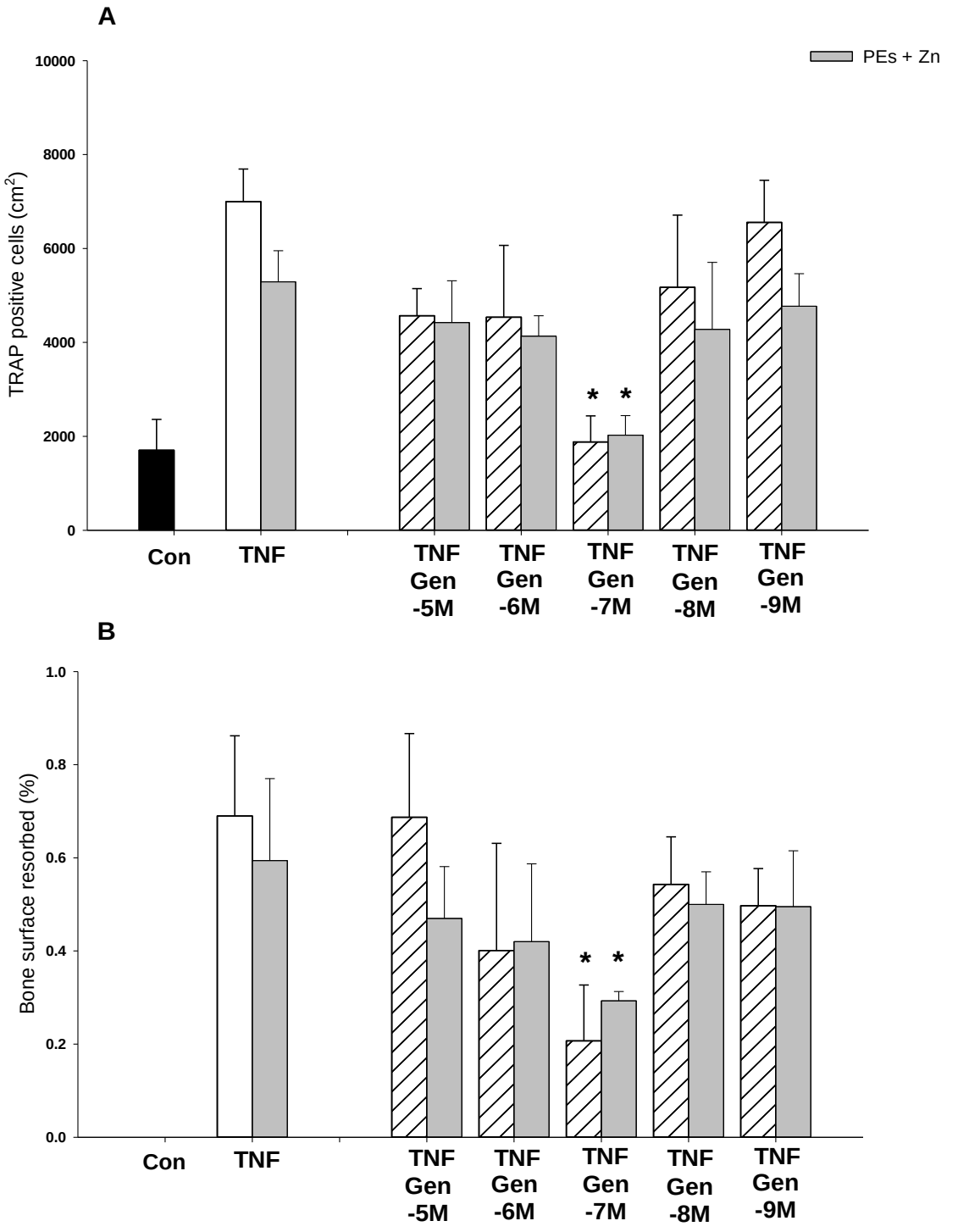

Figure 5.1. Zinc has no effect on the direct anti-osteoclastic action of genistein on TRAP cell formation (A) and bone resorption (B). RAW264.7 cells were incubated in the presence of TNF-α (50 ng/mL) for 4 days plus genistein (10^{-5}-10^{-9} M) in the presence or absence of zinc (10^{-5} M). Osteoclast formation was assessed by TRAP staining, while bone resorption was determined by the percentage of bone surface displaying resorption pits analysed by reflected light microscopy. Values are expressed as mean (± SEM) of three separate experiments. Differences between groups were assessed by one-way analysis of variance. * Values were significantly different versus TNF-α treated group $P < 0.05$.

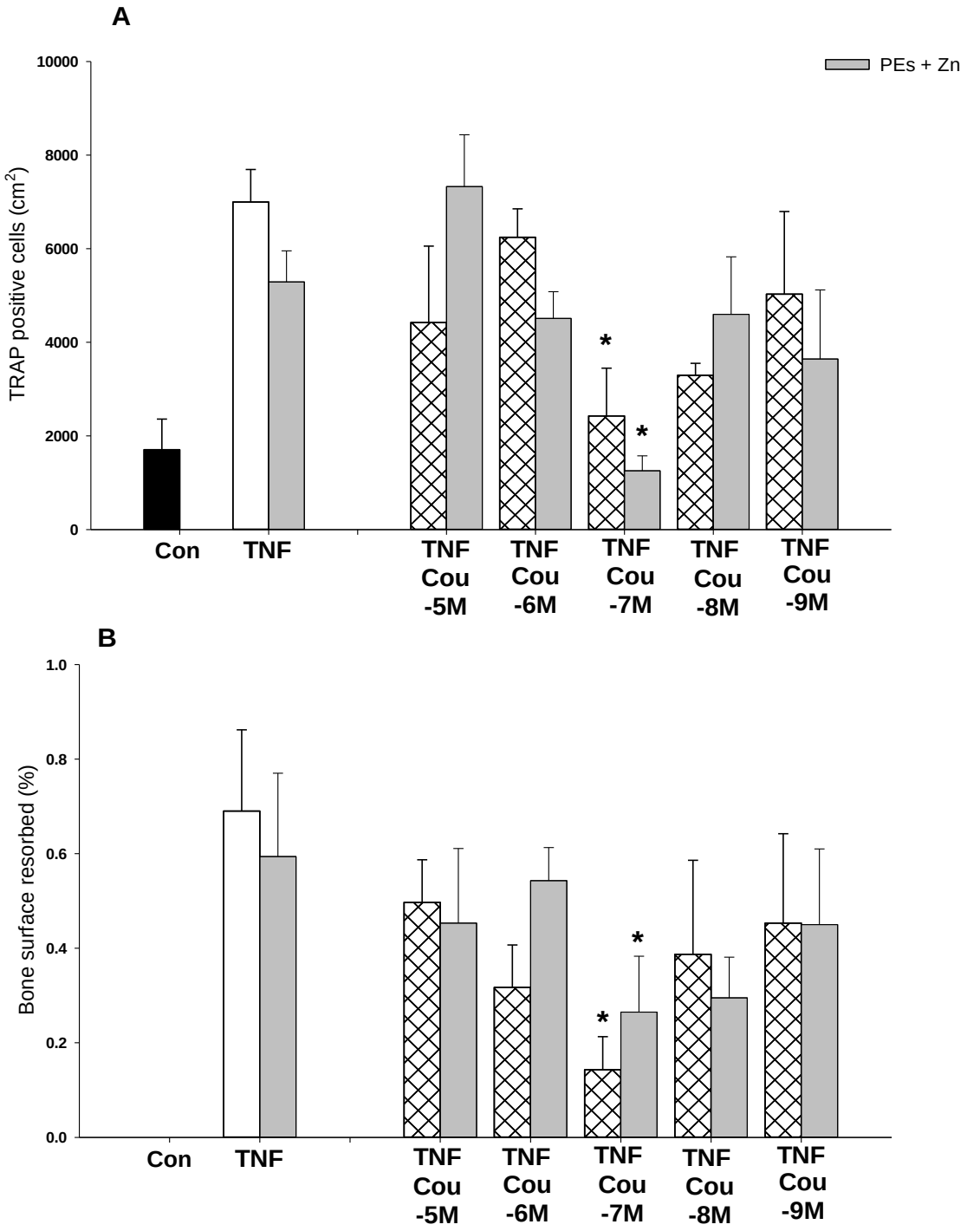

Figure 5.2. Zinc has no effect on the direct anti-osteoclastic action of coumestrol on TRAP cell formation (A) and bone resorption (B). RAW264.7 cells were incubated in the presence of TNF-α (50 ng/mL) for four days plus coumestrol (10^{-5}-10^{-9} M) in the presence or absence of zinc (10^{-5} M). Osteoclast formation was assessed by TRAP staining, while bone resorption was determined by the percentage of bone surface displaying resorption pits analysed by reflected light microscopy. Values are expressed as mean (± SEM) of three separate experiments. Differences between groups were assessed by one-way analysis of variance. * Values were significantly different versus TNF-α treated group $P < 0.05$.

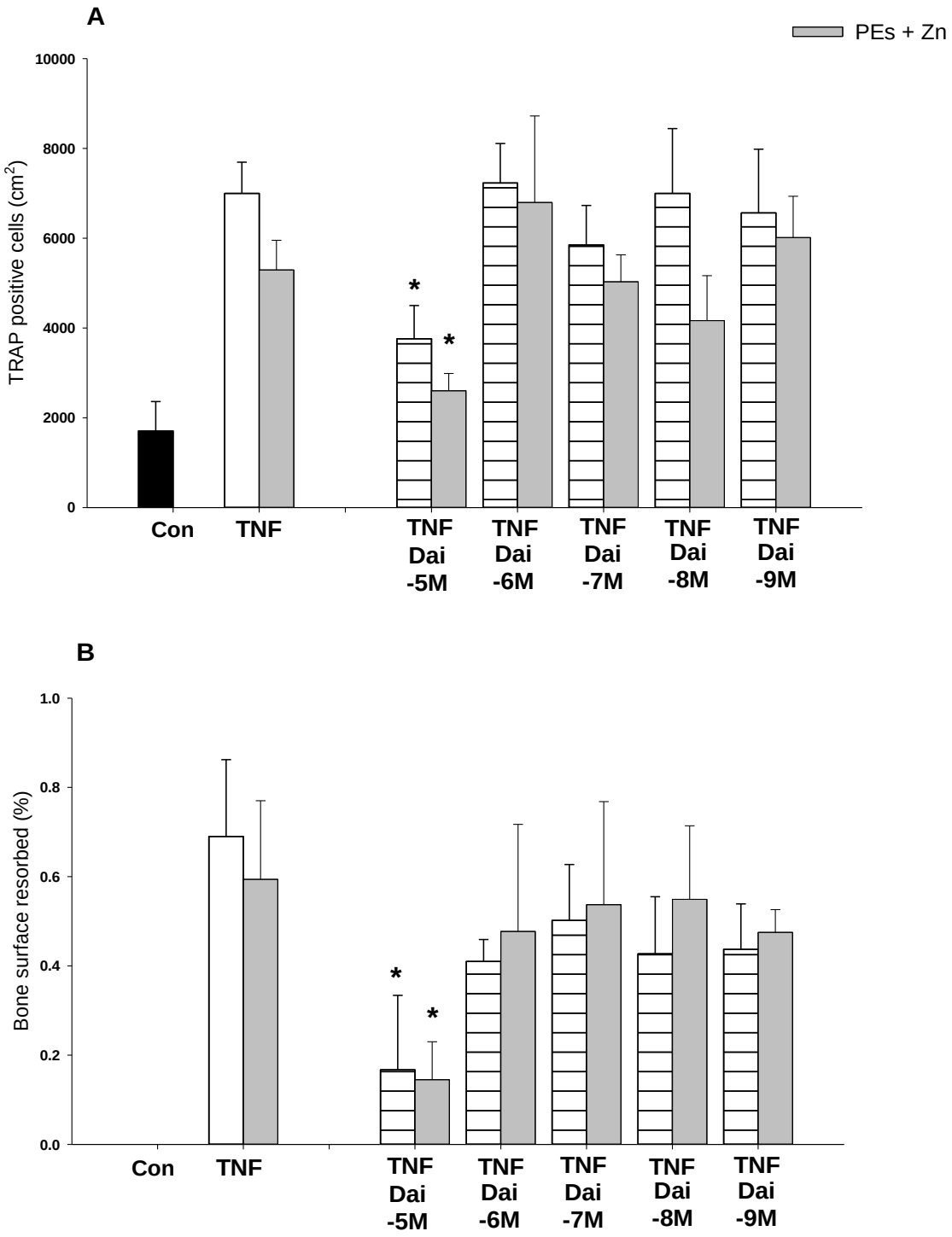

Figure 5.3. Zinc has no effect on the direct anti-osteoclastic action of daidzein on TRAP cell formation (A) and bone resorption (B). RAW264.7 cells were incubated in the presence of TNF-α (50 ng/mL) for 4 days plus daidzein (10^{-5}-10^{-9} M) in the presence or absence of zinc (10^{-5} M). Osteoclast formation was assessed by TRAP staining, while bone resorption was determined by the percentage of bone surface displaying resorption pits analysed by reflected light microscopy. Values are expressed as mean (± SEM) of three separate experiments. Differences between groups were assessed by one-way analysis of variance. * Values were significantly different versus TNF-α treated group $P < 0.05$.

5.3.2 Zinc augments the suppressive action of PEs on osteoblastsic RANKL/OPG ratio

PEs may also indirectly suppress osteoclast formation by modifying expression of the key osteoblasts derived regulators of osteoclastogenesis RANKL and OPG. To assess the effect of combinations of PEs and Zn^{2+} on *RANKL* and *OPG* mRNA ratio, I incubated osteoblasts with Zn^{2+} and the concentration of PEs shown to have the maximal suppressive effect on osteoclastogenesis. I found that *RANKL*/*OPG* expression ratio was significantly reduced by Zn^{2+} (4.16 fold), coumestrol (1.88 fold) and genistein (3.57 fold) in comparison to control. Furthermore, combinations of Zn^{2+} and genistein or coumestrol induced a further significant reduction in *RANKL/OPG* gene expression in comparison to Zn^{2+} or PEs alone (Figure 5.4). In contrast, although daidzein lowered *RANKL/OPG* expression this did not reach statistical significance and in the presence of daidzein the suppressive action of Zn^{2+} was not noted (Figure 5.4).

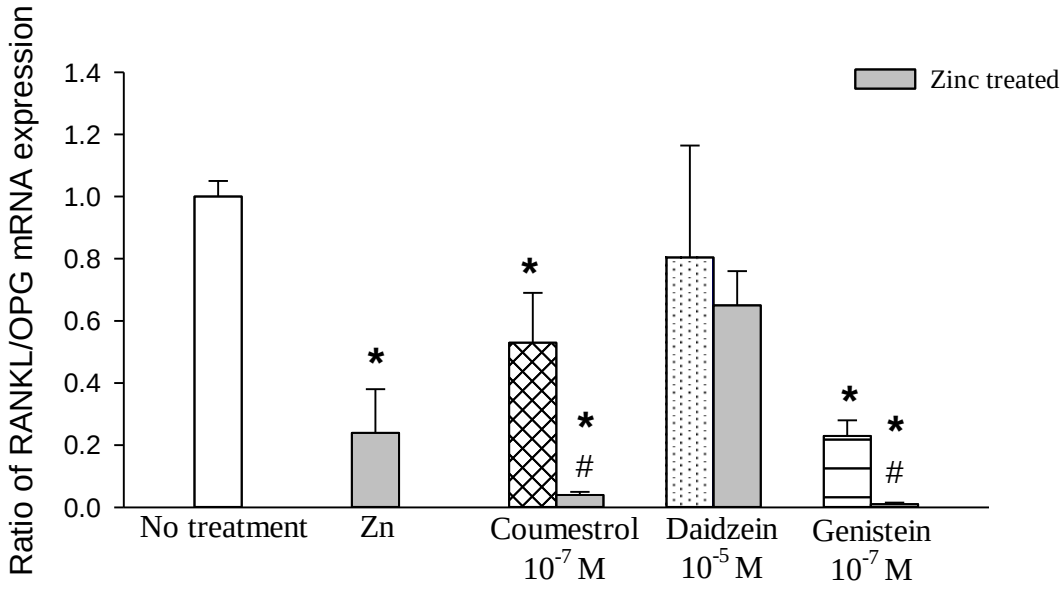

Figure 5.4. PEs and zinc suppressed the osteoblast derived stimulus for osteoclast formation. Human SaoS-2 cells were incubated with coumestrol (10^{-7} M), daidzein (10^{-5} M) or genistein (10^{-7} M) in the presence or absence of zinc (10^{-5} M). Total RNA was extracted and the expression of RANKL and OPG quantified by real time PCR. Data was normalized to *β-actin* and expressed as a ratio of RANKL to OPG expression in comparison to control. Values are expressed as a mean (± SEM) from two separate experiments. Differences between groups were assessed by one-way analysis of variance, * Values were significantly different versus control group $P < 0.05$, # values were significantly different versus zinc relevant PE treated group alone $P < 0.05$.

5.3.3 Zinc augments the stimulatory effect of PEs on osteoblasts mineralisation

The effect of PEs and Zn^{2+} on mineralisation was assessed in cultures of SaoS-2 cells incubated in the presence of differentiation agents (L-AA and β-GP). Genistein, daidzein and coumestrol all enhanced mineralisation, with coumestrol having the most pronounced effect (Figure 5.6). Zn^{2+} alone had no significant effect on mineralisation as assessed by calcein incorporation into mineralised nodules. Coumestrol (10^{-5} to 10^{-7}M) significantly enhanced osteoblastsic 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 (Figure 5.7). Genistein stimulated a significant 1.39 fold increase in calcein incorporation at the highest dose studied (10^{-5} M) (Figure 5.5). The addition of Zn^{2+} augmented the anabolic effect of all PEs and significantly increasing mineralisation compared to cultures treated with PEs alone, coumestrol (10^{-5} to 10^{-7} M), daidzein (10^{-5} to 10^{-6} M) and genistein (10^{-5} to 10^{-7} M).

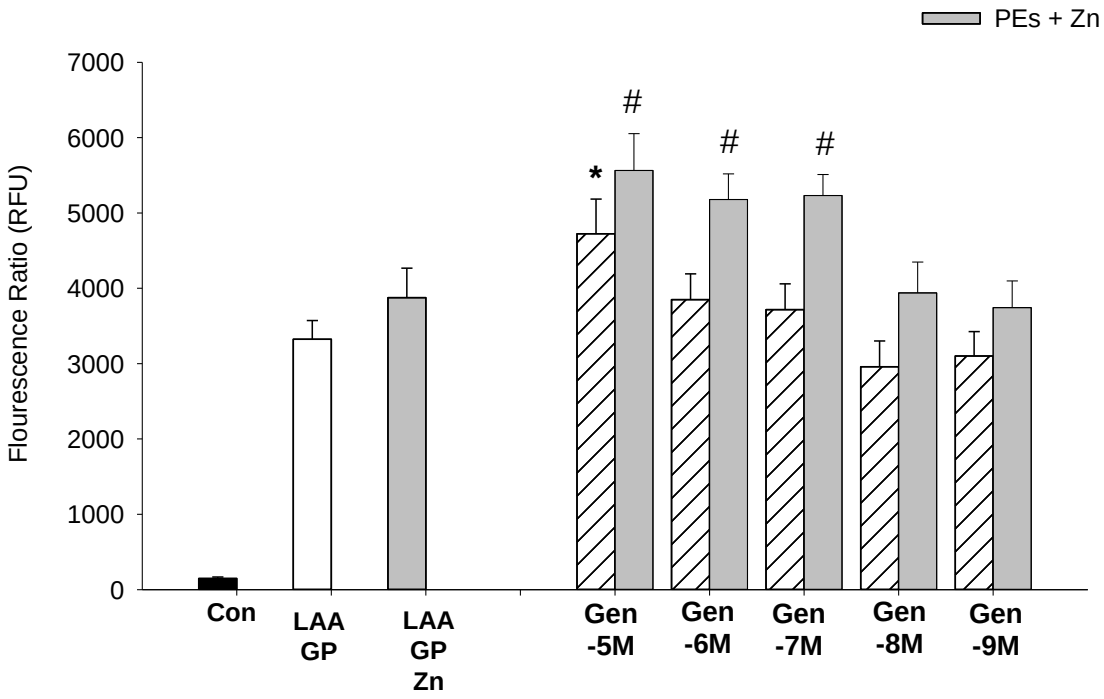

Figure 5.5. Zinc augments the effect of genistein on osteoblasts mineralisation and differentiation. SaoS-2 cells were incubated with genistein (10^{-9}-10^{-5} M) with or without zinc (10^{-5} M) for 18 days. Mineralisation measured using calcein incorporation. Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance. * $P < 0.05$ versus (LAA+GP) group, # $P<0.05$ versus relevant PE treated group.

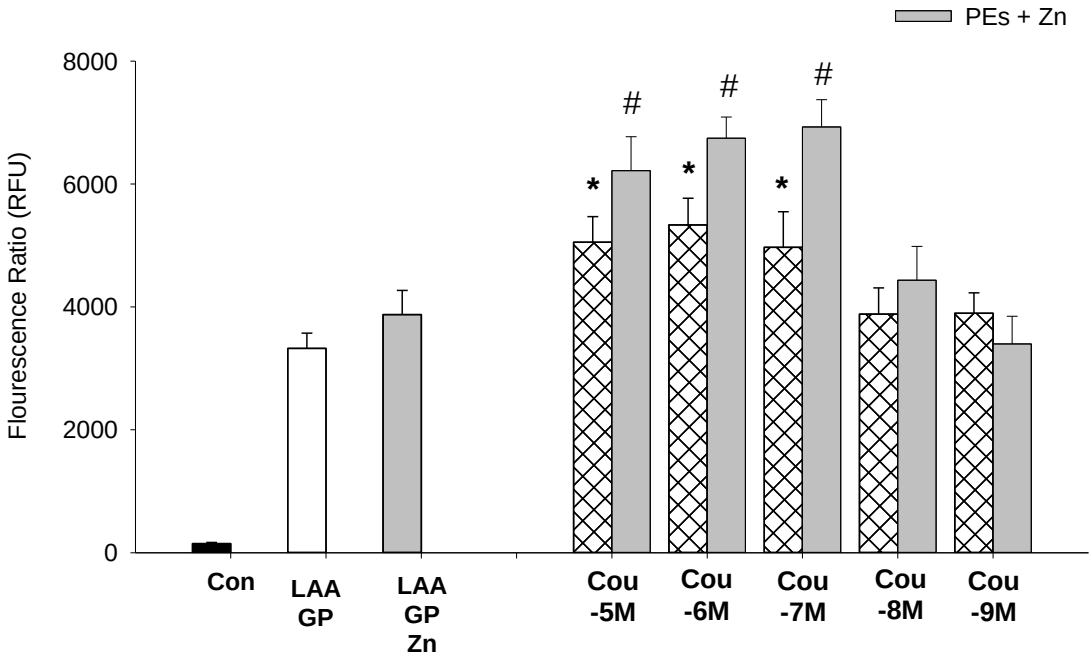

Figure 5.6. Zinc augments the effect of coumestrol on osteoblasts mineralisation and differentiation. SaoS-2 cells were incubated with coumestrol (10^{-9}-10^{-5} M) with or without zinc (10^{-5} M) for 18 days. Mineralisation measured using calcein incorporation. Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance. * $P < 0.05$ versus (LAA+GP) group, # $P<0.05$ versus relevant PE treated group.

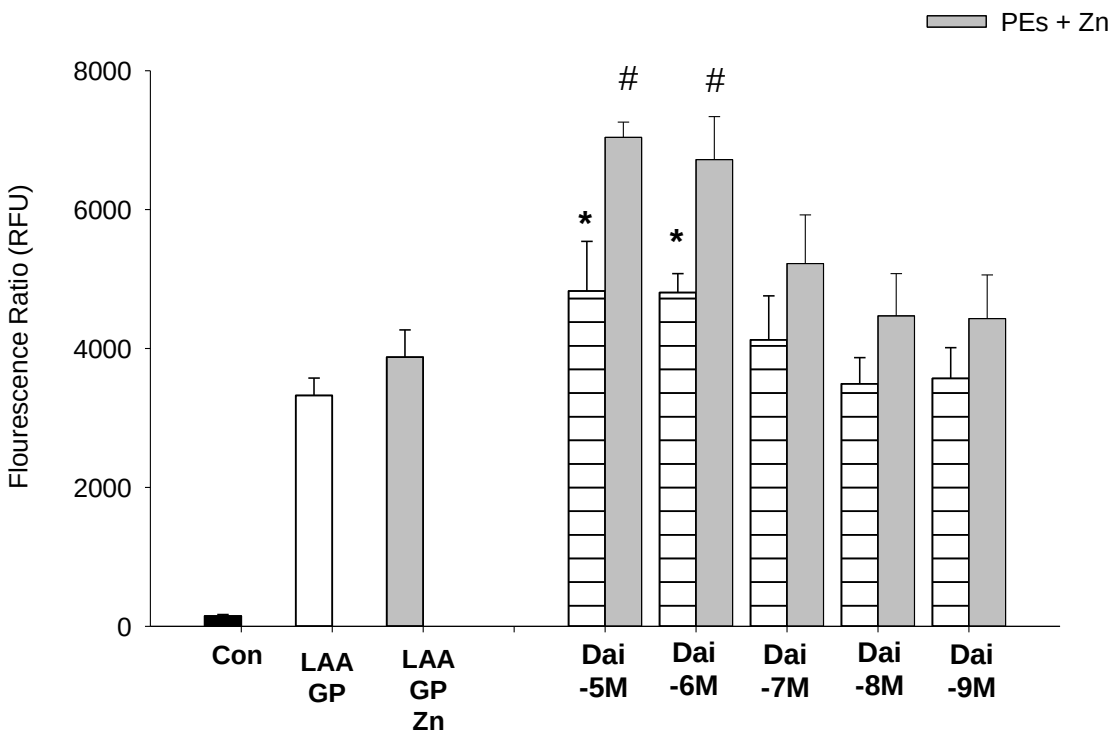

Figure 5.7. Zinc augments the effect of daidzein on osteoblasts mineralisation and differentiation. SaoS-2 cells were incubated with daidzein (10^{-9}-10^{-5} M) with or without zinc (10^{-5} M) for 18 days. Mineralisation measured using calcein incorporation. Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance. * $P < 0.05$ versus (LAA+GP) group, # $P<0.05$ versus relevant PE treated group.

5.3.4 PEs augment bone mineralisation by an ER-dependent mechanism

To assess whether the augmentative action of genistein, coumestrol and daidzein was mediated by an E2 dependent or independent mechanism, SaoS-2 cells were cultured with concentrations of PEs shown to enhance mineralisation in the presence or absence of the E2 antagonist ICI 182,780 (10^{-6} M). The antagonist had no effect on mineralisation in control cultures but prevented the augmentative effect of PEs in the presence or absence of Zn^{2+} (Figure 5.8), suggesting that PEs directly enhance osteoblastsic mineralisation by an ER-dependent mechanism.

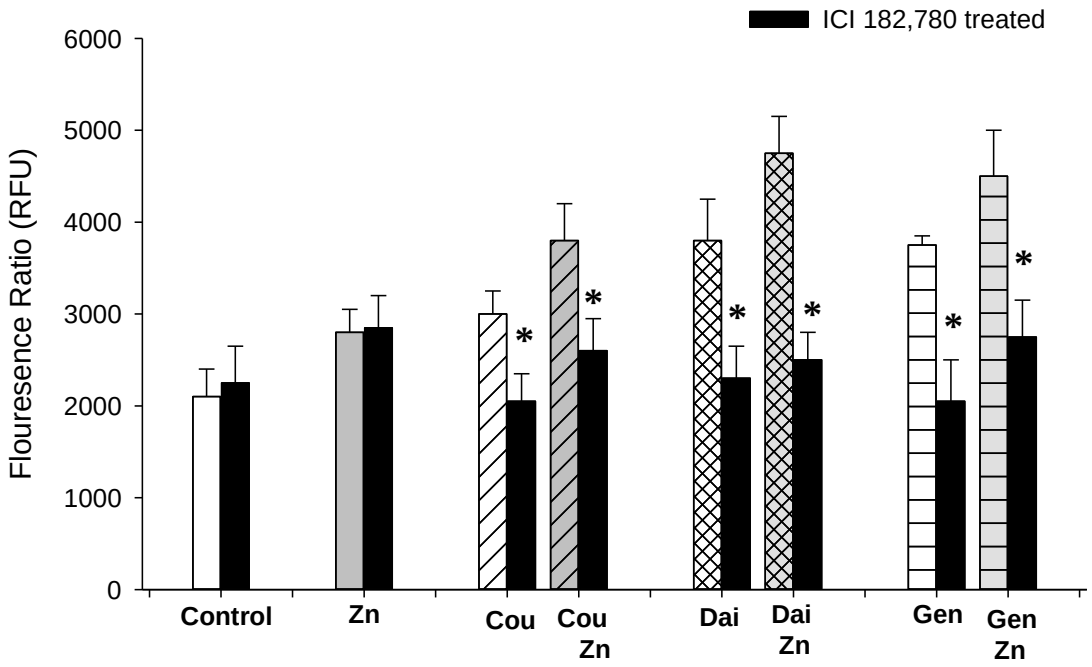

Figure 5.8. The E2 antagonist ICI 182,780 abolished the augmentative effect of PEs on mineralisation in the presence or absence of zinc. SaoS-2 cells were cultured with coumestrol (10^{-7} M), daidzein (10^{-5} M), genistein (10^{-7} M) with or without ICI 182,780 (10^{-6} M). Mineralisation has been suppressed in the presence of ICI 182,780 (10^{-6} M). Values are expressed as the mean (± SEM) from three separate experiments. Differences between groups were assessed by one-way analysis of variance. * Values are significantly different from non-ICI 182,780 treated group $P < 0.05$.

5.3.5 Zinc enhanced the stimulatory effect of PEs on ALP production

To determine the cellular mechanism by which Zn^{2+} and PEs enhanced mineralisation, I examined their effect on osteoblasts 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, which was significantly elevated in the presence of Zn^{2+} for four days. The augmentative action of Zn^{2+} was further enhanced in the presence of genistein (10^{-5} to 10^{-7} M) (Figure 5.9), coumestrol (10^{-5} to 10^{-7} M) (Figure 5.10) or daidzein (10^{-5}M to 10^{-9} M) (Figure 5.11). 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^{2+} treated cultures.

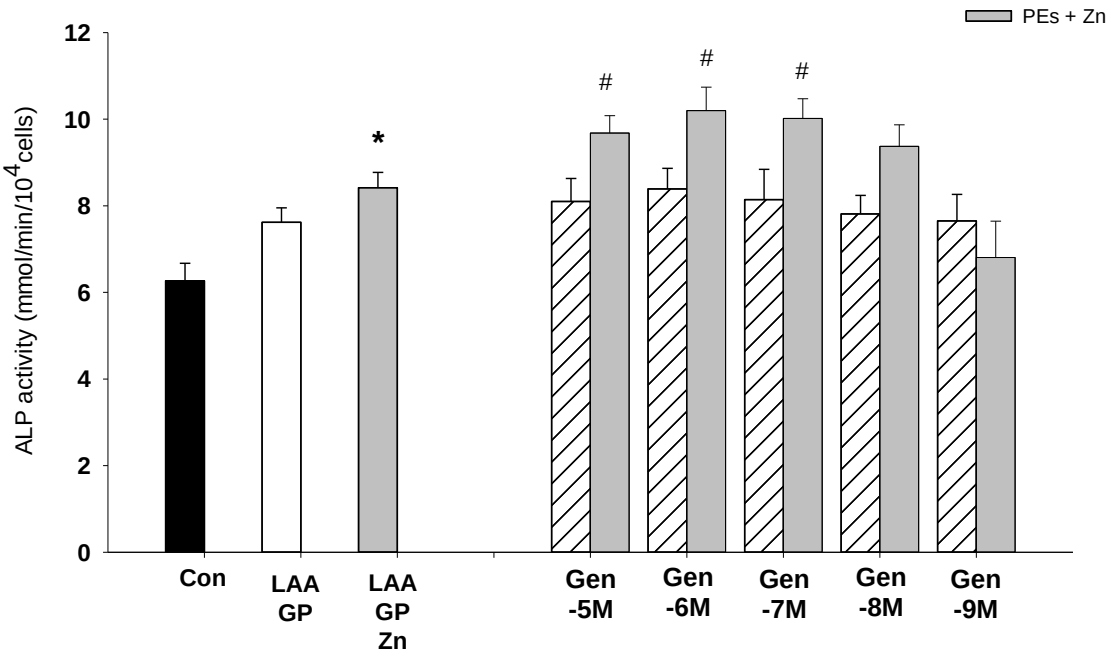

Figure 5.9. Zinc augments the effect of genistein on osteoblastsic ALP activity. SaoS-2 cells were incubated with genistein (10^{-9}-10^{-5} M) with or without zinc (10^{-5} M) for 4 days. Alkaline phosphatase activity was assessed using p-nitrophenol phosphate as a substrate (1mg/ml). Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance. *P<0.05 versus control group, # P<0.05 versus relevant PE treated group.

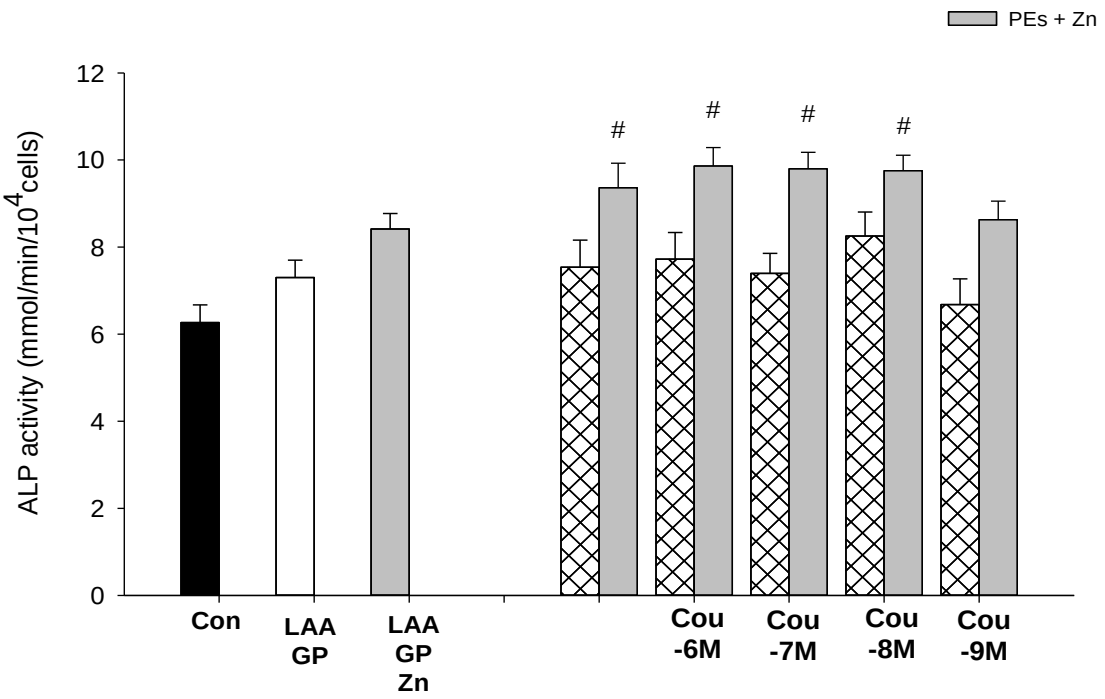

Figure 5.10. Zinc augments the effect of coumestrol on osteoblastsic ALP activity. SaoS-2 cells were incubated with coumestrol (10^{-9}-10^{-5} M) with or without zinc (10^{-5} M) for 4 days. Alkaline phosphatase activity was assessed using p-nitrophenol phosphate as a substrate (1mg/ml). Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance. *P<0.05 versus control group, # P<0.05 versus relevant PE treated group.

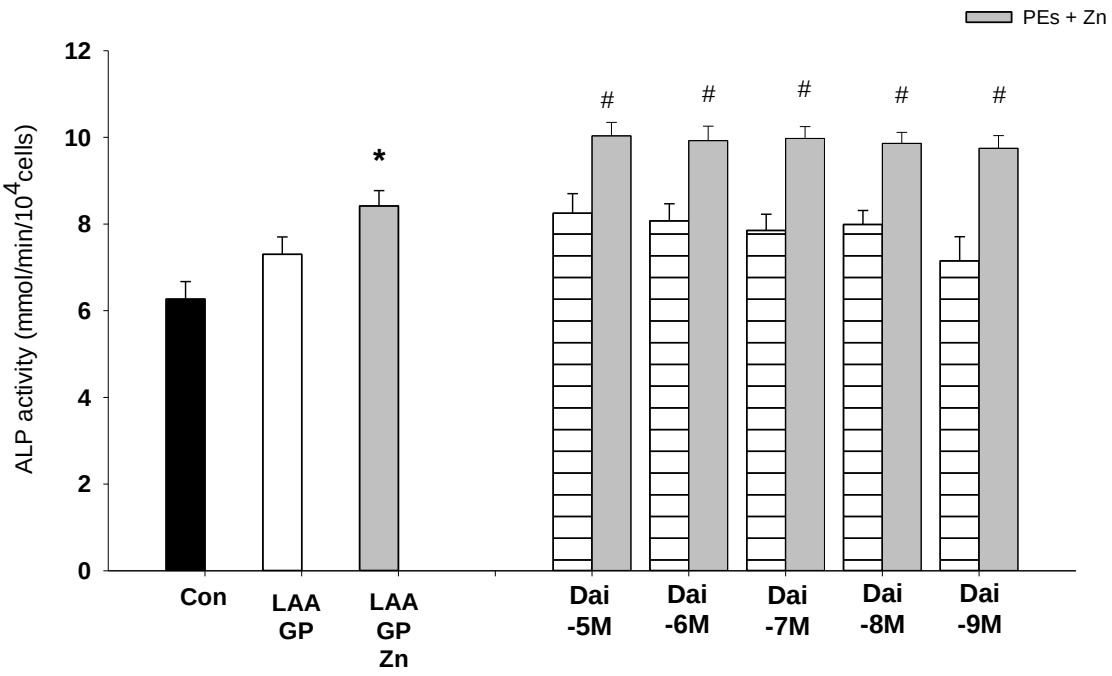

Figure 5.11. Zinc augments the effect of daidzein on osteoblastsic ALP activity. SaoS-2 cells were incubated with daidzein (10^{-9}-10^{-5} M) with or without zinc (10^{-5} M) for 4 days. ALP activity was assessed using p-nitrophenol phosphate as a substrate (1mg/ml). Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance. * $P<0.05$ versus control group, # $P<0.05$ versus relevant PE treated group.

5.3.6 Zinc had no effect on osteoblasts proliferation

Zn^{2+} alone had no effect on osteoblasts number (Figure 5.12). Similarly coumestrol on its own or in combination with Zn^{2+} had no proliferative effect although there was a trend towards lower osteoblasts number in all groups (Figure 5.13). Genistein (10^{-6} to 10^{-9} M) induced a modest but significant increase in cell number and Zn^{2+} increased the effect of the highest genistein concentration examined (10^{-5} M) (Figure 5.12). Concentrations of daidzein shown to enhance mineralisation also had no effect on proliferation although increases in osteoblasts number were observed in the presence of daidzein with or without Zn^{2+} at 10^{-6} and 10^{-9} M (Figure 5.14).

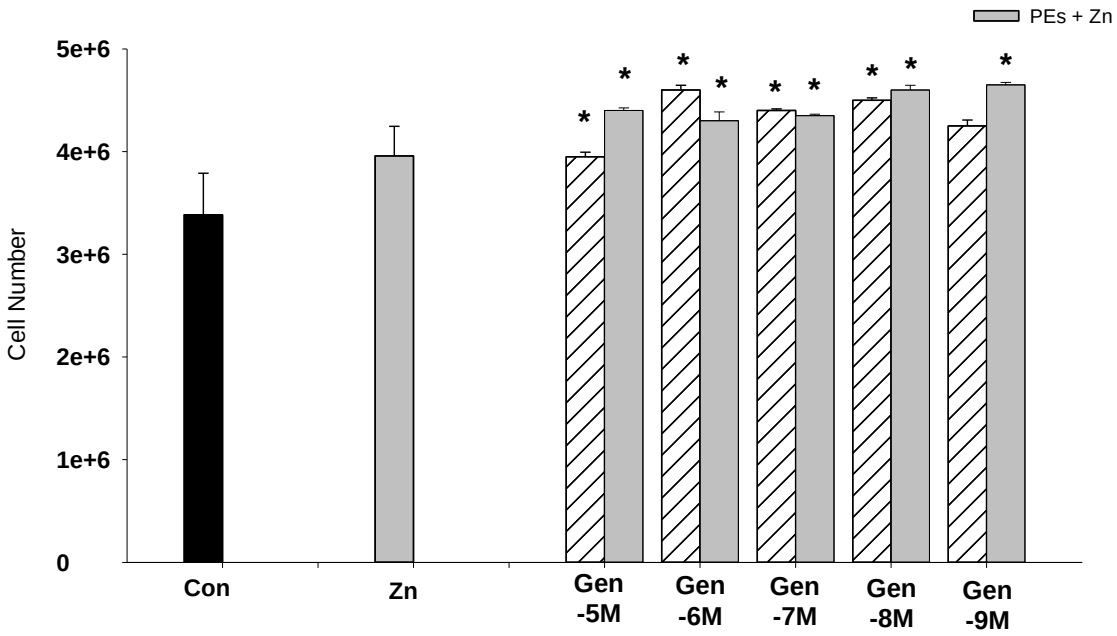

Figure 5.12. Zinc had no effect on genistein's stimulatory effect on osteoblasts proliferation. SaoS-2 cells were incubated with genistein (10^{-5}-10^{-9} M) with or without zinc (10^{-5} M) for 4 days. Proliferation was then assessed using an MTS assay. Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance. * $P<0.05$ versus control group.

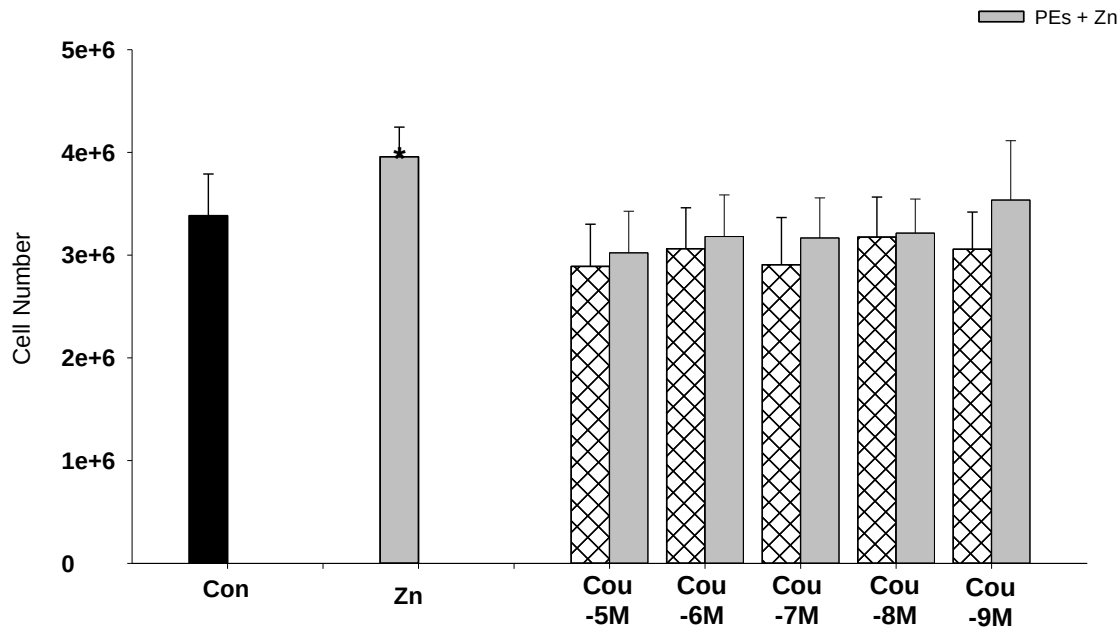

Figure 5.13. Zinc and coumestrol had no effect on osteoblasts proliferation. SaoS-2 cells were incubated with coumestrol (10^{-5}-10^{-9} M) with or without zinc (10^{-5} M) for 4 days. Proliferation was then assessed using an MTS assay. Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance.

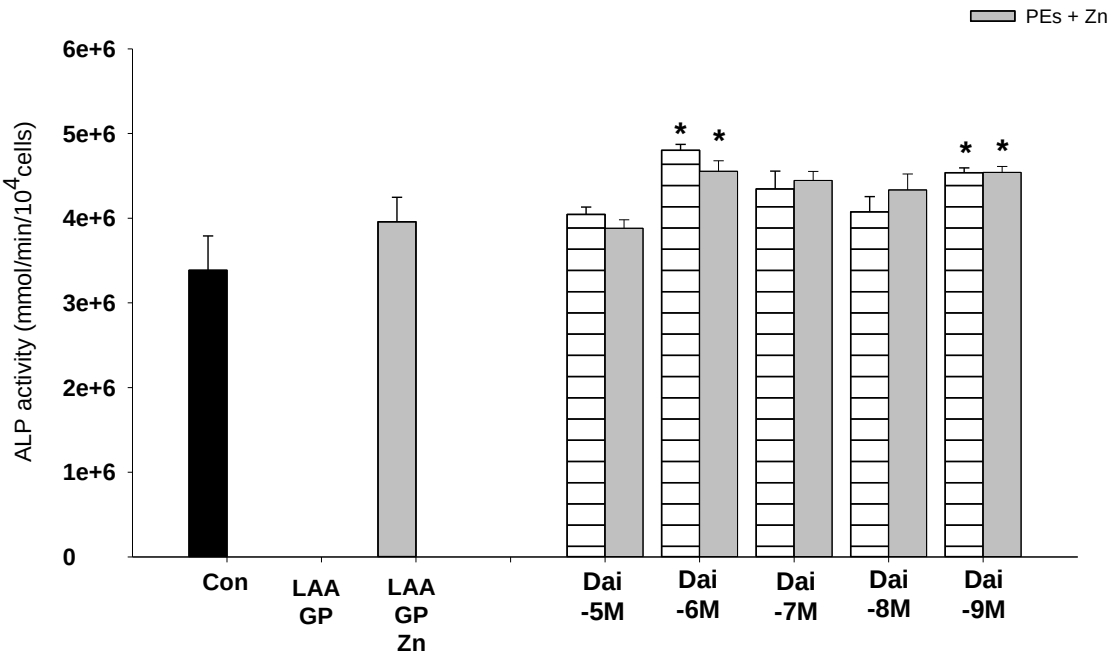

Figure 5.14. Zinc had no effect on the stimulatory effect of daidzein on osteoblasts proliferation. SaoS-2 cells were incubated with daidzein (10^{-5}-10^{-9} M) with or without zinc (10^{-5} M) for four days. Proliferation was then assessed using a MTS assay. Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance. * $P<0.05$ versus control group.

5.3.7 Zinc blunts the augmentative action of PEs 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. Therefore, this study examined the effect of Zn^{2+} alone or in combination with PEs concentrations shown to augment mineralisation on *type I collagen* and *osteocalcin* mRNA expression. Zn^{2+} alone had no effect on *type I collagen* or *osteocalcin* expression (Tables 5.2). In contrast all PEs 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 5.2). PEs also enhanced osteocalcin expression, a marker of mature osteoblast; 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^{2+} blunted the stimulatory effect of PEs on *type I collagen* and *osteocalcin* expression, although mRNA levels were still significantly above that of control (Table 5.2). Thus, in contrast to a positive interaction with PEs on mineralisation Zn^{2+} appears to blunt the stimulatory action of PEs on organic components of bone matrix.

Table 5.2. Zinc blunts the stimulatory effect of PEs on the expression of organic components of bone matrix.

	***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	35048	13799	64	19
Zinc (10^{-5}M)	20017	8045	41	11
Coumestrol (10^{-7}M)	422137 [a]	127678	136 [a]	19
Coumestrol (10^{-7}M) and Zn^{2+}	86339 [a b]	2529	92 [a b]	9
Daidzein (10^{-5}M)	504560 [a]	11805	1066 [a]	389
Daidzein (10^{-5}M) and Zn^{2+}	119396 [a b]	9980	321 [a b]	13
Genistein (10^{-7}M)	257731 [a]	86054	200 [a]	34
Genistein (10^{-7}M) and Zn^{2+}	51177 [b]	12014	96 [a b]	9

a $P<0.05$ versus control, b $P<0.05$ versus relevant PE alone.

5.3.8 Zinc augments the effect of PEs on Runx2 expression but suppresses osterix expression

Osteoblast differentiation is regulated by a network of transcription factors that control gene expression. These include Runx2 and osx which are expressed in a temporally defined manner. Runx2 expression is elevated during early stages of osteoblast differentiation, when it promotes formation of immature osteoblasts from mesenchymal stem cells. Runx2 expression is then downregulated during the formation of mature osteoblasts (Marie, 2008). Whereas osx expression is restricted to mature osteoblasts (Marie, 2008). To examine the potential molecular mechanism mediating the effect of Zn^{2+} and PEs on osteoblast differentiation, I therefore examined the effect of PEs and Zn^{2+} on *RUNX2* and *osx* expression.

In keeping with their lack of effect on mineralisation Zn^{2+} or genistein (10^{-7} M) alone had no effect on *Runx2* or *osx* expression (Table 5.3). However, genistein concentrations shown to promote mineralisation (10^{-5} M) did significantly increase *osx* and *Runx2* expression (data not shown). Similarly, coumestrol (10^{-7} M) and daidzein (10^{-5} M) enhanced *Runx2* and *osx* expression. Interestingly the addition of Zn^{2+} had a differential effect on PEs-induced *Runx2* and *osx* expression. PEs-induced *Runx2* expression was significantly enhanced by Zn^{2+}, whereas Zn^{2+} suppressed the augmentative effect of coumestrol and daidzein on *osx* expression.

Table 5.3. Zinc augments the effect of PEs on *Runx2* and *osterix* expression.

Groups	***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
Zinc (10^{-5}M)	8952	1377	7693	362
Coumestrol (10^{-7}M)	23114 [a]	1678	13856 [a]	1505
Coumestrol (10^{-7}M) and Zn^{2+}	34591 [a]	7006	10291 [b]	443
Daidzein (10^{-5}M)	19705 [a]	1599	39273 [a]	1498
Daidzein (10^{-5}M) and Zn^{2+}	44631 [ab]	8695	17651 [b]	3610
Genistein (10^{-7}M)	7148	778	11049	1492
Genistein (10^{-7}M) and Zn^{2+}	18179 [ab]	1169	10107	12

a $P<0.05$ versus control, b $P<0.05$ versus relevant PE alone.

5.4 Discussion

Bone remodelling the coupled process of osteoclastic bone resorption and osteoblastsic 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^{2+} and PEs have a positive impact on bone cell activity (Karieb and Fox, 2011, Poulsen and Kruger, 2008, Seo et al., 2010, Yamaguchi, 2010); 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*. This study aimed to clarify the cellular mechanism through which these dietary factors may interact and suggests that appropriate combinations of Zn^{2+} and PEs may augment mineralisation and further suppress bone resorption. These results strengthen the data for the use of combinations of Zn^{2+} and PEs 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 osteoblastsic RANKL while decreasing OPG expression (Boyce and Xing, 2008). 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 osteoblastsic RANKL expression decreases and OPG concentrations rise, OPG than 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 is a hallmark of

many osteolytic disorders (Nanes, 2003, Somayaji et al., 2008). 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 PEs suppress osteoclast formation through both mechanisms. Coumestrol, daidzein and genistein directly inhibit osteoclast formation in response to pro-osteoclastic cytokines *in-vitro* as found in chapter four of this study (Garcia Palacios et al., 2005) and decreased osteoblastsic RANKL/OPG ratios have been noted following treatment with a range of PEs (Wu et al., 2009, Chen and Wong, 2006). Genistein and daidzein also reduce osteoblastsic expression of other inducers of osteoclast differentiation including IL-6 (Chen et al., 2002). Zn^{2+} has also been shown to reduce osteoclast formation *in-vitro* (Holloway et al., 1996, Yamaguchi and Weitzmann, 2011, Uchiyama and Yamaguchi, 2007), whereas Zn^{2+} deficiency is associated with increased levels of osteoclast formation and bone resorption (Fong et al., 2009). However, the cellular mechanism by which Zn^{2+} suppresses osteoclast differentiation is unclear, as previous studies used heterogeneous bone marrow cultures (Uchiyama and Yamaguchi, 2007) or have shown inconsistent osteoclastic responses to changes in Zn^{2+} status (Fong et al., 2009, Windisch et al., 2002, Eberle et al., 1999). Similarly, interactions between Zn^{2+} and PEs have not been widely investigated.

To help clarify this I examined the direct effect of Zn^{2+} on homogenous RAW264.7 monocytic cultures and the indirect action on osteoblastsic RANKL/OPG ratios. I noted no direct effect of Zn^{2+} on TNF-α-induced osteoclast formation in RAW264.7 cells and Zn^{2+} 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^{2+} significantly suppressed PTH-induced osteoclast formation and

combinations of Zn^{2+} and genistein decreased RANKL-induced osteoclastogenesis (Uchiyama and Yamaguchi, 2007). The suppressive action noted in these studies may be due to an indirect effect of Zn^{2+} mediated through stromal cells present in bone marrow cultures which are absent from RAW264.7 cultures. In keeping with this I noted that Zn^{2+} alone suppressed osteoblastsic *RANKL/OPG* gene expression ratio and also augmented the suppressive effect of PEs. This assertion is strengthened by the studies of Holloway in which Zn^{2+} suppressed bone resorption in the presence of added osteoblasts (Holloway et al., 1996). Thus Zn^{2+} associated changes in osteoclast number are most likely mediated through an indirect action on osteoblasts rather than a direct effect on monocyte differentiation and appropriate concentrations of Zn^{2+} and coumestrol or genistein may have a more pronounced anti-osteoclastic effect than either alone.

Serum PE concentrations differ between populations and are dependent on an individual's diet. Asians who typically have a soy-rich diet have significantly higher PEs concentrations compared to Westerners (Morton et al., 2002). The range of PEs concentrations examined in my study reflect those measured in Asians (10^{-6}-10^{-7}M) and Westerners (10^{-8} M). The anti-osteoclastic concentration of genistein and coumestrol seen in this study is similar to those shown previously (Karieb and Fox, 2011) and are in the range of levels measured in Asian populations but higher than those achieved by Western diets (Morton et al., 2002). 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 my studies. However, these concentrations could be achieved with daidzein supplementation which generates tissue levels several orders of magnitude higher than dietary sources (Gardner et al., 2009). Serum Zn^{2+} concentration also varies between populations with diets that lack animal sourced foods

leading to a high risk of Zn^{2+} deficiency (Hotz, 2007). The Zn^{2+} concentration used in my studies is similar to previous *in-vitro* experiments and is within normal serum reference ranges reflecting those achieved by healthy Western diets (Yamaguchi et al., 2008, Martín-Lagos et al., 1998). Thus, Zn^{2+} tissue levels at 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, suppression of resorption would only address part of the underlying pathology as this would not prevent the reduction in osteoblasts function (Raisz, 2005). A reduction in resorption would 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 (Shane et al., 2010). An ideal therapeutic strategy would therefore rectify defects in both osteoclast and osteoblast activity. Osteoblastsic 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 (Golub, 2009). 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. I found that Zn^{2+} enhanced the stimulatory effect of coumestrol, daidzein and genistein on osteoblasts mineralisation *in-vitro*. Combinations of coumestrol and Zn^{2+} and genistein and Zn^{2+} 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^{2+} 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^{2+},

coumestrol, daidzein or genistein augment osteoblasts function *in-vitro* enhancing the expression of components of the organic matrix and stimulating mineral deposition.

The augmentative action of Zn^{2+} would at least in part appear to be due to increased expression of the marker of osteoblast differentiation ALP. Osteoblasts derived matrix vesicles contain high ALP levels and mutations in ALP lead to the genetic disorder hypophosphatasia which is characterised by poorly mineralised bone (Fedde et al., 1999). ALP promotes the initial stage of mineralisation by hydrolysing inhibitory pyrophosphate to generate inorganic phosphate needed for the initiation of hydroxyapatite deposition (Golub, 2009). Thus elevated ALP activity would be expected to enhance mineral formation. In contrast, the augmentative effect of PEs 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 osteoblasts number this was only seen at concentrations other than those shown to enhance mineralisation.

To further examine the mechanism by which PEs and Zn^{2+} augment osteoblasts function this study examined the expression of key intracellular regulators of osteoblast differentiation. Osteoblastsogenesis is a sequential process involving multiple transcription factors that stimulate mesenchymal precursors to form immature pre-osteoblasts and ultimately mature osteoblasts (Jensen et al., 2010). The initial stage of osteoblastsic 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 (Otto et al., 1997), whereas heterozygous loss leads to cleidocranial dysplasia in humans and is associated with abnormal osteoblasts development in mice (Otto et al.,

1997, Mundlos et al., 1997). With the formation of mature osteoblasts capable of mineralising osteoid Runx2 expression falls whereas levels of osx increase (Komori, 2010).

Previous studies have shown that Zn^{2+} enhances Runx2 expression (Yamaguchi et al., 2008) but to date no study has examined the effect of Zn^{2+} on osx expression. I found that anti-osteoclastic concentrations of coumestrol (10^{-7} M) and daidzein (10^{-5} M) significantly enhanced Runx2 expression and Zn^{2+} significantly enhanced this response. On the other hand, Zn^{2+} blunted the stimulatory action of coumestrol and daidzein on osx expression. Therefore, Zn^{2+} appears to promote the expression of a transcription factor profile typical of early mature osteoblasts with high Runx2 and low osx levels. This profile is likely to explain the observed changes in organic matrix protein expression. The studies of Liu *et al* 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 (Liu et al., 2001b). Osteocalcin levels are comparatively low in pre-osteoblasts when Runx2 expression peaks, 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^{2+} promoting the formation of early rather than late stages of mature osteoblast differentiation.

My data show that Zn^{2+} augments the indirect anti-osteoclastic action of coumestrol and genistein at concentrations typically generated by soy-rich diets. Interactions between Zn^{2+} and anti-osteoclastic PEs concentrations were also noted for osteoblast differentiation and function. Appropriate combinations of Zn^{2+} and PEs increased ALP activity, extracellular matrix expression and mineralisation. This effect may be due to Zn^{2+} inducing the formation of an early mature stage of osteoblast differentiation. These

findings suggest that PEs have an anabolic action on osteoblast differentiation and bone formation markers, and combinations of Zn^{2+} and PEs may be more effective than either alone. This result strengthens data for the use of combinations of nutritional factors as a supplementary treatment of skeletal disorders.

Chapter Six: The Effect of Phytoestrogens on T Cell-Mediated Osteoclastogenesis

Chapter 6

6.1 Introduction

The interaction between immune cells such as Thelper1 ($CD4^+$) cells and osteoclast is thought to be critical for bone loss in post-menopausal women (Pfeilschifter et al., 2002). Co-culture of murine spleen cells and human T cells augments osteoclast formation and *RANKL* mRNA expression (Horwood et al., 1999), and activated T cells enhance osteoclast formation in RAW264.7 cultures (Wyzga et al., 2004). The ability of T cells to induce osteoclast formation also provides a new insight into the mechanism supporting bone resorption in diseases such as rheumatoid arthritis. The increase in osteoclastogenesis is at least in part dependent on TNF-α (Cenci et al., 2000). TNF-α levels are increased in peripheral blood cells of post-menopausal women and ovariectomised animals, whereas E2 reduces this expression (Pacifici et al., 1991, Ralston et al., 1990, Kimble et al., 1996). Ovariectomy increases TNF levels which E2 prevents (Tyagi et al., 2012). Furthermore, TNF-α-deficient mice or mice lacking T cells are resistant to ovariectomy-induced bone loss (Roggia et al., 2001, Lee et al., 2006b).

This increase in TNF levels is thought to be due to expansion of the T cell population. E2 deficiency modulates T cell activation, proliferation and life span by modifying the cytokine environment in bone ultimately leading to increased MHC II expression and T cell antigen presentation (Li et al., 2011). MHCII expression is stimulated following an E2 dependent increase in IL-7 and decrease in TGF-β level. E2 promotes TGF-β expression as the gene encoding TGF contains several ERE (Gao *et al*., 2004). Thus E2 deficiency decreases TGF-β expression. E2 also represses IL-7 expression as IL-7 levels are elevated in ovariectomized mice (Ryan *et al*., 2005, Weitzmann *et al*., 2002) and IL-7 neutralising antibodies prevent bone loss due to the suppression of TNF-α and IFN-γ production (Ryan *et al*., 2005). Moreover, TGF-β and IL-7 inversely regulate the production of each (Huang, 2002), and therefore reduced TGF-β levels stimulates

further IL-7 production. The net result of these changes is to create an inflammatory state which is characterised by elevated IFN-γ levels. IFN-γ in turn promotes the expression of class II transactivator (CIITA) (Pacifici, 2008) which enhances MHCII levels on APC. The subsequent presentation of unknown antigens to T cells leads to activation, clonal expansion and elevated TNF-α production. The mechanism by which E2 deficiency modulates cytokine-producing T cells is summarised in Figure 6.1. T cells also express RANKL (Kong et al., 1999), and osteopetrosis in RANKL-knockout mice was rescued after crossing with mice expressing RANKL-producing $CD4^+$ T cells (Kim et al., 2000). In addition, T cells also release IL-1, IL-17, IL-4 and IFN-γ (Weitzmann and Pacifici, 2006a, Huang et al., 2006) which are also regulators of bone cell activity (Teitelbaum, 2004, Rifas and Arackal, 2003)

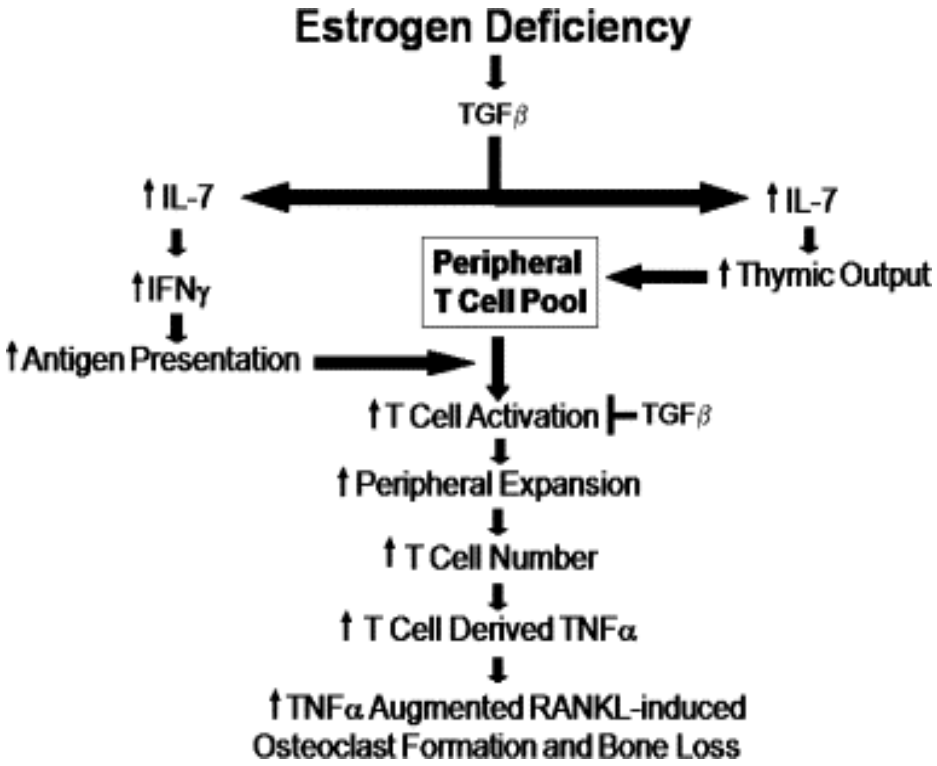

Figure 6.1. Oestrogen deficiency induced TNF-α-producing T cells in bone marrow which caused enhancement of RANKL-induced osteoclastogenesis. From (Pacifici, 2008).

Several studies suggested that PEs might be useful in the prevention of skeleton disorders associated with aging. In the previous chapter data indicated that PEs have a direct beneficial effect on osteoclasts and osteoblasts activity. However data on their potential impact on immune modulation of bone cell activity is sparse. Genistein reduced thymus weight in ovariectomised mice, which was accompanied by a decrease in thymocyte number and T cell apoptosis (Yellayi et al., 2002). Genistein was proposed to effect immune cell function through ER-dependent and independent mechanisms (Suenaga et al., 1998), for instance genistein's tyrosine kinase activity was suggested to inhibit lymphocyte proliferation (Sakai and Kogiso, 2008), and genistein also inhibited B cell activation in OVX mice (Ishimi et al., 1999). Additionally, genistein and daidzein have growth inhibitory effects on leukaemia cells through DNA damage *in-vivo* and *in-vitro* (Yamasaki et al., 2007), and genistein via an ER dependent mechanism induces T-cell leukaemia apoptosis via caspase-3 activation, (Yamasaki et al., 2010).

Further studies are needed to clarify whether this effect of PEs on T cells could further protect against post-menopausal bone loss. Alternatively, PEs could augment the expression of inflammatory cytokines and thereby antagonise their inhibitory action on osteoclast formation. Therefore, the aim of this part of the study is to investigate the effect of PEs on pro-inflammatory cytokine and RANKL production by T cells to enhance our understanding of the potential impact of these compounds on bone remodelling.

6.2 Material and methods

6.2.1 Culture of Jurkat E6.1 cells

Jurkat E6.1 T cells, a human leukemic T cell line were obtained from ECACC, Porton Down, UK (ECACC cat. num. 88042803) and cultured in phenol-red free RPMI supplemented with 10% charcoal stripped foetal calf serum (Autogen Bioclear, UK), 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin (all from Sigma, UK). RAW264.7 monocytes (ATCC, UK, cat. num. TIB-71) were incubated in phenol red free Dulbecco's minimum essential medium supplemented with 10% charcoal stripped foetal calf serum (Autogen Bioclear, U.K.), 2 mmol/l glutamine, 100 IU/ml benzylpenicillin and 100 mg/ml streptomycin (all from Sigma, UK). All incubations were performed at 37^{o}C in 5% CO_2 in a humidified incubator. Cultures were fed every 2–3 days by replacing half of the culture volume with fresh medium. Concanavalin A (Con A) and all other reagents and kits were obtained from Sigma (Poole, Dorset, UK) unless stated. Anti-human TNFα antibodies were purchased from Insight Biotechnology, Wembley, UK.

6.2.2 Real time quantitative PCR analysis of pro-inflammatory cytokine expression in Jurkat E6.1 T cells

To assess the effect of PEs on T cell function, Jurkat cells were treated with and without the T cell activator concavalin A (Con A) in the presence or absence of PEs. T cells were cultured for four days with RPMI phenol red free cell culture medium in six well plates and treated with genistein (10^{-7}-10^{-9} M), coumestrol (10^{-7}-10^{-9} M), or daidzein (10^{-7}-10^{-9} M) in the presence of Con A (10μg/ml). Total RNA was extracted and reversed transcribed with M-MLV reverse transcriptase, and real time-PCR performed on a StepOne PCR system (Applied Biosytems, UK) using the DNA-binding dye SYBR

green as a fluophore as described in chapter three (3.8.1). Primers used for each transcription factor are shown in (Table 6.1).

6.2.3 Cell proliferation assay

Jurkat E6.1 T cells were cultured in 96 well plates at a density of $1x10^4$ cells per well and treated with Con A (10μg/ml) 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) for four days. Proliferation was then assessed using a commercial AQueous one solution cell proliferation assay (Promega, UK) according to manufacturer's instructions, as described in chapter three (3.6).

Table 6.1. Primer sequences

Genes	5´-3´ Forward primer	3´-5´ Reverse primer
Human *β-Actin*	GCGCGGCTACAGCTT CACCA	TGGCCGTCAGGCAGCTCGTA
Human *TNF-α*	GCTCCAGTGGCTGAA CCGCC	AGCACATGGGTGGAGGGGCA
Human *IL-6*	TCAATGAGGAGACTTGCCTGG TGA	TCTGCAGGAACTGGATCAGGAC TT
Human *IL-1β*	ACGCTCCGGGACTCA CAGCA	TGAGGCCCAAGGCCACAGGT
Human *RANKL*	ACAGGCCTTTCAAGGAGCTGTG C	ACCAGATGGGATGTCGGTGGC

6.2.4 The effect of phytoestrogens on the pro-osteoclastic action of T cells

T cells were cultured in RPMI medium and treated with genistein (10^{-7} M), coumestrol (10^{-7} M) or daidzein (10^{-5} M) in the presence of con A (10µg/ml) for four days. Cells were then centrifuged, washed in medium and resuspended in appropriate volumes of RPMI to remove con A and PEs. T cell number was then counted using a Neubauer cell chamber. $1x10^5$ T cells were added to 96 well plates containing RAW264.7 cells ($2x10^4$ cell/well). TRAP+ mono and multinucleated cell formation was evaluated as previously described in chapter three (3.4) after 7 days of incubation. The experiment was performed in triplicate (n=15). To determine if the pro-osteoclastic effect of T cells was mediated through TNF-α production, neutralising mouse anti-human TNF-α antibodies (5µg/ml) was added to replicate wells.

6.3 Results

6.3.1 Phytoestrogens blunt the augmentative effect of activated T-cells on osteoclast formation

To examine the effect of activated T cells on osteoclast differentiation RAW264.7 cells were co-cultured with Jurkat E6.1 which had or hadn't been treated with PEs. No TRAP+ osteoclasts were seen in cultures of T cells whether they had been activated with con A or not. Similarly, osteoclasts were rarely seen in RAW264.7 cells without T cells, while this number was increased following addition of non-activated T cells. Osteoclast formation was further augmented by co-culture with con A activated T cells. Interestingly, the effect of T cell activation on TRAP+ cell number was reversed by incubating T cells with anti-resorptive concentrations of genistein (10^{-7} M), coumestrol (10^{-7} M) or daidzein (10^{-5} M) (Figure 6.2). Osteoclast formation in these cultures was reduced to that seen in cultures containing un-stimulated T cells.

To examine the role of TNF-α in the T cell mediated induction of osteoclast formation, cultures were also incubated with anti-human TNF-α antibody to block TNF activity. Neutralisation of TNF had no effect on osteoclast formation induced by un-stimulated T cells. However there was a significant reduction in TRAP+ cell number with anti TNF-α treatment in cultures containing con A activated T cells. Osteoclast formation in these cultures was reduced to that induced by un-stimulated cells. Addition of antibodies to PEs treated cultures did not further suppress osteoclast formation (Figure 6.2).

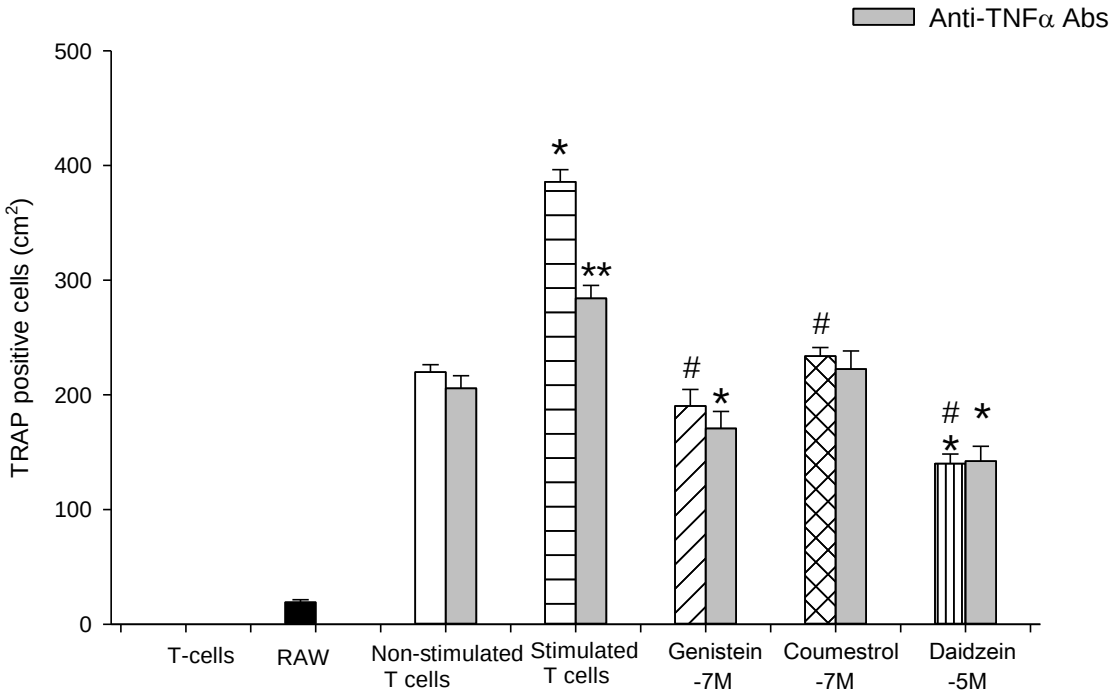

Figure 6.2. Genistein, coumestrol and daidzein blunt $TRAP^+$ cell formation induced by activated T cells in co-culture with RAW264.7 cells. T cells alone cultured in the presence or absence of conA and genistein (10^{-7} M), coumestrol (10^{-7} M) and daidzein (10^{-5}M) and incubated for 4 days. RAW264.7 cells ($2x10^4$) cultured in 96- well plates and Jurkat (T cells) at ($1x10^5$) cell/well added after treatment and incubated for 5 days. Anti-human TNF antibodies were used at (5 μg/ml) to neutralise TNF effect in co-culture experiment. Osteoclast formation was assessed by TRAP staining. Values are expressed as mean (± SEM) of three separate experiments. Differences between groups were assessed by one-way analysis of variance. * Values were significantly different versus non-stimulated T cells treated group $P < 0.05$. # $P< 0.05$ versus stimulated T cells treated group. ** $P<0.05$ versus corresponding group without anti-TNF antibody.

6.3.2 The effect of phytoestrogens on viable T cell number

An MTS assay was used to examine the effect of PEs on con A induced T cell proliferation. The results show that that genistein, coumestrol or daidzein can suppress T cell proliferation at relevant concentrations (Figure 6.3). Genistein at (10^{-5} M) decreased T cell proliferation, while coumestrol at all concentrations decreased T cell number. Daidzein decreased T cell proliferation at (10^{-5} M). This suggests that the anti-osteoclastic effects of daidzein and coumestrol noted in the previous study were at least in part due to an inhibition of viable T cell number. In contrast the anti-osteoclastic (10^{-7} M) concentration of genistein had no effect on cell number suggesting that it inhibits T cell induced osteoclast formation through an alternate mechanism.

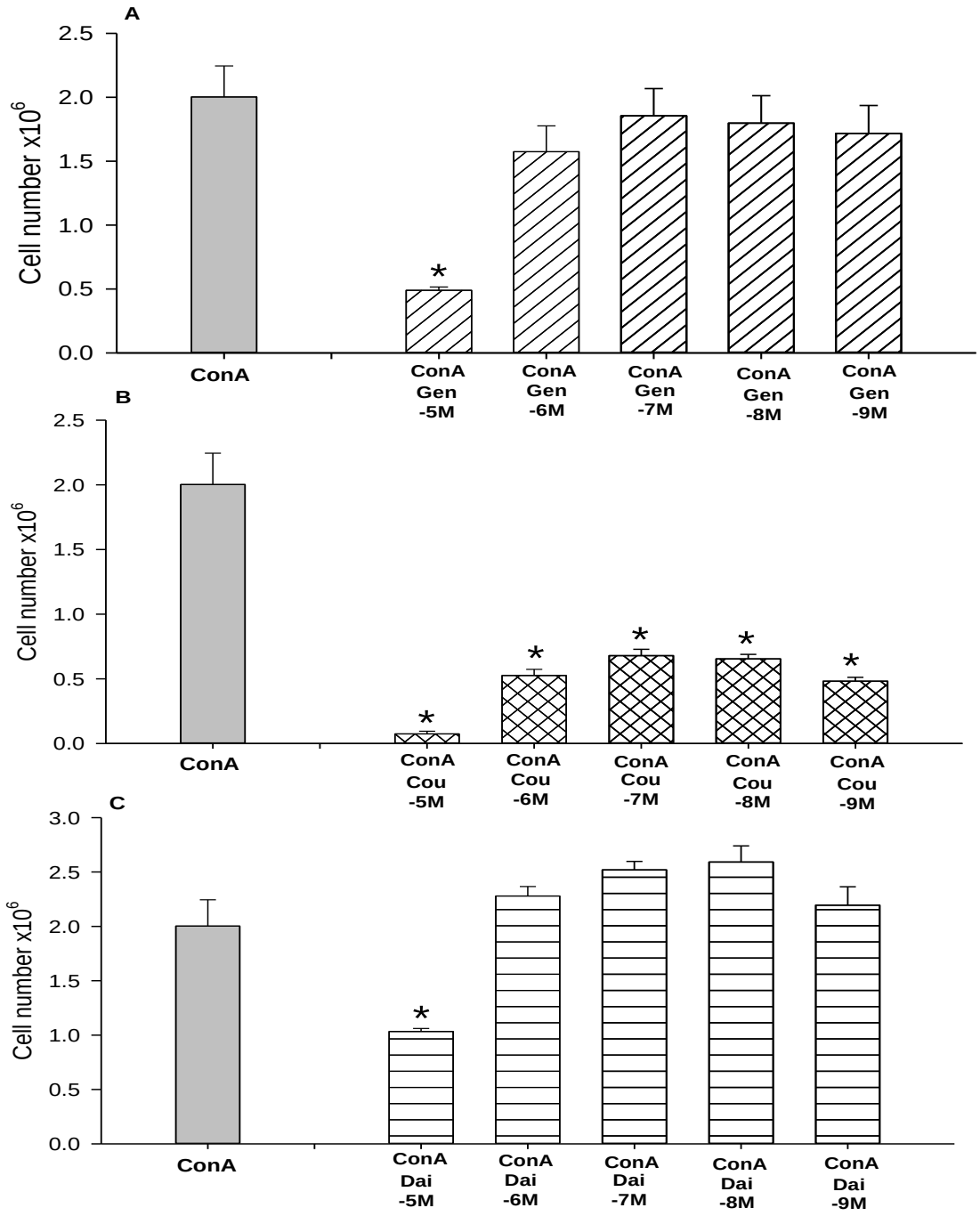

Figure 6.3. Genistein (A), coumestrol (B) and daidzein (C) decreased T cell proliferation. Jurkat E6.1 T cells were incubated in combination of conA (10μg/ml), genistein (10^{-5}-10^{-9} M), coumestrol (10^{-5}-10^{-9} M) or daidzein (10^{-5}-10^{-9} M) for 4 days. Proliferation was then assessed using MTS assays. Values are expressed as a mean (± SEM) from three experiments. Differences between groups were assessed by one-way analysis of variance. * $P<0.05$ versus con A treated group.

6.3.3 Effect of phytoestrogens on pro-osteoclastic cytokine expression

It is well established that T cells express a range of proinflammatory cytokines that may be responsible for inducing bone loss in post-menopausal women. In keeping with this, my study revealed that con A stimulated T-cells exhibited a 2.63 fold increase in TNF-α mRNA expression and this was prevented in the presence of PEs. Genistein (10^{-5}-10^{-9} M), daidzein (10^{-6}, 10^{-7} and 10^{-9} M) and coumestrol (10^{-6}-10^{-9} M) all significantly suppressed con A-induced TNF-α expression (Figure 6.4). In contrast to the predominantly suppressive effect of PE daidzein (10^{-5} M) augmented TNF-α production.

Similar effects of PEs were seen on IL-1β expression. T cell activation induced a significant 2.12 fold increase in IL-1β mRNA which was suppressed in the presence of genistein (10^{-5}-10^{-9} M), daidzein (10^{-6}-10^{-9} M) or coumestrol (10^{-6}-10^{-9} M) (Figure 6.5). The lowest concentrations of daidzein and coumestrol showed a trend towards a less potent action whereas similar to their effect on TNF-α expression the highest concentrations were unable to prevent the increase in T cell IL-1β production.

IL-6 expression was enhanced in activated T cells and this expression was significantly blunted by PEs treatment in a dose-dependent fashion. Genistein (10^{-5}, 10^{-6}, 10^{-8} and 10^{-9}M), daidzein (10^{-6}, 10^{-7}, 10^{-9} M) and coumestrol (10^{-7} and 10^{-9} M) decreased IL-6 expression. Once more, no suppressive effect of daidzein and coumestrol was seen at 10^{-5} M and in this case there was a significant 3.4 and 7.9 fold increase in IL-6 expression (Figure 6.6).

T cells express RANKL and this is reported to stimulate osteoclast formation in RA and E2 deficiency bone loss. Therefore, I examined the effect of PEs on RANKL expression in T cells. As shown in (Figure 6.7), RANKL mRNA expression did not differ in control and activated T cells. Copy number was also low in all groups; nonetheless expression was further reduced in the presence of genistein (10^{-6}-10^{-9} M) or daidzein

(10^{-6} M). Additionally, daidzein and coumestrol at (10^{-5} M) increased RANKL expression as compared to control and activated T cells. However RANKL copy numbers still remained relatively low which raises the question of whether any changes would lead to a significant physiological effect.

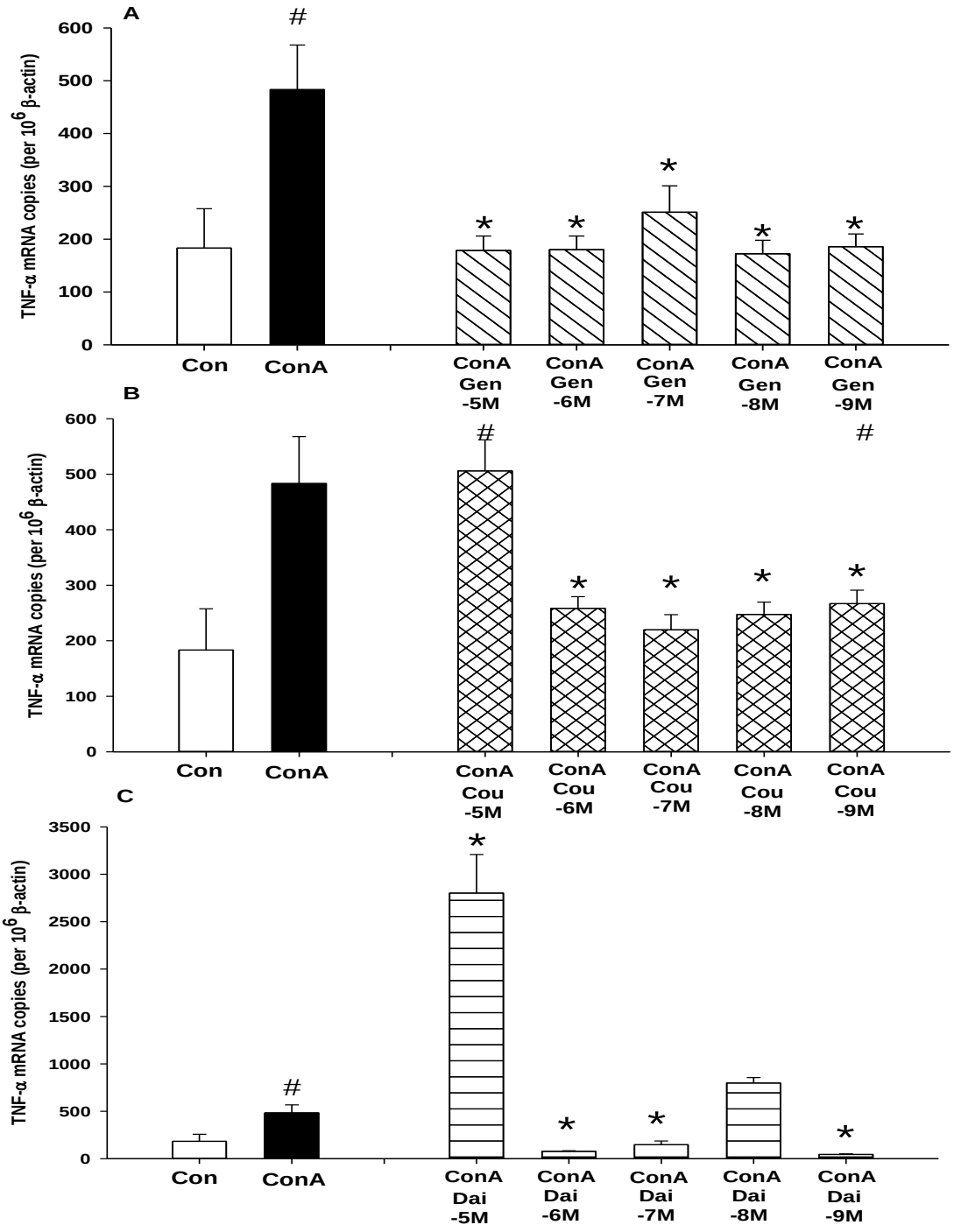

Figure 6.4. Genistein (A), coumestrol (B) and daidzein (C) reduced TNF-α expression in activated Jurkat T cells. Jurkat E6.1 T cells were incubated in combination of con A (10μg/ml), genistein (10^{-5}-10^{-9} M), coumestrol (10^{-5}-10^{-9} M) or daidzein (10^{-5}-10^{-9} M) for 4 days. The expression was assessed by quantitative real time PCR and data normalised to 10^6 copies of β-actin and expressed as mean (± SEM). * $P<0.05$ versus conA treated group, # $P<0.05$ versus control group.

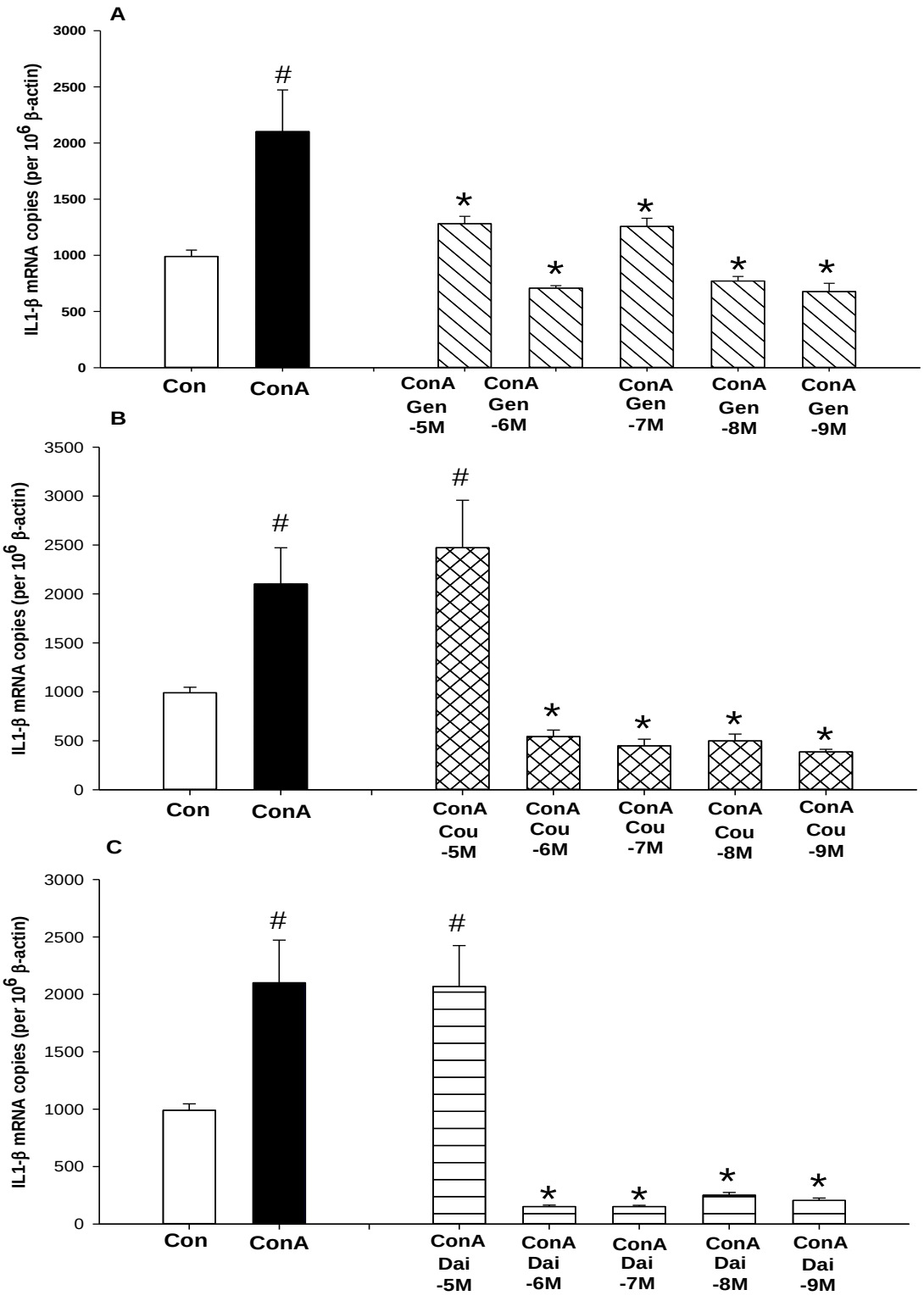

Figure 6.5. Genistein (A), coumestrol (B) and daidzein (C) blunted IL-1β expression in activated Jurkat T cells. Jurkat E6.1 T cells were incubated in combination of con A (10μg/ml), genistein (10^{-5}-10^{-9} M), coumestrol (10^{-5}-10^{-9} M) or daidzein (10^{-5}-10^{-9} M) for 4 days. The expression was assessed by quantitative real time PCR and data normalised to 10^6 copies of β-actin and expressed as mean ± SEM. * $P<0.05$ versus conA treated group, # $P<0.05$ versus control group.

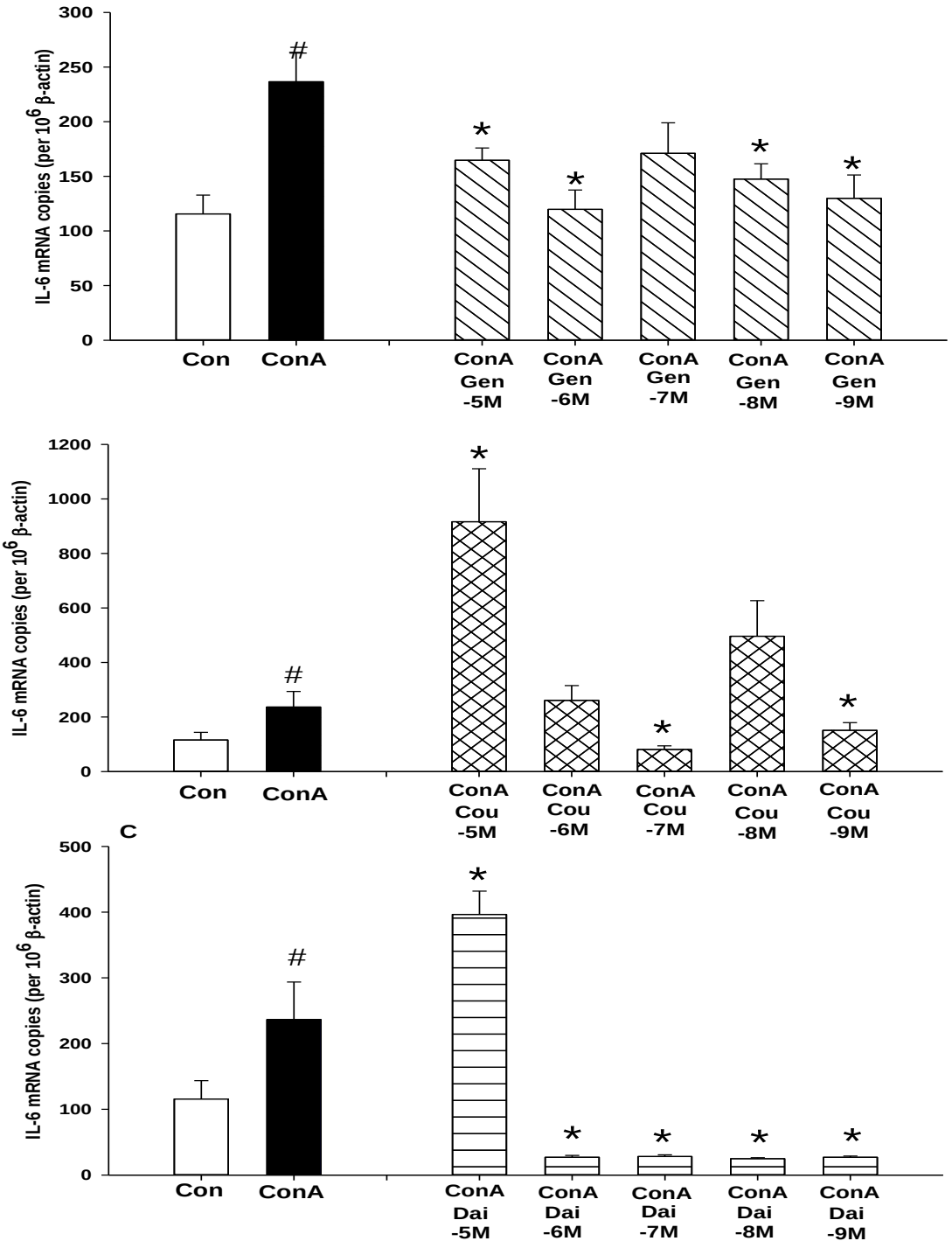

Figure 6.6. Genistein (A), coumestrol (B) and daidzein (C) reduced IL-6 expression in activated Jurkat T cells. Jurkat E6.1 T cells were incubated in combination of conA (10μg/ml), genistein (10^{-5}-10^{-9} M), coumestrol (10^{-5}-10^{-9} M) or daidzein (10^{-5}-10^{-9} M) for 4 days. The expression was assessed by quantitative real time PCR and data normalised to 10^6 copies of β-actin and expressed as mean (± SEM). * $P<0.05$ versus conA treated group, # $P<0.05$ versus control group.

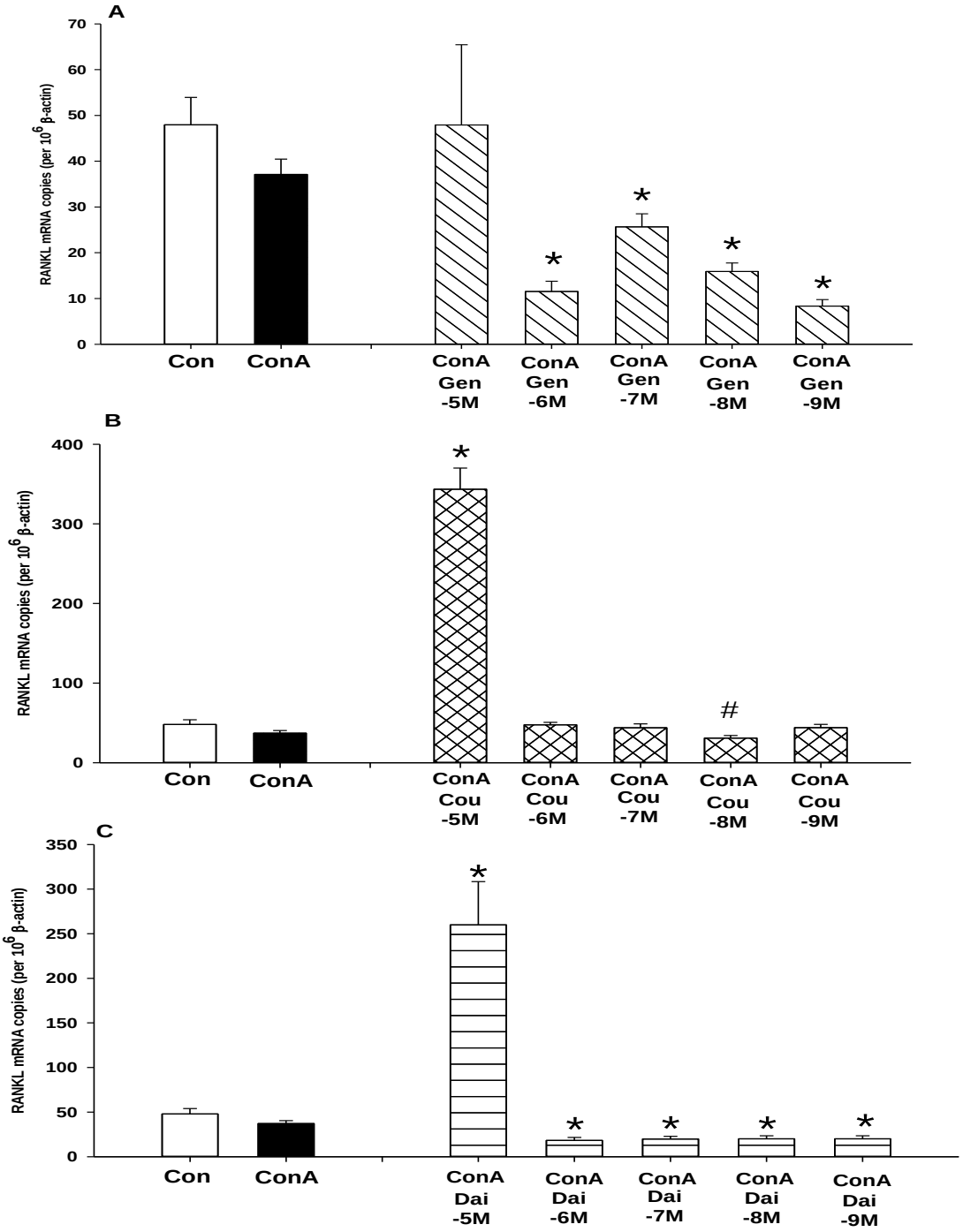

Figure 6.7. Genistein (A), coumestrol (B) and daidzein (C) inhibited RANKL expression in activated Jurkat T cells. Jurkat E6.1 T cells were incubated in combination of conA (10μg/ml), genistein (10^{-5}-10^{-9} M), coumestrol (10^{-5}-10^{-9} M) or daidzein (10^{-5}-10^{-9} M) for 4 days. The expression was assessed by quantitative real time PCR and data normalised to 10^6 copies of β-actin and expressed as mean ± SEM. * $P<0.05$ versus con A treated group, # $P<0.05$ versus control group.

6.4 Discussion

There is accumulating evidence indicating that bone resorption is modified by immune cells and their products (Pacifici, 2010). E2 deficiency augments bone resorption at least in part by up regulating proinflammatory cytokines production, whereas TNFα, IL-1β and IL-6 expression is suppressed by E2 in peripheral blood cells isolated from post-menopausal women (Rogers and Eastell, 2001). E2 deficiency accelerates the proliferation of T-cells including $CD4^+$ and $CD8^+$ (Roggia et al., 2001, Weitzmann and Pacifici, 2007) whereas HRT decreases $CD8^+$ T lymphocyte number (Kumru et al., 2004). T and B lymphocytes may also express RANKL in inflammatory diseases (Kawai et al., 2006).

The previous chapters showed that PEs have an inhibitory effect on osteoclast formation. However, this effect could be overridden if PEs promote T cell proliferation or augment pro-osteoclastic cytokine production. Therefore, this part of the study aimed to examine the effect of PEs on T cell-induced osteoclast formation, T cell number and pro-osteoclastic cytokine expression using Jurkat E6.1 T cells as a model. Co-culture of T lymphocytes with RAW264.7 cells enhanced osteoclast formation and con A stimulated T cells augmented this effect, similar to previous reports (Wyzga et al., 2004, Kawai et al., 2006). This augmentative action of activated T cells was significantly blunted by genistein (10^{-7} M), coumestrol (10^{-7} M) and daidzein (10^{-5} M).

The nature of the T cell derived stimulus promoting osteoclast formation in un-stimulated T cells is not known, but would not appear to be due to an effect of TNF-α, as neutralising TNF-α antibodies had no effect on osteoclast differentiation. This may result from the ability of un-stimulated cells to express IL-6 and low levels of RANKL which could augment osteoclast formation. However, RANKL expression was comparatively low in un-stimulated cultures and may not represent a major pro-

osteoclastic stimulus. Alternatively, T cells may promote the production of other pro-osteoclastic cytokines or up-regulate RANK expression in RAW cells themselves which could act in an autocrine manner to promote differentiation. Low levels of RANKL have also previously been shown to augment RAW cells M-CSF production leading to elevated RANK expression and augmentation of osteoclast formation (Islam et al., 2008). A further potential mechanism is that T cells may induce pro-osteoclastic cytokines expression in the monocytes themselves, as T cells have been suggested to induce macrophage TNF-α, IL-6 and IL-1β secretion in rheumatoid arthritis (Beech et al., 2006). The ability of T cells to modify monocytic cytokine synthesis may be dependent on a reciprocal interaction in which the monocytic induction of T cell CD40L expression promotes monocytic IL-6 and TNF expression (Li et al., 2011). This may be the case but is difficult to assess in the current co-culture experiments.

In contrast TNF-α appears to mediate the augmentative effect of con A as neutralising TNF-α antibodies significantly reduced the osteoclastogenic stimulus provided by activated T cells, although osteoclast formation did not completely return to that of un-stimulated T cell cultures. Con A also enhanced the expression of a range of other pro-osteoclastic inflammatory cytokines although their importance in the pro-osteoclastic action is secondary to that of TNF, as neutralising TNF-α antibodies restored osteoclast number to near un-stimulated levels. The change in cytokine profile is consistent with the results of Kawai et al. (2006), which showed that activated T lymphocytes expressed significantly greater levels of TNF-α, IL-1β and IL-6 followed by increases in the ability of T cells to induce osteoclast formation. Surprisingly no increase in RANKL expression was noted in my studies as T cell RANKL expression has been suggested to be one mechanism promoting osteoclastogenesis in inflammatory disorders (Horwood et al., 1999). The reason for this is unclear as RANKL expression is increased in Jurkart

cells by T cell activators such as bacterial exotoxins (Belibasakis et al., 2008). Thus the lack of RANKL expression in my studies may represent a limitation of con A activation in contrast to more physiological stimuli.

The suppressive effect of genistein (10^{-7} M), coumestrol (10^{-7} M) and daidzein (10^{-5} M) on T cell-induced osteoclast formation is mediated through separate cellular actions. Genistein at this concentration had little effect on T cell viability whereas it reduced con A stimulated TNF-α, IL-1β and RANKL expression. Higher concentrations (10^{-5} M) however decreased T cell number as reported previously (Yamasaki et al., 2007), which is thought to be mediated through an ability to cause mitochondrial damage (Baxa et al., 2005), although this concentration was not examined in the co-cultures due to its previously demonstrated direct stimulatory effect on TNF-α-induced osteoclast formation. In light of the significant effect of anti-TNF-α antibodies on osteoclastogenesis, it is likely that the ability of genistein to reduce TNF-α expression represents the most important aspect of this effect, although this does not preclude a further contribution from decreased IL-6 or RANKL expression, as genistein treated cultures had fewer osteoclast than co-cultures of activated T cells administered TNF-α antibodies. On the other hand, the anti-osteoclastic action of coumestrol may arise from a combination of decreased cell viability and reduced TNF-α, IL-6 and IL-1β expression although surprisingly no greater effect on osteoclast formation was noted in comparison to genistein. In contrast the anti-osteoclastic effect of daidzein was mediated through a significant decrease in the number of viable T cells rather than suppression of cytokine production, as daidzein augmented TNF-α, IL-6 and RANKL expression. This increase in pro-osteoclastic cytokines expression however did not compensate for the decrease in T cell number, an assertion that is supported by the inability of anti TNF-α antibodies to further decrease osteoclast formation in these cultures. Thus, while individual T cells

may synthesise more TNF-α, the net concentration generated is lower due to the smaller pool of viable T cells. Similar effects of daidzein on immune cell number and cytokines levels have previously been reported; daidzein suppressed ovariectomy-induced increases in T lymphocyte number, which was associated with lower TNF-α expression. This study found that daidzein reduced TNF-α expression through effects on CD4+ CD28null T cell proliferation (Tyagi et al., 2011). Similarly ovariectomy-induced RANKL expression has been shown to be blunted in the presence of daidzein (Tyagi et al., 2011). The mechanism thorough which PEs reduce cytokines expression is not known and could potentially be meditated through several effects. It may be ER dependent or alternatively it could be caused by the antioxidant action of PEs which could modify the redox status and inhibit TNF-α secretion. The effect of genistein could also be mediated through its ability to suppress tyrosine kinase signalling which plays an important role in inflammation (Duan et al., 2003).

In conclusion, the data shows an inhibitory effect of genistein, coumestrol and daidzein on activated T cell-induced osteoclast formation. This effect is observed at concentrations shown to directly inhibit osteoclast formation and promote bone matrix formation in previous chapters. This provides evidence that a further mechanism through which PEs may inhibit bone loss *in-vivo* is via a reduction in T lymphocyte driven remodelling. PEs could therefore prove useful in the attenuation of bone destruction in not only post-menopausal osteoporosis but also in other inflammatory disorders such as rheumatoid arthritis.

Chapter Seven : General Discussion

7.1 Discussion

Bone is a dynamic tissue that is constantly remodelled in response to changes in mechanical loading, serum calcium and micro-damage. Bone remodelling is dependent on the co-ordinated activity of osteoclasts, osteoblasts and osteocytes. During a normal remodelling cycle local and systematic factors orchestrate a balance between bone formation and resorption, such that there is little net loss of bone mass (Nanes, 2003, Kwak et al., 2005, Weitzmann and Pacifici, 2005). This balance is disrupted in many skeletal diseases including post-menopausal osteoporosis where a fall in circulating E2 is associated with decreased bone mass and elevated fracture risk. The fall in E2 generates an imbalance between bone formation and resorption; such that there is an increased loss of bone during each remodelling cycle. E2 has been shown to regulate bone remodelling through several mechanisms, it can directly modify osteoclast life-span and via the modification of cytokines production by osteoblasts and immune cells it can stimulate osteoclast formation and activity (Pfeilschifter et al., 2002, Weitzmann and Pacifici, 2006a).

Several anti-resorptive therapies have been developed to try and limit bone resorption in post-menopausal women. These include HRT, however following the publication of large scale studies indicating that HRT is associated with a greater risk of developing cancer and cardiovascular disease HRT is not now typically prescribed for post-menopausal osteoporosis (Chlebowski et al., 2010, Chlebowski et al., 2003). Alternatives to HRT have been sought and several publications suggest that PEs could have a beneficial effect on bone mass with fewer side effects. Asian populations whose diet is rich in PEs in comparison to Western populations have a lower incidence of fractures, breast cancer and cardiovascular disease (Morton et al., 2002, Peeters et al., 2003). Consumption of PEs has been shown to improve bone density in animal models

(Lee and Choi, 2005, Yuebin et al., 2006, Uchiyama and Yamaguchi, 2007). These positive actions have been postulated to be mediated via effects on both osteoclast and osteoblasts activity. However the molecular mechanism through which PEs act is not clear, similarly the mechanism through which a fall in circulating E2 promotes osteoclast formation is still open to debate. One potential mechanism is increased TNF-α production, TNF-α has been shown to induce bone loss associated with ovariectomy through direct stimulation of osteoclast formation and indirectly by regulating the production of various pro-inflammatory cytokines (Kimble *et al.*, 1997). Despite the central role of TNF-α in menopausal bone loss no study had examined the effect of PEs on TNF-α-induced osteoclast formation, therefore this study aimed to examine this and to determine the cellular mechanism by which PEs may modify TNF-α-induced bone resorption.

Chapter four's results provide new evidence that PEs directly suppress TNF-α-induced osteoclast differentiation via inhibition of *c-fos*-dependent NFATc1 expression. The mechanism leading to a reduction in *c-fos* transcription is uncertain, but this could arise as a consequence of a direct ER mediated effect on the *c-fos* gene or indirectly via modification of an upstream regulator. The PE-induced reduction in *c-fos* would in turn be expected to prevent NFATc1 reaching levels sufficient to enable osteoclast differentiation, maintaining precursors in a non-committed state or allowing them to differentiate towards alternative macrophage lineages. Furthermore, PEs also inhibited *DC-STAMP* expression, an essential regulator of mononuclear osteoclast aggregation and cell fusion. PEs also decreased *NFkB* expression which may also effect osteoclast differentiation signalling. On the other hand in contrast to some earlier studies PEs had no effect on osteoclast viability. These results assert the positive relationship between PE administration and a reduction in bone resorption, and also provide new evidence

that PEs in addition to suppressing RANKL-induced osteoclast formation also blunt TNF-α-induced osteoclast formation. This inhibitory effect is mediated via an action on the differentiation pathways promoting the formation and fusion of osteoclast precursors rather than modifying the pool of precursors available to form osteoclast or reducing the life span of mature osteoclast themselves. This anti-osteoclastic effect was seen at concentrations of genistein and coumestrol generated by consuming a diet containing high soy content, whereas to achieve effective daidzein levels would require dietary supplementation. This may make geneistien and coumestrol a more attractive strategy than daidzein.

In addition to the direct inhibitory effect of PE on osteoclast formation PEs may also indirectly reduce the osteoblastsic stimulus for resorption. I found that genistein and daidzein in the presence of Zn^{2+} suppressed osteoblastsic RANKL/OPG ratios and may therefore reduce osteoclast differentiation. In contrast to PEs Zn^{2+} had no direct effect on TNF-α-induced osteoclast formation and did not modify the direct suppressive action of PEs on osteoclastogenesis.

Osteoblast and osteoclast activity is tightly coupled during each remodelling cycle, such that bone resorption is spatially linked to a subsequent formative phase. This coupling is lost in post-menopausal women, which contributes to the bone loss during both the initial and later stages of the disorder. An ideal therapy would rectify this imbalance preventing excessive bone loss during each cycle; furthermore an ability to augment formation beyond normal levels could restore bone that had already been lost. Thus the effect of a therapy on osteoblasts activity is arguably as important as its action on osteoclast function; therefore this study examined the effect of PEs on osteoblast differentiation and bone matrix formation. The results of chapter five indicate that PEs stimulate osteoblast differentiation; increasing ALP activity and enhancing organic and

non-organic bone matrix formation including hydroxyapatatite formation and *type I collagen* and *osteocalcin* mRNA expression. Thus, PEs directly increase bone formation *in-vitro*. An ability to decrease resorption and at the same have the capacity to enhance bone formation *in-vivo* would be beneficial in maintaining or restoring bone after E2 deficiency. The effect of PEs on osteoblast and osteoclast activity appear to be mediated through an ER dependent mechanism as both actions were abolished in the presence of the E antagonist ICI 182,780.

Several studies suggest that other nutritional factors such as vitamin D, Zn^{2+} and calcium may reduce the effect of E2 deficiency (Li et al., 2011, Uchiyama and Yamaguchi, 2007). Zn^{2+} plays an important role in supporting osteoclast and osteoblast function; Zn^{2+} has been shown to inhibit osteoclast formation whereas Zn^{2+} deficiency is associated with reduced osteocalcin and type I collagen expression (Sun et al., 2011). However no study has examined if combinations of PEs and Zn^{2+} could improve bone formation; thus this study examined if Zn^{2+} and PEs could improve osteoblast differentiation and bone nodule formation. It was found that Zn^{2+} augmented the stimulatory effect of PEs on osteoblast differentiation and bone nodule formation, while no substantial effect of these factors on osteoblasts proliferation was noted. Furthermore, PEs and Zn^{2+} enhanced *Runx2* expression, a transcriptional factor responsible for regulating the initial aspects of osteoblast differentiation. However, Zn^{2+} blunted the positive action of PEs on t*ype I collagen* and *osteocalcin* expression although levels were still significantly above control. This suggests that the stimulatory effect of Zn^{2+} is predominantly mediated at the initial stages of osteoclast differentiation, increasing *Runx2* and augmenting the early stages of bone formation. These findings assert that zinc has an additive effect on the stimulatory effect of PEs on osteoblast differentiation and bone matrix formation. Thus, appropriate combination of PEs and zinc may

increase bone formation *in-vivo*. The concentrations of PE shown to have this anabolic effect were similar to those that inhibited osteoclast formation and therefore the generation of these concentrations *in-vivo* would appear to be potentially desirable.

Aberrant T cell number and TNF-α production is central to the pathogenesis of E2 dependent bone loss (Tyagi et al., 2012, Weitzmann and Pacifici, 2006b). The impact of PEs on T cell formation and activity has not been extensively studied. If PEs stimulate T cell proliferation or promote cytokine production then this could antagonise their inhibitory effect on osteoclast differentiation. I therefore examined the effect of PEs on T cell function. All PEs prevented the augmentative effect of activated T cells on osteoclast formation. However their mechanism of action varied, genistein reduced con A stimulated TNF-α, IL-1 and RANKL expression, coumestrol decreased cell viability and cytokine expression whereas the inhibitory effect of daidzein was mediated via suppression of viable T cell number. This likely reflects differences in the mechanism of action of these compounds or differing affinity for ER. However, the concentrations of genistein, coumestrol and daidzein displaying these inhibitory actions were similar to those that reduced osteoclast and increased osteoblast differentiation. This further suggests that concentrations of genistein and coumestrol that can be achieved by consuming a soy-rich diet could have a beneficial effect on bone cell activity through multiple actions. In contrast there would be insufficient levels of daidzein from the diet to generate concentrations able to modify osteoblast, osteoclast or T cell activity and therefore supplements would have to be taken.

While this *in-vitro* data suggests that dietary interventions may be an alternative strategy further *in-vivo* data is needed to clarify their beneficial effect on bone cell activity and immune status in animal models and humans. In light of their lower affinity for ER it may be the case that PEs exerts a weaker effect on bone metabolism in

comparison to E2, however this may be sufficient during the early stage of E2 deficiency-dependent osteoporosis to reduce the rate of bone loss and prolong the time before bone mineral density falls into the osteoporotic window. In addition *in-vivo* experiments would enable the study of routes of administration, effective concentrations, and the safety of long term usage. For instance PEs may modify endogenous sex hormone production or impact on the progression of ER positive tumours.

In conclusion this study provides novel and important results regarding the mechanisms by which PEs regulate bone cell activity and suggest that PE-rich diets may aid in the treatment of hormone-dependent disease. They directly inhibit the pro-osteoclastic stimulus provided by T cells, directly and indirectly inhibit osteoclast formation and also have a direct stimulatory effect on osteoblast formation. If similar effects are elicited *in-vivo* this would be expected to have a beneficial effect on skeletal health in post-menopausal osteoporosis and osteolytic inflammatory disorders such as rheumatoid arthritis.

7.2 Future studies

Several areas still need further investigation to clarify the precise effect of PEs and zinc on bone cell activity. PEs have multiple actions and thus the understanding of their potential beneficial effect is far from complete. *In-vivo* mouse and rat models of bone loss and remodelling would greatly increase the robustness of the data. They would also enable the further refinement of doses necessary to modify osteoblast and osteoclast activity during normal and inflammatory-induced remodelling. It is important to choose suitable routes of administration and also enable control of the doses administered; therefore these studies would use oral gavage as a route of administration. This data would also provide a starting point for initial concentrations ranges for human studies.

The results of this study showed that PEs exert their agonist effects in part through an ER-dependent mechanism. PEs differ in their ER binding affinity, therefore the assessment of relative binding affinity with the ligand-ER binding and ER-promoter binding assays will provide information about binding affinity for ERα and ERβ. In addition, utilising ERα and ERβ-knockout mice will give crucial information about the contribution of ERα and ERβ to the regulatory action *in-vivo*. Moreover, measurement of serum levels of PEs and other steroidal hormones in animals after controlled administration will give insights about the daily amounts required to generate serum PEs concentrations in humans relevant to their biological actions noted in this study. However, the bioavailability of these compounds in humans varies between individuals, which pose a further challenge for testing the activity of PEs *in-vivo*. For instance, many PEs are metabolised *in-vivo* by intestinal bacteria, for instance daidzein is metabolised to equol and O-desmethylangolesin which has a greater oestrogenic activity than daidzein. The formation of these metabolites depends on the presence of these bacteria in the gut flora and therefore the ability of individuals to metabolise PEs should incorporated into any investigation. In addition, the determination of risks and other side effects which may occur with treatment will provide information on safety profiles.

Several minerals and vitamins are describing as having a positive effect on bone. This study found that Zn^{2+} had an additive effect on PE-augmented osteoblast differentiation and mineralisation while having no detrimental action on osteoclast formation. Therefore, it would be interesting to see if combinations of these factors were able to blunt the loss of bone in mouse modules of ovariectomy-induced resorption. Effects on osteoclast and osteoblast formation and activity could be ascertained using dynamic and static histomorphometry and the mechanical competence of long bones assessed by measuring ash content and mechanical failure properties.

In addition to Zn^{2+} several other vitamins such as vitamin K2 have a beneficial effect on bone formation and resorption. Investigation of combinations of these factors would be of interest to establish further beneficial effects on remodelling and immune cell activity. In addition many PEs-rich foods contain more than PE, and thus investigation of the effect of combined PEs compound on bone cells differentiation and activity *in-vitro* and *in-vivo* would be useful.

The immune system has an important role in post-menopausal and inflammatory bone loss and the current studies found that PEs reduced pro-osteoclastic cytokines expressed by T cells such as TNF-α. Therefore it would be interesting to see the effect of PEs in the presence or absence of Zn^{2+} or vitamins on circulating and local levels of inflammatory cytokine production in animal models of inflammatory disease such as collagen-induced arthritis and bacterial osteomyelitis. This could then be related to changes in osteoclast and osteoblast activity to identify relationships between cytokine levels and bone cell activity to determine the contribution of direct and indirect PE actions. Ultimately, this could be resolved using T cell deficient mouse models such as nude mice, which lack all circulating T cells; this would enable the direct comparison of the response to PE in with or without T cell inputs.

In conclusion, further experiments to test for the potential beneficial effect of PEs on bone and immune cells are needed to understand the precise effect of these compounds on bone health, establish effective concentration ranges *in-vivo* and determine any risks associated with long-term use.

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Appendix

ARTICLE
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Phytoestrogens Directly Inhibit TNF-α-Induced Bone Resorption in RAW264.7 Cells by Suppressing *c-fos*-Induced *NFATc1* Expression

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ABSTRACT

TNF-α-induced osteoclastogenesis is central to post-menopausal and inflammatory bone loss, however, the effect of phytoestrogens on TNF-α-induced bone resorption has not been studied. The phytoestrogens genistein, daidzein, and coumestrol directly suppressed TNF-α-induced osteoclastogenesis and bone resorption. TRAP positive osteoclast formation and resorption area were significantly reduced by genistein (10^{-7} M), daidzein (10^{-5} M), and coumestrol (10^{-7} M), which was prevented by the estrogen antagonist ICI 182,780. TRAP expression in mature TNF-α-induced osteoclasts was also significantly reduced by these phytoestrogen concentrations. In addition, in the presence of ICI 182,780 genistein and coumestrol (10^{-5}–10^{-6} M) augmented TNF-α-induced osteoclast formation and resorption. However, this effect was not observed in the absence of estrogen antagonist indicating that genistein's and coumestrol's ER-dependent anti-osteoclastic action normally negates this pro-osteoclastic effect. To determine the mechanism mediating the anti-osteoclastic action we examined the effect of genistein, coumestrol, and daidzein on caspase 3/7 activity, cell viability and expression of key genes regulating osteoclast differentiation and fusion. While anti-osteoclastic phytoestrogen concentrations had no effect on caspase 3/7 activity or cell viability they did significantly reduce TNF-α-induced *c-fos* and *NFATc1* expression in an ER dependent manner and also inhibited NFATc1 nuclear translocation. Significant decreases in *NFκB* and *DC-STAMP* levels were also noted. Interestingly, constitutive *c-fos* expression prevented the anti-osteoclastic action of phytoestrogens on differentiation, resorption and *NFATc1*. This suggests that phytoestrogens suppress TNF-α-induced osteoclastogenesis via inhibition of *c-fos*-dependent NFATc1 expression. Our data provides further evidence that phytoestrogens have a potential role in the treatment of post-menopausal and inflammatory bone loss directly inhibiting TNF-α-induced resorption. J. Cell. Biochem. 112: 476–487, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: TNF-α; OSTEOCLAST; PHYTOESTROGENS; NFATc1

Post-menopausal osteoporosis is characterized by low bone mass and increased fracture risk. Worldwide, osteoporotic fractures are a major health concern especially in countries with aging populations. Prior to menopause osteoblastic bone formation and osteoclastic bone resorption are balanced such that there is little net bone loss during each remodeling cycle. Estrogen deficiency disrupts this equilibrium increasing bone turnover and skewing remodeling in favor of resorption. Recent evidence suggests that this is at least in part due to elevated levels of the potent pro-inflammatory cytokine TNF-α [Weitzmann and Pacifici, 2007]. Several studies show increased TNF-α production by bone marrow and T cells following estrogen deficiency in mice [Cenci et al., 2000; Grassi et al., 2007]. Furthermore, mice lacking T cells or TNF-α receptors are resistant to ovariectomy-induced bone loss [Roggia et al., 2001]. Human peripheral blood mononuclear cells (PBMC) also display estrogen dependent changes in TNF-α production; PBMC from post-menopausal or oophorectomised individuals synthesize elevated levels of TNF-α [D'Amelio et al., 2004], whereas estrogen replacement suppresses PBMC TNF-α production in-vivo [Bernard-Poenaru et al., 2001]. In addition to promoting post-menopausal bone loss TNF-α is also a key regulator of osteolysis associated with chronic inflammatory conditions such as rheumatoid arthritis [Boyce et al., 2005]. TNF-α promotes osteoclast differentiation by several actions, it augments receptor activator of NFκB ligand (RANKL) induced osteoclastogenesis and also directly stimulates osteoclast formation from human or mouse monocytes [Komine et al., 2001; Fox et al., 2008] and has been suggested to activate osteoclast formation independent of RANKL signaling [Kudo et al., 2002]. Thus, TNF-α has a central role in post-menopausal bone loss directly promoting

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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

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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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