CHAPTER II

REVIEW OF LITERATURE

Menopause

According to the World Health Organization (WHO), osteoporosis is the second leading health problem in the developed world in terms of numbers, owing to the ageing of the western female population (Anonymous, 1994). Ovarian hormone deficiency is a major risk factor for osteoporosis. A sharp decrease in ovarian estrogen production is the predominant cause of rapid bone loss during the first decade after menopause. The incidence of fractures is related to the age of the population, and to skeletal and extra-skeletal factors. Epidemiology studies suggested that the low incidence of osteoporosis and heart disease in postmenopausal Asian women compared to American women is attributable to their higher intake of soybean-base foods (Anderson and Garner, 1997).

Bone

Bone is a mineralized tissue composed of several cell types which is continuously undergoing a process of renewer and repair termed ' bone remodelling' (Figure 2.1). The two major cell types responsible for bone remodelling are osteoclasts, which is resorb bone, and osteoblast which is form new bone.

Osteoclasts are multinucleated cells of haemopoietic origin, which differentiate from precursors in the monocyte/macrophage lineage, in response to co-ordinated expression of lineage-specific regulatory molecules including c-fos, M-CSF (macrophage colony-stimulating factor), RANK (receptor activator of NFkB), RANK ligand (RANKL) and osteoprotegerin (OPG)(Gildeon *et al.*, 2000 and Teitelbaum, 2000).

Osteoblast, on the other hand, are cells of mesenchymal origin which differentiate from marrow stromal cells in response to activation of the transcription factor Cbfa1 (Macdonald *et al.*, 1987 and Patricia *et al.*, 2000). During the bone remodelling cycle, old or damaged bone is removed by osteoclasts, by the secretion of acid and proteolytic enzymes onto the bone surface. Subsequently the osteoclast migrate away from the area of bone undergoing resorption and undergo apoptosis. They are replace by osteoblasts, which lay down new bone matrix in the form of osteoid becomes calcified to form mature bone. During bone formation, some osteoblasts become embedded within the bone matrix, and differentiate into osteocytes, a third cell type unique to bone.

Osteocytes interconnect with one another and with cells on the bone surface through a series of long cytoplasmic processes that run through cannaliculi in the bone matrix. It is thought that osteocytes act as sensors of mechanical stress in the skeletal, by detecting and responding to changes in fluid flow which run through cannaliculi in bone.

Bone remodeling

Bone remodeling is a dynamic, lifelong process in which old bone was removed from the skeletal and new bone is added. It consists of two distinct stages, resorption and formation that involve the activity of special cells called osteoclast and osteoblast. Usually, the removal and formation of bone are in balance and maintain skeletal strength and integrity (Figure 2.1).

Once the osteoblast is activated, cytokine synthesis and secretion result in recruitment and differentiation of osteoclasts at the remodeling surface. These specialized bone-resorbing cells secrete protons and proteases that dissolve the mineral matrix and break down collagen. Matrix-bound growth factors are released by this process, and these factors couple osteoblastic activity to osteoclastic resorption. The remodeling cycle is complete when bone mass is restored (Manolagas and Jilka , 1995).

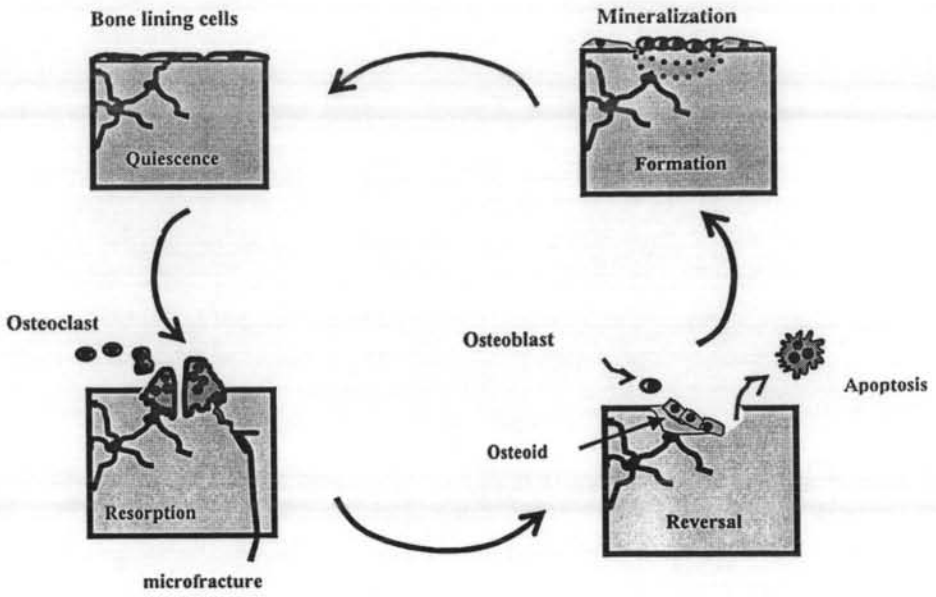

Figure 2.1 The bone remodeling cycle. The bone remodeling cycle of resorption, reversal and bone formation is responsible for renewal and repair of old and damaged bone. (Modified from Van't Hof and Ralston, 2001)

Bone remodeling is regulated by several systemic hormones such as parathyroid hormone (PTH), 1,25 dihydroxyvitamin D3, sex hormones and calcitonin, as well as by local factors such as NO, prostaglandins, growth factors and cytokines (Rob *et al.*, 2001). It is currently believed that many of the factors which regulate bone remodeling by influencing local expression of RANK, RANKL and OPG, (Lacey *et al.*, 1998) which together form a paracrine system that plays an essential role in regulating osteoclast differentiation and function (Figure 2.2) Osteoclasts differentiate from haemopoetic precursors in the monocyte lineage in response to activation of RANK, by its ligand RANKL, expressed on stromal cells. This reaction is blocked by OPG which act as a decoy receptor for RANKL. Osteoblasts differentiate from mesenchymal precursors in bone marrow in response to activation of the osteoblast-specific transcription factor Cbfa 1 (Roux and Orcel, 2000).

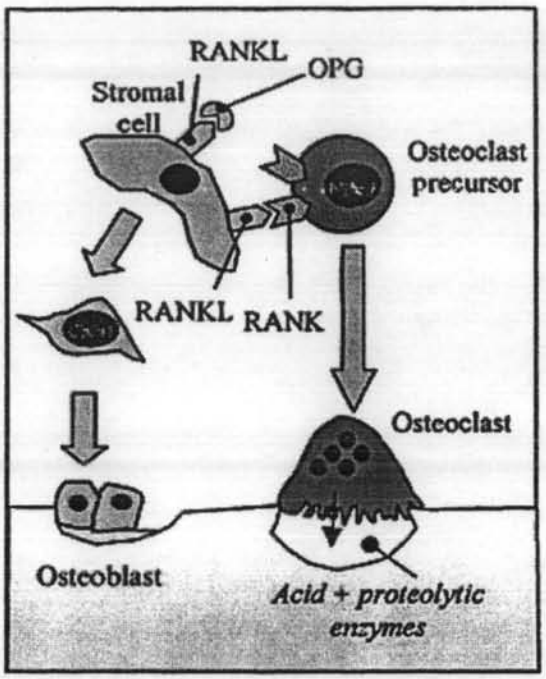

Figure 2.2 Osteoclast differentiate from mesenchymal precursor in bone marrow. (Roux and Orcel, 2000.)

Bone remodeling markers

During bone remodeling, osteoblasts synthesize numerous peptides, growth factors, and cytokines essential for coupling bone resorption to formation. Bone-specific proteins (eg, osteocalcin, bone-specific alkaline phosphatase, procollagen-I extension peptide) are synthesized by mature osteoblasts and find their way from the skeleton into the circulation and it has been shown to correlate with the bone turnover rate.

Osteocalcin is one of the most abundant non-collagenous proteins of bone matrix, synthesized by osteoblasts. The serum concentration of osteocalcin reflects the rate of osteoblast synthesis of osteocalcin. However, only approximately 50% of newly synthesized osteocalcin is released into circulation while the remaining 50% is incorporated into hydroxyapatite. Osteocalcin contains three γ -carboxylated glutamic acid residues and the degree of carboxylation (i.e. number of residules carboxylated) seem to influence mineralization. Serum osteocalcin concentration has correlated with both active bone formation and resorption. Taken the other finding together, the obvious elevation of osteocalcin is directly related with the active bone formation. These peptides

can be measured by sensitive radioimmunoassays or enzyme-linked immunosorbent assays.

Alkaline phosphatase (ALP) is an enzyme that is also expressed by osteoblasts and is another marker of osteoblast activity. ALP is a membrane-bound protein with enzymatic activity expressed by liver, bone intestine, kidney and placenta. Two major circulating informs of tissue alkaline phosphatase, the liver isoenzyme and the bone isoenzyme, are posttranslationally different in their glycosylation pattern. In bone, alkaline phosphatase anchored to the outer plasma membrane of osteoblast by a glycan linkage to phosphatidyl-inositol. Several possible roles for alkaline phosphatase in bone formation have been proposed. The enzyme hydrolyzes phosphate-ester and provide supplemental phosphate for deposition in hydroxyapatite, destroys local inhibitors of mineral crystal growth, or acts as a calcium-binding protein or Ca^{2+} ATPase. Skeletal alkaline phosphatase can be released to circulation by action of a glycan-inositol phosphase specific hydrolase which cleaves the molecule at the inositol-PO_4 linkage site. Its level can increase as a consequence of increased release from bone cells during the process of bone formation.

Similarly, osteoclasts induce bone degradation, releasing skeletal-specific matrix products into the interstitium. These products enter the circulation and often clear the kidney without being metabolized. These fragments include collagen cross-links, which are small amino acids that bridge collagen fibrils and add support to the tertiary collagen structure. Cross-links are added during the final stages of collagen synthesis and are the first segments to be hydrolyzed by proteases during bone resorption (Rosen, 1996).

Thus, bone remodeling can be assessed by the measurement of biomarkers of bone turnover in the blood or urine. The level of these markers may identify changes in bone remodeling within a relatively short time interval (several days to months) before changes in bone mineral density can be detected.

Osteoporosis

Osteoporosis is characterized by a disturbance in the relationship of resorption to synthesis, so that too much bone is resorbed. This results in bone loss and skeletal fragility. The increasing of bone turnover cause of remodeling imbalance, in which the amount of bone formed within individual remodeling units is less than that resorbed due either to an increase in resorption, decrease in formation, or these are increased concomitantly (Figure 2.3). The most common cause of osteoporosis in women is the decrease in estrogen that accompanies menopause. Estrogen loss is associated with evaluated bone resorption caused by rise in osteoclast number, which is driven by increase in the cytokines that regulate osteoclast generation as follows: RANK (receptor for activator of nuclear factor-kB) ligand; TNF-α (tumor necrosis factor–α); interleukin-1 (IL-1), IL-6, IL-11, M-CSF (macrophage-colony stimulating factor); and prostaglandin E (Pacifici R., 1998). RANK ligand (RANKL), its receptor RANK, and a neutralizing soluble receptor that blocks RANK activity called osteoprotegerin (OPG) have been shown to fully control osteoclast formation in mice (Teitelbaum, 2000). Production of all of these cytokines is either directly or indirectly suppressed or regulated by estrogen.

These mechanisms of bone loss can be quantitatively assessed using histomor - phometric techniques. The administration of two, time-spaced doses of a tetracycline compound before bone biopsy enables identification of actively forming bone surfaces (Frost, 1969) and calculation of bone turnover and activation frequency. The amounts of bone formed and resorbed within individual bone remodeling units can also be measured; the former is known as the wall width (Darby and Meunier,1981) and is a measure of osteoblast function.

Diagnosis of osteoporosis is also based on physical method, such as Dual emission x-ray absorptionmetry, ultrasonography, bone ash and on biochemical markers.

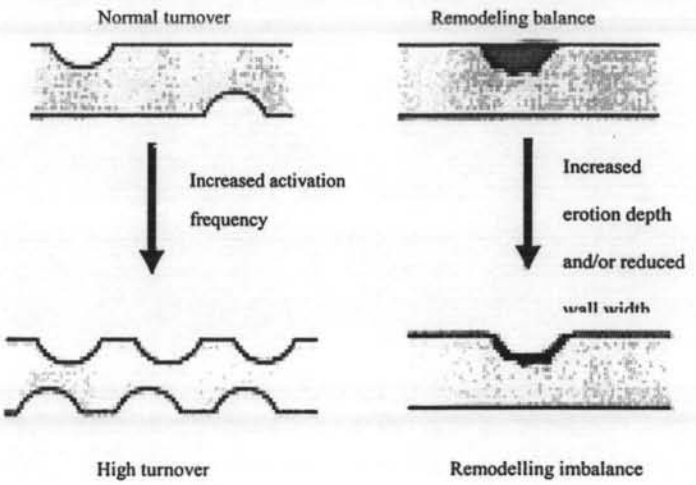

Figure 2.3 Mechanisms of bone loss in osteoporosis. (From Compston, The skeletal effects of estrogen depletion and replacement: histomorphometry studies. In: *Annual Review of the Management of Menopause*, edited by Studd J. Carnforth, Lancs, UK: Parthenon, 2000, 287-296.)

Bone circulation

Blood flow within bones is unique in two ways: the blood circulate within the close cavity in which pressure must remain constant, a feat achieved in part thank to the considerable distensibility of the intraosseous vessels and, above all, veins; the intraosseous circulation allows traffic of minerals between the blood and bone tissue and send the blood cells produced within the bone marrow into the systemic circulation. In contrast, the arterioles and capillary within bones have the same anatomic structure as those lacated elsewhere in the body and are susceptible to arteriosclerosis, arthritis or thrombosis. The mechanisms that regulate blood flow within bone are incompletely understood, probably because they are difficult to study in vivo. The cytokine and growth factor that regulate intraosseous angiogenesis also regulate bone remodeling, and close link exist between the blood supply to bone and bone formation and resorption: most diseases characterized by increased bone resorption are associated with increased

bone vascularization, whereas arteriosclerosis may contribute to the development of osteoporosis (Michel Laroch, 2002).

Angiogenesis and bone

Angiogenesis is an essential component of bone development and repair. During endochondral ossification in late embryonic development and in rapid post natal growth, a major event is the invasion of the cartilage of the growth plate region of long bones with new capillaries from existing blood vessels (Gerber and Ferrara, 2000). After growth plate closer in adults, the angiogenic switch can transiently be turned on during bone remodeling in response to fracture or other pathological conditions such as osteoarthritis. Administration of angiogenesis inhibitor completely prevents fracture healing by suppressing formation of both callus and periosteal woven bone (Hausman *el al.*, 2001). Blood supply and formation of new vasculature might also be important for skeletal integrity (i.e., during bone remodeling).

A variety of growth factors and cytokines including VEGF, the fibroblast growth factor (FGF) family, insulin growth factor-1 (IGF-1), epidermal growth factor (EGF), platelet-derived growth factor-A (PDGF-A), and the transforming growth factor-β (TGF-β) family are involved in bone angiogenesis (Garcia-Ramirez *et al.*, 2000). Among these factors, the endothelial cell-specific mitogen VEGF plays a key role for normal and abnormal angiogenesis. All member of the VEGF family have the common ability to stimulate endothelial cell migration and proliferation, proteolytic activity, and capillary morphogenesis (Ferrara *et al.*, 2003). The functional significance of VEGF for bone formation was determined in animal studies. Administration of a soluble VEGF receptor chimeric protein in juvenile mice almost completely suppressed blood vessel invasion at the growth plate and inhibited endochrondal bone formation (Gerber *et al.*, 1999)

New blood vessel formation is crucial for establishing the conduit that allows a variety of cells essential for bone morphogenesis such as chondoclasts, osteoblast, and

osteoclasts to migrate into the growth plate. To develop and maintain normal bone tissues, osteoblastic matrix deposition and osteoclastic resorption must be closely coordination. A perturbation of this balance can result in skeletal abnormalities characterized by decrease (osteoporosis) or increase (osteopetrosis) bone mass.

Vessel and bone

Recently, have been investigated the role of estrogen in bone angiogenesis in association with bone cellular activity changing during the process of ovariectomy-related bone loss. The ovariectomized (OVX) rat has been widely used as an animal model for postmenopausal bone loss (Kalu, 1991). In this model, it is well established that the development of osteopenia is associated with increased bone turnover. Meanwhile, estrogen treatment prevents cancellous osteopenia in OVX rats by reducing bone resorption activity. Multiple lines of evidence suggest that estrogen directly modulates angiogenesis via effects on endothelial cells. Under physiological conditions, angiogenesis is routinely observed in the uterus in association with fluctuation in the levels of circulating estradiol and other sex steroids. Estradiol has been shown to accelerate functional endothelial recovery after arterial injury (Losordo, 2001). On the other hand, 17-β estradiol inhibits growth factor induced smooth muscle cell proliferation and migration.

In relatively healthy older women, it has been suggested that an increased rate of bone loss at the hip and calcaneus in associated with decreased blood flow in the lower extremities and bone mineral decreases in the leg with unilateral occlusive arterial disease. Macroscopic and histological evidence of the dependence of bone on an adequate vascular supply include the finding that vascularized bone grafts, the interruption of blood supply to bone results in avascular necrosis, osteocyte survival requires a less than 0.1 mm proximity to nutrient vessels (Ham, 1952), and a close correlation exists between the rate of osteonic bone deposition and the vascular surface area (Marotti and Zallon, 1980). Parfitt (2000), reported that stressed that bone vessels

might be involved in osteoprogenitor migration toward the newly activated bone multicellular units during bone remodeling. Barou *et al.*, 2002, have been developed a technique allowing simultaneous visualization of blood vessels and bone cellular activities and found evidence for a relationship between vessel number and histodynamic bone formation parameter within the tibial metaphysic of male rats. Despite these consistent observations, relationships between bone vascular events and bone cellular activity changes in the pathophysiology of estrogen deficiency have been only rarely investigated. Femoral bone blood flow is markedly increased in OVX rats as well as the proliferative capability of osteoblast-like cells cultured from marrow and trabecular bone (Chikazu *et al.*, 2001). Laroche *et al.* (1996) showed, although without specific staining, that the area of the intraosseous sinusoidal capillaries increased after ovariectomy in the rat model, in association with an increase in resorption surfaces and osteoid surfaces leading to 40% decrease in trabecular bone volume in lumbar spine. Changes in functional properties of resistance arteries in ovariectomized rabbits are limited to metaphyseal vessels, with no change noted in diaphyseal vessels isolated from the same femur.

Estrogen is an important hormone in female body. This steroid travels far, interacts with multi organ systems, and plays a pivotal role in the sentinel physiologic events that occur during women's life. Estrogen has several therapeutic uses including contraceptive applications, treatment of menopausal symptoms, cardiovascular disease and the prevention of osteoporosis.

Estrogen and vascular function

Many studies indicate that estrogen exerts beneficial effects on the circulatory system. It is clear that estrogen reduces atherosclerosis by reducing low-density lipoproteins (LDL) and inflammatory processes in the vasculature, and may also act as an antioxidant; however, these effects account for only a portion of the total

cardiovascular benefit of estrogen. Estrogen is also a vasodilator and hypotensive agent, and can induce vascular relaxation by stimulating release of endothelium-derived vasodilatory substances (e.g., nitric oxide [NO]) or by acting directly on the vascular smooth muscle (VSM). Recent evidence indicates that calcium and potassium channels in VSM cells play an important role in mediating estrogen-induced relaxation of many vascular beds, but elucidating the signal transduction mechanisms coupling estrogen receptor (ERα and/or ERβ) activation to generation of second messengers (Richard, 2002).

Experimental studies have well documented that estrogen enhances blood flow in the female reproductive tract (Killam *et al.*, 1973; Rosenfeld *et al.*, 1973), and vasodilatory actions of estrogen on a variety of other tissues have also been described (Collins, 1998). It is now apparent that estrogen can modulate vascular tone, and hence, organ blood flow, by targeting both endothelial cells and/or vascular smooth muscle (VSM) cells. An important key to helping unlock the mysteries of estrogen action was the cloning of distinct estrogen receptors, ERα (Walter *et al.*, 1985) and ERβ (Kuiper *et al.*, 1996), and then demonstrating the functional importance for the novel ERβ in mice lacking the classical ERα receptor (Iaflati *et al.*, 1997).

More recent studies demonstrate that vascular endothelium and myocytes express both ERα and ERβ, but this expression seems heterogeneous with regard to vascular bed and gender. For example, RT-PCR studies have identified mRNA for both ERα and ERβ in human VSM from the coronary artery, iliac artery, aorta, or saphenous vein, but expression of ER tends to predominate in females (Hodges *et al.*, 2000). In rats, ERβ immunoreactivity appears more abundant in VSM and endothelium from the aorta, tail and uterine arteries, whereas ERα is found mainly in the uterine vasculature (Anderson *et al.*, 2001). A current challenge facing investigators is to better define the functional roles of ER subtypes and their impact on vascular physiology and pathophysiology. The potential therapeutic impact of this research is substantial, as indicated by the flurry of interest in synthesis and characterization of selective ER modulators (SERMS). It is

hoped that more selective ER agonists/antagonists will one day maximize the beneficial effects of estrogen action (e.g., cardiovascular protection, bone preservation) while minimizing potential undesirable side effects (e.g., cancer, feminization in males).

NO: mediator of estrogen-induced vasodilation

Many studies indicate that important vascular effects of estrogens are mediated via NO (Van Buren *et al.*, 1992; Rosenfeld *et al.*, 1996; Mendelsohn and Karas, 1999). For example, aorta from females release more NO than vessels from males (Hayashi *et al.*, 1992), suggesting a role for estrogen in NO production. Consistent with this finding is the fact that NO production is increased during pregnancy (Conrad *et al.*, 1993) and during the preovulatory phase of the menstrual cycle when estrogen levels are highest (Kharitonov *et al.*, 1994). The mechanism of how estrogen enhances NO production is not completely defined. Estrogen may stimulate release of NO from vascular cells by mechanisms dependent upon (Weiner *et al.*, 1994; Binko and Majewski, 1998) or independent of (Lantin-Hermoso *et al.*,1997; Caulin-Glaser *et al.*, 1997) gene expression. Estrogen-stimulated NO production may promote anatomical recovery of intimal injury by accelerating re-endothelialization and/or inhibiting VSM proliferation (Haynes *et al.*, 2000).

Estrogen increases eNOS activity in human aortic endothelial cells (Hishikawa *et al.*, 1995). Moreover, Goetz *et al.* (1999) reported that estrogen stimulated the dissociation and translocation of eNOS (Type III) from the plasma membrane to intracellular sites near the nucleus of cultured aortic endothelial cells within only 5 min, thereby suggesting a nongenomic mechanism of eNOS activation. Similarly, chronic or acute estrogen treatment stimulated eNOS activity in the endothelium of uterine arteries, but estrogen also activated nNOS (Type I) expressed solely in medial smooth muscle cells (Salhab *et al.*, 2000). Could NO derived from VSM play a role in mediating acute vascular effects of estrogen? There is evidence suggesting that this may indeed be the

case. NO produced in primary myocytes from porcine coronary arteries in the absence of the endothelium mediates estrogen-induced stimulation of BK_{Ca} channels (Darkow *et al.*, 1997). Furthermore, it has been recently demonstrated that estrogen increases the intensity of NO-induced fluorescence in cultured human coronary myocytes grown in the absence of the endothelium, and that this estrogen-stimulated NO production is prevented by inhibiting NOS activity (White *et al.*, 2002). The role of VSM-generated NO in mediating estrogen-induced vascular relaxation remains to be established; however, it is highly probable that this "autocrine" NO production could mediate the ability of estrogen to relax vessels with dysfunctional endothelium found in atherosclerosis and thrombotic or mechanical injury. Thus, there is strong evidence to suggest that estrogen can stimulate NO production in the vascular wall by both an endothelium-dependent and -independent action.

Estrogen and bone

Estrogen is major hormonal regulator of bone turnover in women. It clearly inhibits bone remodeling, inhibits bone resorption and enhances bone formation. Effect of estrogen on stromal/osteoblastic cells which support osteoclastogenesis, has been reported. Thus estrogen deficiency is associated with an increase in this cell population (Jilka *et al.*, 1998). Increased synthesis of Macrophage-colony stimulating factor (M-CSF) and osteopontin which are osteoclast precursor has been reported in vitro and in ovariectomized animals (Felix *et al*, 1994; Kimble *et al.*, 1996). The key, essential molecule for osteoclast development is receptor activator of NF-kB ligand (RANKL) which is expressed on the surface of bone marrow stromal/osteoblastic precursor cells, T-cell ,as well as B-cells (Eghbali-Fatourechi *et al.*, 2003). RANKL binds its cognate receptor, RANK, on osteoclast lineage cells, and it is neutralized by the soluble decoy receptor, osteoprotegerin (OPG), which is also produced by osteoblastic lineage cells (Simonet *et al.*, 1997) Recently, it has also been shown that estrogen increases level of OPG mRNA and protein in osteoblastic cells (Hofbauer *et al.*, 1999). In addition,

estrogen plays an important role in the regulation of osteoclast activity. The cytokines IL-1, IL-6, TNF-α and M-CSF have all been shown to inhibit apoptosis in osteoclasts (Hughes *et al.*, 1997, Jimi *et al.*, 1997), whereas TGF-β, the production of which is decreased in estrogen deficiency states, stimulates apoptosis (Hughes *et al.*, 1994). Estrogen may also directly stimulate apoptosis by decreasing expression of NF-kB-activated genes that normally suppress apoptosis (Jimi *et al.*, 1996). Interestingly, the reverse effect has been reported for osteocytes, acute estrogen withdrawal in human being associated with increased apoptosis of osteocytes (Tomkinson *et al.*, 1997).

Reports on the effects of estrogen on DNA synthesis and proliferation and bone matrix protein production have produced conflicting results, possibly as a result of differences in the in vivo systems investigated and, in particular, the stage of differentiation of osteoblast in these systems (Oursler, 1998). Thus, in osteoblastic cells, for which estrogen acts as a mitogen, increased expression of alkaline phosphatase and type I collagen has been reported (Majeska, 1994; Zang *et al.*, 1994), whereas in cells that show no proliferative response to estrogen, stimulation of type I collagen and osteocalcin expression have been demonstrated without the increase in alkaline phosphatase (Keeting *et al.*, 1991). Estrogen also increases PTH responsiveness in osteoblastic cells (Ernst *et al.*, 1989; Fukayama, and Tashjian, 1989) and increases expression of IGFBP-4, as well as reducing its proteolytic breakdown (Kassem *et al.*, 1996).

Estrogen, nitric oxide and bone

Estrogen deficiency is one of the most important factors in the pathogenesis of osteoporosis, although the mechanisms by which estrogen prevent bone loss are incompletely understood (Katharin *et al.*, 1998). The major effect of estrogen is stimulates eNOS activity and mRNA levels in endothelial cells (Hayashi *et al*, 1995) and osteoblasts (Amour and Ralston, 1998).

Nitric oxide is a highly reactive molecule, and it has many potential molecular targets. NO is a major second messenger for estrogen action in bone, and a key mechanism by which anabolic estrogen effects on osteoblasts and other mesenchymal cells can reduce osteoclastic activity (O'Shaughnessy *et al.*, 2000). Preincubation with a physiologic concentration of 17β-estradiol (10^{-12}-10^{-18}M) over 8 hours significantly enhanced the activity of eNOS in endothelial cells of cultured human umbilical vein and of bovine aortas. 17β-estradiol also enhances the release of NO as measured by an NO selective meter and nitrate/nitrite, metabolites of NO, from endothelial cells (Hayashi *et al.*, 1995). Moreover, 17β-estradiol have been found to stimulate eNOS activity and mRNA levels in cultured human osteoblasts-like cells (Katharine *et al.*, 1998).

Samuels and co-workers (2001) found that estrogen-induced osteogenesis in intact female mice was partially, but significantly, inhibited by co-administration of the nonspecific NOS inhibitor, L-NAME. In contrast, co-treatment with the iNOS inhibitor, amino guanidine had no discernible effect on this response, suggesting that estrogen-induced osteogenesis preferentially involved eNOS. eNOS is the predominant constitutive isoform of NOS expressed in bone, raising the possibility that NO derived from the eNOS pathway plays a role in mediating the effects of sex hormones in bone . However, in the same studies, no change in eNOS mRNA levels was observed following estrogen administration, as assessed by reverse transcription-polymerase chain reaction (RT-PCR). These results suggest that eNOS plays a role in mediating estrogen-induced bone formation in intact female mice, possibly as a consequence of posttranscriptional regulation of eNOS activity by estrogen.

Specifically, transgenic studies have shown that NO, as produced by the endothelial nitric oxide synthase (eNOS), mediates the anabolic estrogen response (Armour *et al.*, 2001) and mechanical strength response (Nomura and Takano-Yamamoto, 2000) in bone. However, under most circumstances osteoblast and endothelial cells are the major NO source in bone (Helfrich *et al.*, 1997), and NO has inhibitory effects on osteoclasts (MacIntyre *et al.*, 1991; Van't Hof and Ralston, 1997).

Since NO is a diffusible gas, osteoclast response to osteoblastic NO is logically to be expected, and indeed this has been demonstrated (Lowik *et al.*, 1994).

The osteoclast also produces NO, probably mainly by the inducible NO synthase (iNOS); a knockout mouse lacking iNOS had a profound defect in IL-1-stimulated osteoclast activity (Van't Hof *et al.*, 2000), that iNOS is important for autocrine osteoclastic NO activity, although other NO producing cells including osteoblasts will obviously produce overlapping signals in vivo. The importance of NO as a regulator of bone mass is highlighted by animal and human studies show that bone loss in progressive osteoporosis or in response to estrogen withdrawal is reduced by NO donors (Jamal *et al.*, 1998; Hukkanen *et al.*, 2003). Wimalawansa *et al.* (1996) also reported that nitroglycerine, a nitric oxide donor, alleviated bone loss induced by ovariectomy in rats and that in the presence of N^G-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase (NOS). Hukkanen *et al.* (2003) also showed that transdermal delivery of the NO donor NG counteracted the development of skeletal osteopenia associated with ovariectomy-induced estrogen deficiency. The effect of transdermal NG on bone metabolism is likely to include both a local bone cell-mediated and vasoreactive response of bone. This is consistent with earlier studies in the guinea pig demonstrating estrogen–induced regulation of the constitutive NOS enzymes, epithelial NOS (eNOS) and neuronal NOS (nNOS) and with the inhibitory effect of high nitric oxide concentrations on osteoclastogenesis and osteoclast activity (Evans and Ralston, 1996). Interestingly, function of estrogen have been demonstrated in bone endothelial cells in vitro (Brandi *et al.*, 1993), supporting a role for estrogens in angiogenesis and hence, potentially, access of osteoclasts to remodeling bone surfaces (Parfitt, 1998).

The role of estrogen in the regulation of osteoclast activity is thus mediated via effects on osteoclast number and activity. The former action is determined both by direct cytokine-induced effects on osteoclast proliferation and differentiation and by modulation of the stromal/osteoblastic cell population that supports osteoclastogenesis. Changes in osteoclast activity are probably mediated predominantly through effects on

apoptosis.

Earlier studies have demonstrated that estrogen maintains bone mass by inhibition of bone resorption, which leads to a reduction in bone turnover. Estrogen may also inhibit osteoclastic bone resorption (Hayashi *et al.*, 1995) and this has been variously attributed to the suppression of cytokine production in the bone microenvironment and modulation of systemic calciotropic hormones such as parathyroid hormone and vitamin D.

Recently, it has been reported that estrogen exhibits antiinflammatory activity by preventing the induction of iNOS and other components of the inflammatory reaction (Salvatore *et al.*, 2003). These data indicate that in WT mice the observed induction of iNOS has functional relevance, because it leads to overproduction of nitric oxide and accumulation of highly reactive molecules, triggering a local inflammatory reaction. Estrogen depletion results in increased iNOS synthesis in stromal cells and osteoblasts, leading to NO accumulation in the bone microenvironment. This, inturn, results in increased formation of peroxynitrites and other inflammatory compounds. The components of the inflammatory reaction, and possibly NO itself via NF-kB activation, induce osteoclast differentiation. These effects may be amplified by circulating inflammatory compounds (cytokines) that accumulate in blood vessels after the increase in iNOS, induced by the lack of estradiol, and permeate to the bone (Figure 2.4). These inflammatory foci attract cytokines, well known actors in the mechanical of osteoclastogenesis.

Taken together, these findings indicate that both the anti bone resorptive and anabolic effects of estrogen on bone could be mediated in part by NO.

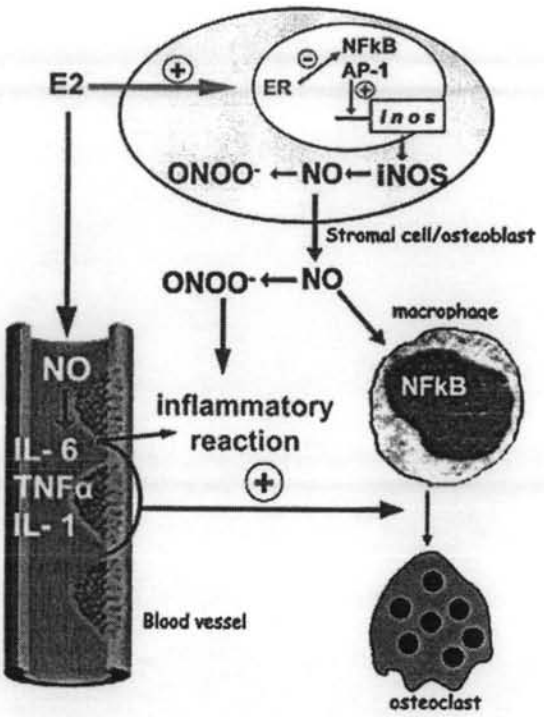

Figure 2.4 Proposed model for the actions of estrogen depletion on bone. (Salvatore *et al.*, 2003)

Estrogen deficiency and inflammation

Many evidences suggesting that the decline in ovarian function with menopause is associated with spontaneous increases in proinflammatory cytokines. Bone is a rich source of cytokines and growth factors (Figure 2.5) and also other mediators such as prostaglandins and nitric oxide. In addition, cells in the bone microenvironment play a major role in the regulation of bone remodeling, both as a source of bone cell precursors and by the production of bone active cytokines and growth factors. IL-6 also stimulates bone resorption, although by different mechanisms. Its production in bone is increased by other bone-resorbing cytokines and systemic hormones (for example, PTH) (Feyen *et al.*, 1989), and it also acts synergistically with these agents, increasing their bone resorptive effects (De La Mata, 1995). The effects of IL-6 in vivo may be modulated by the circulating levels of IL-6 soluble receptor.

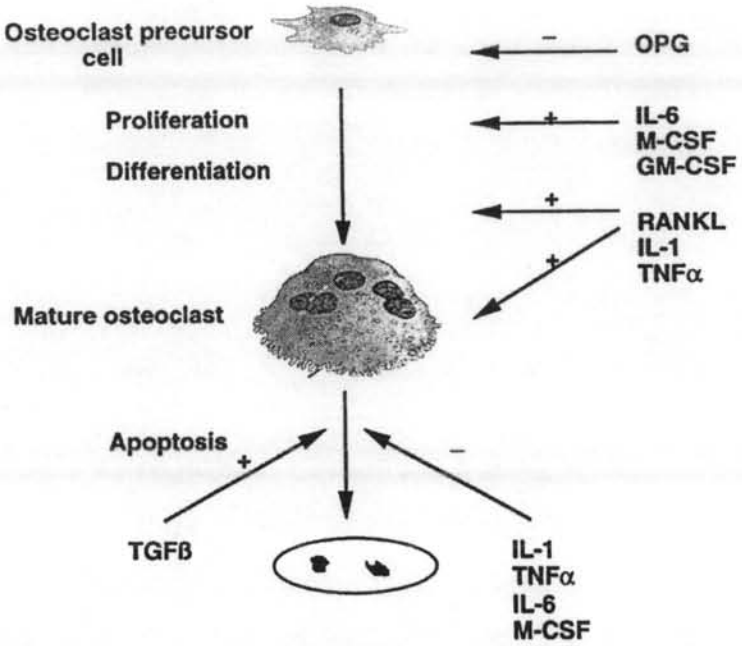

Figure 2.5 Effects of cytokines on osteoclast production and activity. TGF-β, transforming growth factor-β; IL, interleukin; TNF-α, tumor necrosis factor-α; M-CSF, macrophage-colony stimulating factor; GM-CSF, granulocyte/macrophage-colony stimulating factor.

The phenomenon is lead to slow blood flow and decrease perfusion pressure into tissue area. Spontaneous increases in the expression and secretion of the proinflammatory cytokines IL-1, IL-6 and TNFα were first noted several years ago in cell cultures of circulating monocytes, bone marrow macrophages and osteoblasts (Binbin and Shifeng, 2003). However, several studies distinct mechanisms by which estrogen deficiency enhances the function of key pro-inflammatory pathways. For example, estrogen deficiency has been shown to enhance the cell response to some cytokines by up-regulating receptor numbers and cofactors required for cytokine signal transduction and cellular action (Lin *et al.*, 1997). Estrogen-mediated down-regulation of IL-6 expression may involve inhibitory effects on NF-kB pathways. Estrogen also exerts an inhibitory influence on AP -1 mediated transcription of the TNF-α gene (An *et al.*,

1999). Finally, recent studies have pointed to the ability of estrogen to modulate inflammation-mediated release of pro-inflammatory cytokines from macrophages. For example, physiologic concentrations of 17β-estradiol have been shown to specifically inhibit inflammation-mediated release of two pro-inflammatory cytokines, TNF-α and MIF (macrophage migration inhibitory factor) from macrophages (Lambert *et al.*, 2004).

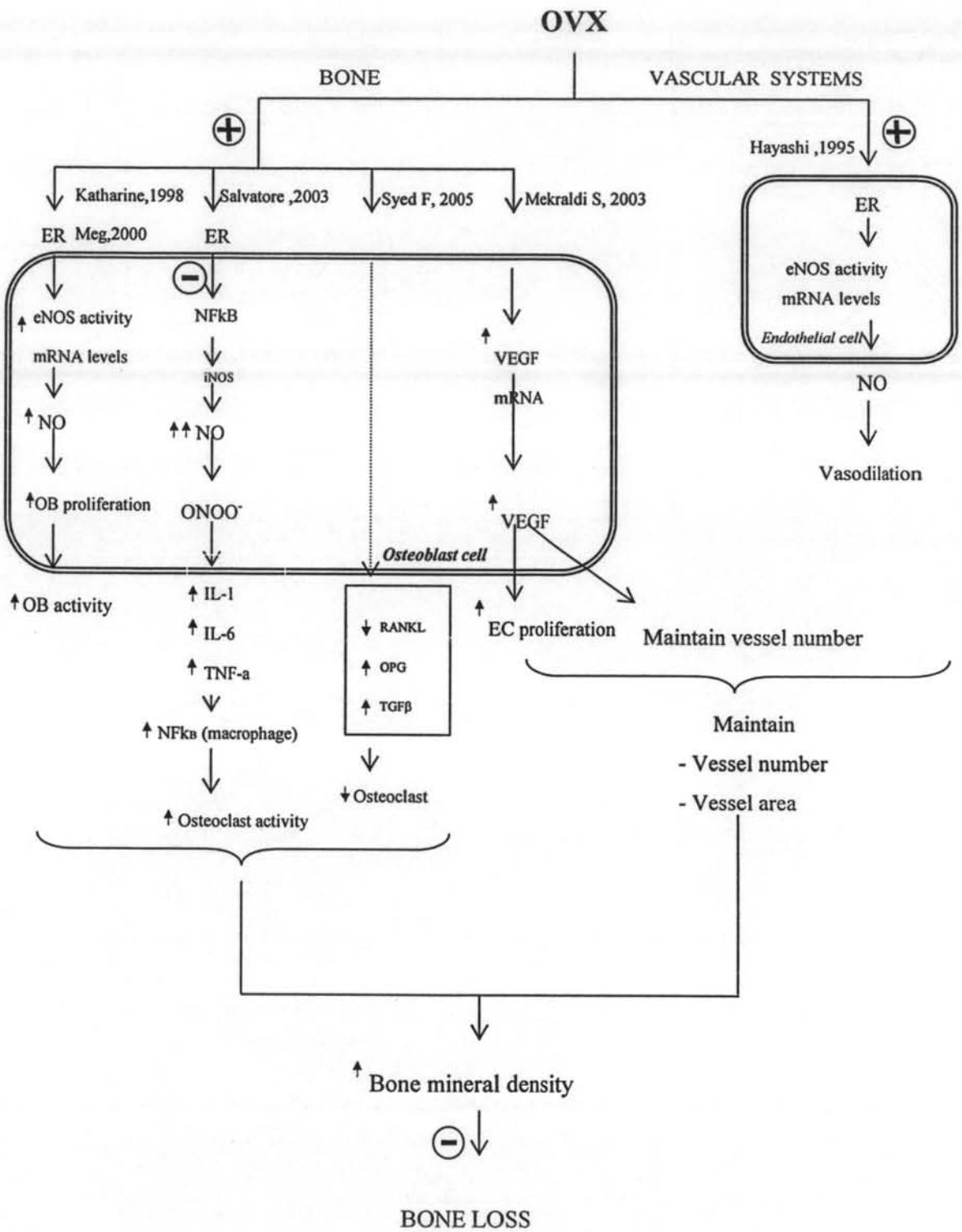

Figure 2.6 Effects of estrogen on osteoblast and endothelial cell.

Estrogen and selective estrogen receptor modulators (SERMs)

Estrogen replacement therapy has long been considerated the first line therapy for preventing osteoporosis in women. Treatment with estrogens clearly inhibits bone loss as well as bone turnover and in creases bone mineral density (BMD). In early postmenopausal women, estrogen increase spine BMD by 3 to 4 %, as well as hip BMD to an extent similar to that induced by alendronate (Hosking *et al.*, 1998). In late postmenopausal women, the effect is less pronounced. The Women's Health Initiative (WHI), suggested that estrogen reduce the risk of hip fractures by over 50% while subjects are on HRT (Michaelsson *et al.*, 1998). However, besides their effects on bone, estrogens affect many other tissues including breast and uterus; undesirable side effects have limited the long-term use of estrogen in the United States and in many other countries. Estrogen treatment is associated with a well-estrablished increase in the risk of uterine cancer. There is also a 20 to 50 % increase the risk of breast tumors, which deters many women from receiving HRT, especially those with a family history of breast cancer (who are currently advised not to take estrogen).

Phytoestrogens

Phytoestrogens are naturally occurring non-steroidal plant-derived substances that exhibit estrogen-like biological activity. Structure of the major phytoestrogens are diphenolic compounds that share similarities with endogenous hormones. They can bind to the estrogen receptors and functionally, they may exert both estrogenic effect, such as stimulation of uterus growth and inhibition of bone loss, and anti-estrogenic activities, such as inhibition of breast cancer cell growth (Terreaux, 2003). Increasing numbers of women are using phytoestrogen products to treat menopausal symptoms (Tarkan, 2000)

They have been categorized in three main classes, according to their chemical structures:1) isoflavones: found in soy, lentils and other legumes, the active ingredients are genistein and daidzein, 2) lignans: found in flaxseed and other seed oils, the active

ingredients are enterodiol and enterolactone; and 3) coumestans: found in red cover, sunflower seeds, and bean sprouts, the active ingredient is coumestrol. Isoflavones have up to now received the most attention, especially genistein and daidzein, found in soy. Because many women and their health care providers lack confidence in the benefits of ERT/HRT along with fear of increased risk of breast cancer and/or ERT/HRT side effects, fewer than one in three menopausal women choose traditional ERT/HRT. Today, isoflavones are considerated as the most important class of phytoestrogens that occur frequently in human diet.

Genistein

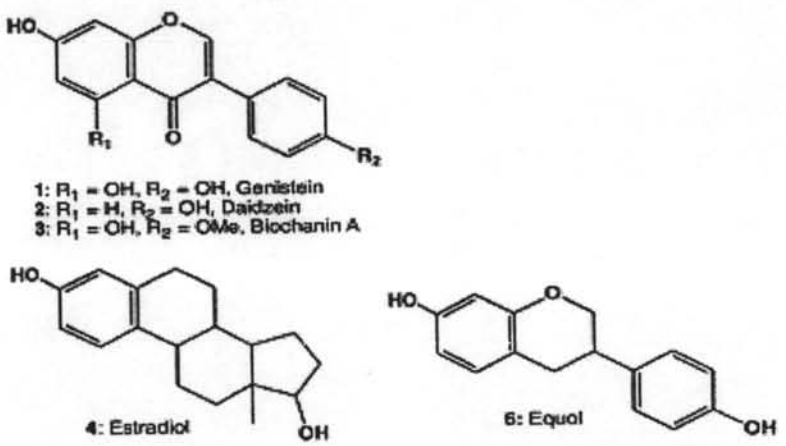

Figure 2.7 Structures of isoflavone phytoestrogens in relation to estradiol.(Dixon and Ferreira, 2002)

Genistein shares structural features with the potent estrogen estradiol-17, particularly the phenolic ring and the distance between its 4′- and 7- hydroxyl groups (Figure 2.7). These features confer ability to bind estrogen receptors and sex hormone binding proteins, it have classically been considered weak estrogens because their binding affinity for the ERα is less than 1 percent of that of estradiol. However, the binding affinity of genistein to ERβ, although less than the binding affinity of estradiol,

may be significantly greater than the binding affinity to ERα . Genistein can exert both estrogenic and anti-estrogenic activity, the latter by competing for receptor binding by estradiol. Genistein is also known to act as inhibitor of tyrosine kinase, important enzyme in many cellular activities that do not involve the ER. Therefore, not all of the actions of genistein are necessarily a result of its interaction with the ER. The potent estrogen equol (a major metabolite of dietary isoflavonoids formed by the gastrointestinal flora) (Figure 2.7) and genistein can displace bound estrogen and testosterone from human sex steroid binding protein. Thus, genistein and other phytoestrogens could potentially affect clearance rates of androgens and estrogens and therefore the availability of the hormones to target cells. It should be noted that genistein binds differentially to human and estrogen receptors (Barnes *et al.*, 2000), and this should be carefully considered when extrapolating the results of phytoestrogen administration experiments in animals to hormone-related diseases in humans.

Results of epidemiological studies have suggested that high dietary intake of isoflavones and/or flavonols may contribute to a low incidence of heart disease in Japanese women. These effects may result from inhibition of low density lipoprotein oxidation by isoflavones, an effect that may be enhanced by food sources rich in vitamin C (Hwang *et al.*, 2001). Genistein also appears to improve plasma lipids, resulting in lowered LDL cholesterol, the ratio of total cholesterol to HDL cholesterol, and the ratio of LDL to HDL cholesterol, in pre-menopausal women (Merz Demlow *et al.*, 2000). In rats, the hypocholesterolemic effect of a soy diet may involve interactions between the isoflavones and soy protein (Peluso *et al.*, 2000), whereas, in cholesterol fed rabbits, attenuation of atherosclerosis by isoflavones does not require the presence of soy protein.

Estrogen deficiency in post-menopausal women can lead to unpleasant symptoms such as hot flushes and vaginal dryness, with a long-term increased risk of bone loss in addition to cardiovascular disease. An isoflavone-rich diet may help approximately two thirds of post-menopausal women to better cope with hot flushes, in addition to potentially reducing the risk of cardiovascular disease, which is elevated in post-

menopause. Squadrito *et al.*, (2000) reported that genistein (0.2 mg/kg/day,sc) for 28 days enhanced the circulating levels of the phytoestrogen and affected NOS activity and endothelial dysfunction to the same extent. In 2003, Khemapech *et al.*, reported that genistein supplementation (0.25 mg/kg BW/day) for 21 days was also able to restore Ach-induced endothelium-dependent vasodilatation in ovariectomized rats. These results supported by videomicroscopic visualization have clearly provided evidence for such a positive outcome of genistein on the derangement of endothelium-dependent vasodilation caused by a lack of estrogen.

Soy isoflavones positively help maintenance of bone mass in ovariectomized rodents. One study has indicated that isoflavone-rich soy protein may attenuate bone loss in the lumbar spine of post-menopausal women, and that this effect is due to isoflavones rather than to soy protein (Alekel *et al.*, 2000). Fanti *et al.* (1998) reported that genistein reduced bone loss in short-term ovariectomized rats. Animals were administrated with genistein 5 or 25 mg/kg BW/day for 21 days. Administration of genistein was associated with higher bone formation rate per tissue volume and with a trend toward a higher number of osteoblasts per bone perimeter. The concentration of serum osteocalcin is increased conversely the production of TNF-α. Ishimi *et al.* (2000) reported that treatment with genistein (0.7 mg/kg/day, sc) for 14 days in ovariectomized mice prevented trabecular bone loss without hypertrophic effects on the uterus. The recent studies (Binbin and Shifeng *et al.*, 2003) has also indicated that genistein prevents bone resorption diseases by inhibiting bone resorption and stimulating bone formation. After ovariectomized rat was treated with genistein (45 mg/kg BW/day, po) for 84 days, bone mineral density and the serum levels of alkaline phosphatase, acid phosphatase, osteocalcin increased significantly, while the serum level of IL-1and TNF-α was decreased. In addition, Yoon-Bok *et al.*, (2004) examined a potential role of soy bean phytoestrogen (6.25 mg/kg/day) for 16 weeks in ovariectomized rat. They found that isoflavone prevented bone loss and increased activity of both serum alkaline phosphatase and tartrate-resistant acid phosphatase, which are bone formation markers. These studies

have suggested that soy consumption can prevent postmenopausal cardiovascular disease and bone loss.

Effects of genistein on endothelial function and bone mineral density

In table 1, lists of experiments indicate the effects of genistein supplementation on ovariectomized animals. In the evaluation of animal studies, protective effects on endothelial function and bone loss were considered following doses, duration of treatment, endothelial function, bone mineral density and bone marker enzymes.

Table 2.1 Representative animal studies examining the effects of genistein on endothelial function (EF) and bone mineral density (BMD) and biomarkers of bone formation and bone resorption.

Author(s)	Animal model	Treatment genistein (mg/kgBW)	Treatment (duration)	EF/BM	Bone marker enzymes
Squadrito *et al.*, 2000	OVX Sprague-Dawley rats	0.20, sc	28 days	Prevent endothelial dysfunction	-
Khemapech *et al.*, 2003	OVX Wistar rats	0.25, sc	21 days	Prevent endothelial dysfunction	-
Anderson et al., 1998	OVX rats	0.50, P.O.	14 days	Increase femoral ash	Not assessed
Fanti et al., 1998	OVX rats	0.05,0.25, sc	21 days	Both doses had similar tibial BMD as sham	Increase serum OC; no change in resorption markers
Ishimi et al., 2000	OVX mice	0.70, sc	14 days	Prevent trabecular bone loss	Not assessed
Binbin Li, And Shifeng 2003	OVX rats	0.45, P.O.	84 days	Prevent femoral bone loss, decrease IL-1and TNF-α	Increase serum OC and ALP

EF; Endothelial function OC; Osteocalcin, ALP; Alkaline phosphatase

Estrogen deficiency accelerates the development of osteoporosis and this can be prevented by a classical hormone replacement therapy (Riggs, 2002 and O'Connell *et al.*, 1998). The OVX rat is an excellent model to study estrogen deficiency-induced osteoporosis, since it develops severe osteoporosis within a few weeks after ovariectomy and reduction of bone structure parameters of more than 50% (Westerlind *et al.*, 1997). Other animals that have been studied as models of estrogen deficiency-induced bone loss include mice, ferrets, dog, sheep, swine, and monkeys. These species vary in their skeletal responsiveness to estrogen depletion and are less well established than the rat model (Kimmel, 1996). Therefore, this study will use the rat model to study the effect of genistein on several bone parameters.