CHAPTER V

DISSCUSSION

Osteoporosis, a common pathology in postmenopausal women, is widely recognized as a major public health problem. The most dramatic expression of the disease is represented by fractures of the proximal femur, the number of which increases as the population ages (Schapira *et al.*, 1995). Osteoporosis is the result of an imbalance in bone remodeling. The imbalance in bone remodeling means bone resorption exceeds bone formation.

Reproductive hormones play crucial roles in bone health, including E_2 in women and testosterone in men. These hormones help to control the balance in bone remodeling. When women reach their menopausal age whereas E_2 levels begin to decline, the risk for osteoporosis is then increased. Hormone replacement therapy (HRT) is commonly prescribed to prevent osteoporosis. However, many women do not want to take HRT because of its possible to increased risk of breast cancer. Therefore, several investigators have turned their interests on roles of phytoestrogens.

Genistein, one major component of phytoestrogens, has a structure and physiological activity similar to estrogen. It has a wide variety of pharmacological effects including the benefit for estrogen replacement therapy (ERT), and as an anti-oxidant agent. Genistein has been indicated for its similar effect as E_2 on ***cardiovascular protection*** without any estrogen–dependent risk of reproductive organs and side effects. The previous study has shown that genistein at dose of 0.25 mg/kgBW/day could prevent endothelial dysfunction in OVX rats (Khemapetch *et al.*, 2003). They also showed that the effects of genistein on mesenteric endothelial-dependent vasodilatation could be mediated through both L-arginine/NO and cyclooxygenase pathways. Their findings indicated that genistein could induce endothelial-dependent vasodilation similar to 17β-

estradiol (Siriviriyakul *et al.*, 2006). At present several studies have demonstrated more for protective role of genistein in the vasculature (Anthony *et al.*, 1996; Honore *et al.*, 1997; Kapiotis *et al.*, 1997; Williams *et al.*, 1998; Yamakoshi *et al.*, 2000; Pan *et al.*, 2001).

Therefore, our interest is to investigate whether genistein can prevent bone microvascular dysfunction or not and if it can that means it may be able to attenuate the consequent bone loss.

5.1. Effect of genistein on bone mineral content

Our results have demonstrated that there is an E_2- depletion induced lost of tibial bone mineral content significantly at 6 weeks, not significantly difference at 3-wk, after ovariectomy ($OVX_{veh(3wk)} = 98.68\pm0.26^{NS}$; $OVX_{veh(6wk)} = 98.69\pm0.24$, $p< 0.05$). However, genistein supplementation (0.25 mg/kg/day) could prevent lost of tibial bone mineral content ($p< 0.05$), with a completely preserve as much as $Sham_{veh}$ value at 6 weeks of experimental period.

By using different techniques of densitometry to observe bone histomorphometry, Helfrich and Ralston, 2003, have reported that ovariectomy-induced significant bone loss in 12-16-wk-old female rats within 6 weeks period. Whereas, an experiment in 8-12-wk-old females mice model has demonstrated bone lost significantly as early as 3-wk after ovariectomy.

The possible mechanism reported by several studies suggested that a lack of E_2 induced lost in : bone weight, bone mineral density, and bone mineral content. The reduction may relate to the endothelial isoform of nitric oxide synthase (eNOS) activity (Riggs *et al.*, 1969 and Kalu *et al.*, 1989). The eNOS isoform seems to play a key role in regulating osteoblast activity and bone formation because eNOS knockout mice has become osteoporosis due to defective bone formation. In addition, the NO derived from the eNOS pathway acts as a mediator of E_2 in bone (Samuels *et al.*, 2001). Interestingly, the result of Yamaguchi (2006) has indicated that the preventive effect of genistein on

bone loss was mediated by eNOS pathway as E_2 does.

These findings have remarked two important matters: a). the imbalance of bone remodeling seems to exist after endothelial dysfunction b). the same dose of genistein supplementation seems to exert its preventive effects on both targets, bone cells and bone microcirculation.

5.2. Effects of genistein on serum E_2 levels and uterine weight

Our study demonstrated that serum E_2 levels were significantly reduced since 1-wk OVX_{veh} when compared to $Sham_{veh}$ operated rats. 17β-estradiol replacement markedly increased serum E_2 to the levels resembling those observed in $Sham_{veh}$ group (Table 4.2) ($p< 0.05$). In contrast genistein supplementation seem to have no effect on serum E_2 levels either 1-wk or 3-wk OVX_{gen} (Table 4.2).

To confirm the effect of E_2, the uterus was weighted In Table 4.2, both 1-wk and 3-wk of OVX_{veh} and OVX_{gen}, displayed decreased uterus weights as compared to $Sham_{veh}$ and OVX_{E2} groups. These findings suggested that genistein could not exert its agonistic effect to endogenous E_2 in uterus.

There are two kinds of estrogen receptors, alpha (ER_α) and beta (ER_β), and that they are found in almost every organ of the body (Kuiper *et al.*, 1997). However, different tissues have different proportions of the two receptors, with the predominance of ER_β in bone and vascular tissues. Moreover, genistein has a fairly weak affinity for ER_α, but strong affinity for ER_β. With these two reasons, they help to explain our results of bone and vascular protective effects of genistein. ER_α is the receptor subtype predominantly expressed in the uterus and it have previously shown that the estradiol-mediated increase of uterine weight is solely mediated via ER_α (Linberg *et al.*, 2002). Therefore, our findings showed that genistein could not exert its agonistic effect to endogenous estrogen in uterus. Moerover, isoflavone-rich soy protein and isolated isoflavones have been reported for their no affect on endometrial cell proliferation (Duncan *et al.*, 1999 and Upmalis *et al.*, 2000) as well.

5.3. Effect of genistein on bone formation markers

In our results, the activities of both bone metabolic markers, osteocalcin and alkaline phosphatase, were significantly increased after 1-wk and 3-wk ovariectomy. The assays of bone biochemical markers are commonly used as tools in assessment of any alteration of bone remodeling or bone turnover. Actually, osteocalcin and alkaline phosphatase are commonly used as indicators for bone formation. Similar findings of transient increase in serum concentrations of osteocalcin and alkaline phosphatase were reported in OVX_{veh} by (Kalu, 1991 and Marie *et al.*, 1993). Since our results of bone mineral content determined for both tibia and femur bones showed no significant change during this 1-wk and 3-wk ovariectomy. From the previous study, it reported that the number of mesenchymal osteoblast progenitor in the murine bone marrow was increased two- to three fold between **2 and 8 wk** after ovariectomy and returned to control levels by **16 weeks** (Jilka, 1998). The level of these bone 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. Therefore, we got to believe that this increased in bone formation markers were specifically addressed only on biochemical respond changes, not yet imply to bone loss directly.

In addition, it should be noted that in OVX rats and mice, long-term stimulation of osteoclastogenesis leads to a compensatory increase in bone formation that limits the net loss of bone (Kimble *et al.*, 1996). We also believed that these observed increases in bone formation markers during the early period of 1-3 weeks after ovariectomy were indicated only the "compensatory process" as well. However, we suggest that bone resorption markers should be determined in the future.

Interestingly, our results showed that these biochemical markers identified changes in bone remodeling could be significantly attenuated by either treatments of 17β-estradiol or genistein (Figure. 4.3.1 and 4.3.2).

Eventually, the results of this part suggested that genistein could mediate its preventive effect similar to E_2, therefore, it implied that they might act on the same receptor in bone cells. Bone cells contain both receptors, but their distributions within bone are not homogeneous. In humans, ER_α is the predominant isoform in cortical bone, while ER_β is the predominant specie in trabecular bone.

It is now recognized that 17β-estradiol prevents bone loss through effects on bone marrow and bone cells, which result in decreased osteoclast formation, increased OC apoptosis, and decreased capacity of mature osteoclasts to resorb bone (Manolagasan Jilka, 1995). 17β-estradiol modulates osteoclast apoptosis and osteoclast activity both directly (Kameda, 1997 and Oursler, 1991) and indirectly, through regulation of growth factors and prostaglandins (Hughes, 1996 and Kawaguchi, 1995). Conversely, inhibition of osteoclast formation results primarily from the ability of 17β-estradiol to regulate the production of pro- and anti-osteoclastogenic cytokines. Among them the referred pro-inflammatory cytokines were including: IL-1, IL-6, TNF-α, M-CSF, and osteoprotegerin (Manolagas and Jilka, 1995; Kimble *et al.*, 1996; Pacifici, 1996; Lorenzo, 1998; and Hofbauer *et al.*, 1999).

Therefore, in the following session, we will present our findings for the effects of genistein on OVX-induced changes of pro-inflammatory factors; **TNF-α and IL-6**.

5.4. Effect of genistein administration on TNF-α and IL-6

In our experiment, the serum levels of TNF-α significantly increased in both groups of 1-wk and 3-wk OVX_{veh} (Table 4.3.2 and Figure 4.3.3). However, during this early period, 1-wk and 3-wk after ovariectomy, the change of cytokine IL-6 has not yet indicated. There are a lot of evidences suggesting that the decline in ovarian function with menopause is associated with spontaneous increase in pro-inflammatory cytokines including, TNF-α, IL-1, IL-6, and IL-7. (Manolagas and Jilka, 1995; Kimble *et al.*, 1996; Pacifici, 1996; Lorenzo, 1998; Hofbauer *et al.*, 1999; Salvatore *et al.*, Huang *et al.*,

2005 ; and Weitzmann and Pacifici, 2006).

The increased levels of TNF-α in the bone marrow of OVX animals and in the conditioned media of peripheral blood cells of postmenopausal women is well documented (Ben-Hur *et al.*, 1995; Fujiki 2002; Pacifici, 1991; Ralston *et al.*, 1990; and Shanker *et al.*, 1994), although the cells responsible have not been identified conclusively. Recent studies on highly purified bone marrow cells have revealed that OVX increases production of TNF-α by T cells but not monocytes (Hughes, 1996), and that earlier identifications of TNF-α production by monocytes were likely due to T cell contamination of monocytes purified by adherence. Thus the OVX-induced increase in TNF-α levels is likely to be due to T cell TNF-α production.

Interestingly, in our experiment, both E_2 and genistein supplementations (OVX_{gen}, OVX_{E2}) could significantly prevent this increased TNF-α level at 3-wk as compared to OVX_{veh} (Table 4.3.2 and Figure 4.3.3). **Therefore, our finding has suggested that genistein has an estrogen-like action on suppressing the OVX-upregulated inflammatory response.** However, the mechanism of how E_2 represses as such spontaneous increase in pro-inflammatory cytokines has not yet been clarified, both genomic and nongenomic (or nongenotropic) have been referred to (Srivastava, 2001 and Kousteni, 2003).

Several investigations have confirmed this anti-inflammatory response of genistein (Fanti *et al.*, 1998; Binbin and Shifeng, 2003). For instance, the effect of *genistein* on lipopolysaccharide-induced *in vitro* production of TNF-α was tested in monocytic cells. Production of TNF-α was markedly elevated in OVX_{veh} as compared with the $Sham_{veh}$ rats, but this was inhibited by *genistein* in the OVX rats. (Fanti *et al.*, 1998)

Our results did not defined any changes of serum IL-6 concentration during the early period of 1-wk and 3-wk ovariectomy, as which they are different from other studies (Roodman, 1992 and Okumura, 2004). The reason might be resided with the different experimental period used. As our study performed during the transient period

or during the early change as 1-wk and 3-wk after ovariectomy, therefore, the production of intermediate form of cytokine, IL-6, may not increased as much yet.

Weitzmann and Pacifici (2006) have made an exclusive review that demonstrated the bone loss induced by E_2 deficiency which was due to multiple roles of hormones and cytokines that converge to disrupt the process of bone remodeling. They suggested that E_2 deficiency would lead to T cell- released IFN-γ, and also upswing in reactive oxygen species (ROS). The combined effect of IFN-γ and **ROS** markedly enhances antigen presentation, amplifying T cell activation and promoting release of the osteoclastogenic factors RANKL and TNF-α (Cenci, 2000; Cenci *et al.*, 2000). TNF-α further stimulates stromal cell and osteoblast, RANKL and M-CSF production, in part via IL-1 upregulation, driving osteoclast formation. The important summary was suggested in their article as shown in **Figure** 5.1. They implied that TNF-α and IL-7 further exacerbate bone loss by blunting bone formation through direct repressive effects on osteoblasts.

Actually, the idea of free radicals has been previously documented for their roles with TNF-α in bone loss resulting from ovariectomy, however, the different molecule, nitrogen reactive specie, RNS, has been referred to (Chen, 2003 and Salvatore, 2003). It was indicated that iNOS was a key signaling molecule in determining the imbalance of bone remodeling caused by E_2 depletion. Because iNOS which is a major inflammatory enzyme produced the accumulation of nitric oxide (NO), highly reactive molecules, and then resulted in triggering a local inflammatory reaction on bone and microvascular environment. (Schmidt *et al.*, 1992; Fox and Chow, 1998; Salvatore, 2003)

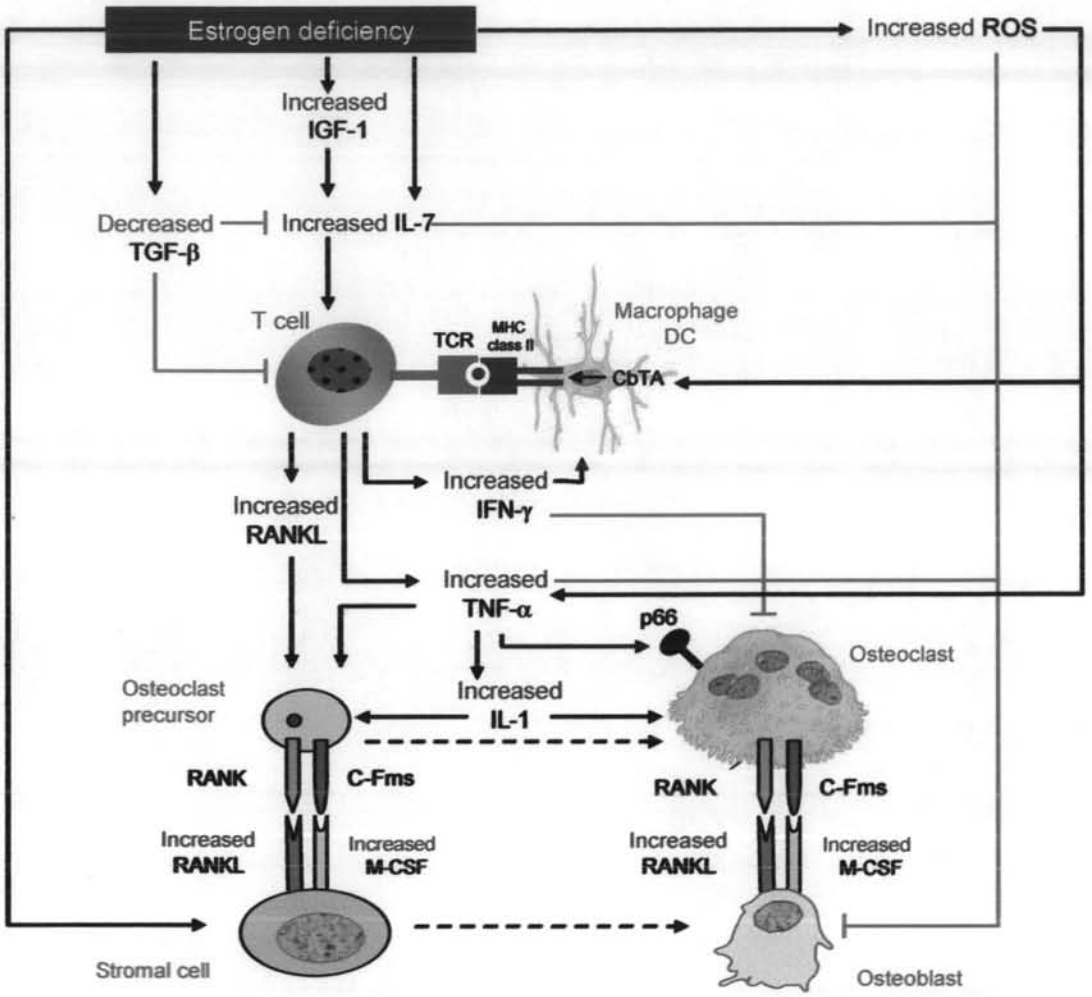

Figure 5.1 **The schematic representation showed the main mechanisms and** feedback interactions by which estrogen deficiency led to inflammatory **response and then consequently caused bone loss. (Modified from Weitzmann and Pacifici , 2006)**

At this point of view, we may give a simple conclusion that genistein supplementation could prevent the OVX-induced increase in serum TNF−α and then consequently inhibit bone loss may be contributed by both crucial roles of genistein: estrogenic and anti-oxidant.

5.5. Effect of genistein administration on serum VEGF concentration

In current study, serum VEGF level was significantly decreased in OVX_{veh} compared to $Sham_{veh}$ at both 1-wk and 3-wk after ovariectomy. Interestingly, this OVX-induced decreased serum VEGF was significantly prevented by both E_2 and genistein supplementation (Table 4.4 and Figure 4.4.1). Invasion of capillaries into the metaphyseal end of the growth plate was known to be an important step in endochondral bone formation, resulting in bone growth (Brighton, 1978). On the other hand, the contemporaneous expansion of the capillary network, providing microvasculature, is believed to be essential for the process of bone repair/remodelling. VEGF which is mediated vascular adaptation has been documented as a required factor for bone adaptation (Yao *et al.*, 2004 and Yen *et al.*, 2005). They also indicated that estrogen directly modulated angiogenesis via VEGF through its effect on endothelial cells and osteoblasts.

Recently, the experimental result by Yen and his co-workers (2005) indicated that diosgenin, phytoestrogen extracted from the root of wild yam, could up-regulates VEGF-A and promoted angiogenic activity in MC3T3-E1 cells in a HIF-1α–dependent manner involving the activation of Src kinase, PI3K/Akt, and p38 MAPK through an estrogen receptor. Yen et al. (2005) proposed the mechanism shown in **Figure** 5.2, which the idea was supported by previous study of Ferrara N, 2001. They found that VEGF receptor expression existed not only on vascular endothelial cells, but also osteoblasts. VEGF receptor-1 (VEGFR-1) expressed in osteoblasts massively. Harada *et al.*, (2003) also found that osteoblastic activity closely related to angiogenesis because VEGF mRNA was expressed in osteoblasts.

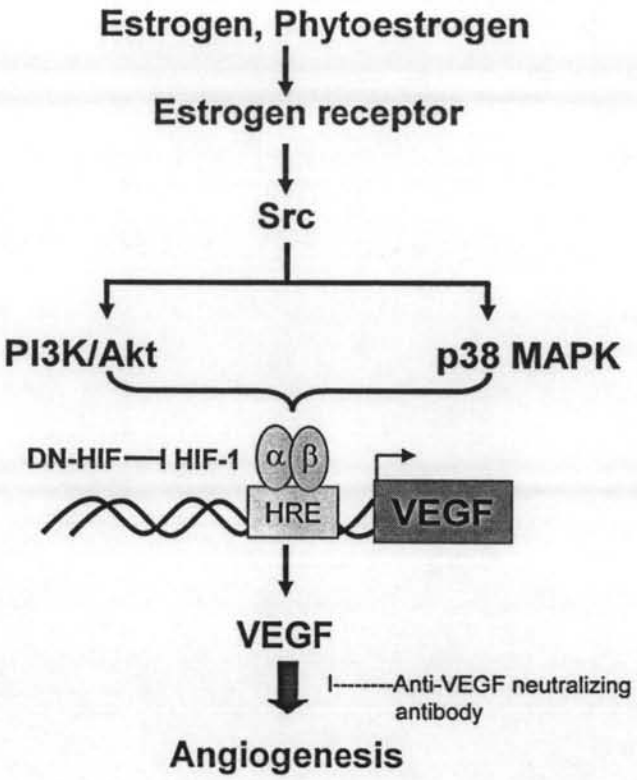

Figure 5.2 The propose mechanism of estrogen and phytoestrogens up-regulate VEGF-A and promote angiogenic activity through an estrogen receptor (Modified from Yen, 2005).

Finally, at this part it may conclude that our findings therefore have added more important evidence that genistein can also restore serum VEGF level in early phase of E_2 depletion. In other word, it may imply that genistein could up-regulate VEGF-A and promoted angiogenic activity through an estrogen receptor as well.

5.6. Effect of genistein administration on bone capillary density

Together with this result of VEGF, the same agreement for the benefit effects of genistein has been demonstrated by our result of bone capillary density. It should be noted that since one week after ovariectomy, the decrease in capillary density in OVX_{veh} was already existed. It continued to decrease until the 3-wk experimental period. Interestingly, our results also demonstrated that genistein could attenuate loss of

capillary density similar to E_2 supplementation.

Capillary density is the simplest indicator of the distribution of vessels and tissue perfusion. 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 (Vogt *et al.*, 1997) and bone mineral decreases in the leg with unilateral occlusive arterial disease. Moreover, macroscopic and histological evidence of the dependence of bone on an adequate vascular supply demonstrated that vascularized bone grafts, the interruption of blood supply to bone resulted in avascular necrosis. Osteocyte survival required a less than 0.1 mm proximity to nutrient vessels (Ham AW, 1952), and a close correlation exists between the rate of osteogenic bone deposition and the vascular surface area (Marotti G and Zallon AZ, 1980). Parfitt (2000), reported that stressed bone vessels might be involved in osteoprogenitor migration toward the newly activated bone multicellular units during bone remodeling. Barou O *et al.*, (2002), who has developed a technique allowing simultaneous visualization of blood vessels and bone cellular activities, found evidence for a relationship between vessel number and histodynamic bone formation parameter within the tibial metaphysis of male rats. Despite these consistent observations, relationships between bone vascular events and bone cellular activity changes in the pathophysiology of E_2 deficiency have been only rarely investigated.

In 2003, Mekraldi and co-workers (2003) reported that E_2 deficiency induced changes in bone vascularization which might be involved in bone loss mechanism. They evaluated bone and vessel histomorphometry in secondary spongiosae by infusing vessels with a mixture of India ink/barium sulfate after 7 and 14 days of OVX. They found that after 7 days expression of VEGF decreased concomitant with a decrease in the bone vessel number and possible area. 17β-estradiol administration for 7 or 14 days prevented not only the OVX-induced changes in bone remodeling but also the morphological alterations observed in bone vessels. It also prevented the alterations in the VEGF gene expression modified by OVX.

In our study, we also showed that genistein could prevent the reduction of capillary density since 1 week and 3- wk after ovariectomy and also prevent the decrease in VEGF level, like estrogen did. Therefore, it may be said that genistein could attenuate as such loss of capillary density similar to E_2 supplementation.

Similar to the previous studies (Khemapech *et al.*, 2003 and Siriviriyakul *et al.*, 2006), we also showed that genistein could prevent endothelial dysfunction and bone loss at 3-wk and 6-wk, respectively. *Therefore, it made us to believe that the abnormal bone remodeling may have a possible closed link to bone vascularization.*

5.7. The relationship between serum VEGF, bone capillary density, and bone mineral content

The positive linear relationships were significantly indicated for the following parameters: a) between VEGF and bone capillary density, b) between VEGF and bone mineral content, and c) between bone capillary density and bone mineral content. These findings importantly remark a **functional link** between bone microcirculation and bone remodeling.

Several lines of evidence in the literature have demonstrated ***the important causal link between angiogenesis and bone formation activity;*** for instance in the cases of : long bone development, healing of a cortical bone defect, and osteoblast differentiation and chemotactic migration (Tatsuyama K *et al.*, 2000, Street J *et al.*, 2002, Deckers MM *et al.*, 2000)(Wang DS *et al.*, 1997 and Mayr-Wohlfart U *et al.*, 2002).

Indeed, osteoblasts metabolically demanding cells with high levels of energy consumption and therefore require an adequate blood supply. They are also thought to be sources of vasoactive factors, such as nitric oxide, which are local regulators of blood flow. Moreover, Dulak *et al.* (2000) reported that endogenous NO enhances VEGF expression by vascular smooth muscle cells. The induction of VEGF synthesis by NO

may be of great importance in the maintenance of vascular homeostasis. Because NO and VEGF reciprocally enhance their synthesis, this interaction may play a significant role in maintain bone microvasculature after E_2 depletion.

Even though the effects of NO on proinflammatory cytokines and cytokine-dependent tissue activities are still controversy, it has been suggested that iNOS pathway is essential for IL-1-induced bone resorption(Van't Hof *et al.*, 2000).

In contrast, NO donors have been rather shown to reverse ovariectomy-induced bone loss in rats, and nitrate use appears to protect postmenopausal women against bone loss as effectively as E_2 use (Jamal *et al.*, 1998; Wimalawansa, 1996; Wimalawansa, 2000; Wimalawansa, 2000).

E_2 depletion had caused bone microvascular dysfunction as indicated by increased TNF-α and enhanced capillary density rarefication. Our findings demonstrated that abnormalities in microvascular geometry are detectable in ovariectomized rats. Since one week after ovariectomy was found decrease of capillary density in OVX_{veh}. Interestingly, at 3-wk after ovariectomy capillary density was significantly higher in genistein treated than OVX_{veh}. Furthermore, an increased of capillary density was related to an increased of serum VEGF level as well as a reduced of osteocalcin, alkaline phosphatase and TNF-α. In addition, there were consistent with restore bone mineral content after E_2 depletion.

Therefore, the complex interaction between bone and microcirculation enables us to evaluate physiological and pathological processes of bone given better understanding for a correlation between blood supply and bone remodeling in postmenopausal. Moreover, the cytokines and growth factors that regulate intraosseous angiogenesis also regulate bone remodeling, and close links exist between the blood supply to bone and bone formation and resorption. (Laroche *et al.*, 2002)

As regard the impact of microvasculature on bone physiology, in any healthy organs, vascular system provides a major component of homeostasis by exchanging heat, metabolic molecules, and also cells. In addition, oxygen delivery is determined mainly

by blood flow perfusion and hematocrit. Exchange of other nutrients, waste, hormones and even cytokines is determined mainly by vessel supply and permeability.

Based on our results, in particular from the test of relationships, it **might** be simply concluded that during E_2-depletion, the pathogenesis of osteoporosis is mutlicellular basis involving : various hematopoietic and immune cells, bone cells (OB and OC), endothelial cells, and smooth muscle cells. Even though the complete mechanisms have not been clarified but at least they are potentially included the activation and inhibition effects of E_2 with : those cells transcription factors, nitric oxide activity (eNOS and iNOS), antioxidative effects (ROS and reactive nitrogen species (RNS)), plasma membrane actions, and changes in immune cell functions.

5.8. The proposed mechanism for our findings on protective roles of genistein in E_2 depletion caused bone loss

Up to our knowledge, we believe that our data should be the first one that implied the *in vivo evidence based* demonstrating the correlation between microvascular function and bone remodeling during the early phase of E_2 depletion. In particular, our results have shown that E_2 depletion caused bone microvascular dysfunction **prior** the bone loss, even biochemical marker changes, osteocalcin level. Therefore, the key point of this finding was actually demonstrated the close link between the microvascular function and bone remodeling based on the potential actions of E_2.

Regarding the mechanism of how these phenomenon occur, we would like to make remark that the abnormalities after ovariectomy could be attenuated by administration of genistein, effectively for both microvascular dysfunction and bone loss.

Therefore, it is concluded that genistein might have a potential partly to use as a therapeutic agent in postmenopausal women in the future. And finally based on the overall results and discussion above, we would like to propose the possible mechanism displayed how genistein could exhibit the preventive effect during the early phase of ovariectomized rat model (**Figure 5.3**).

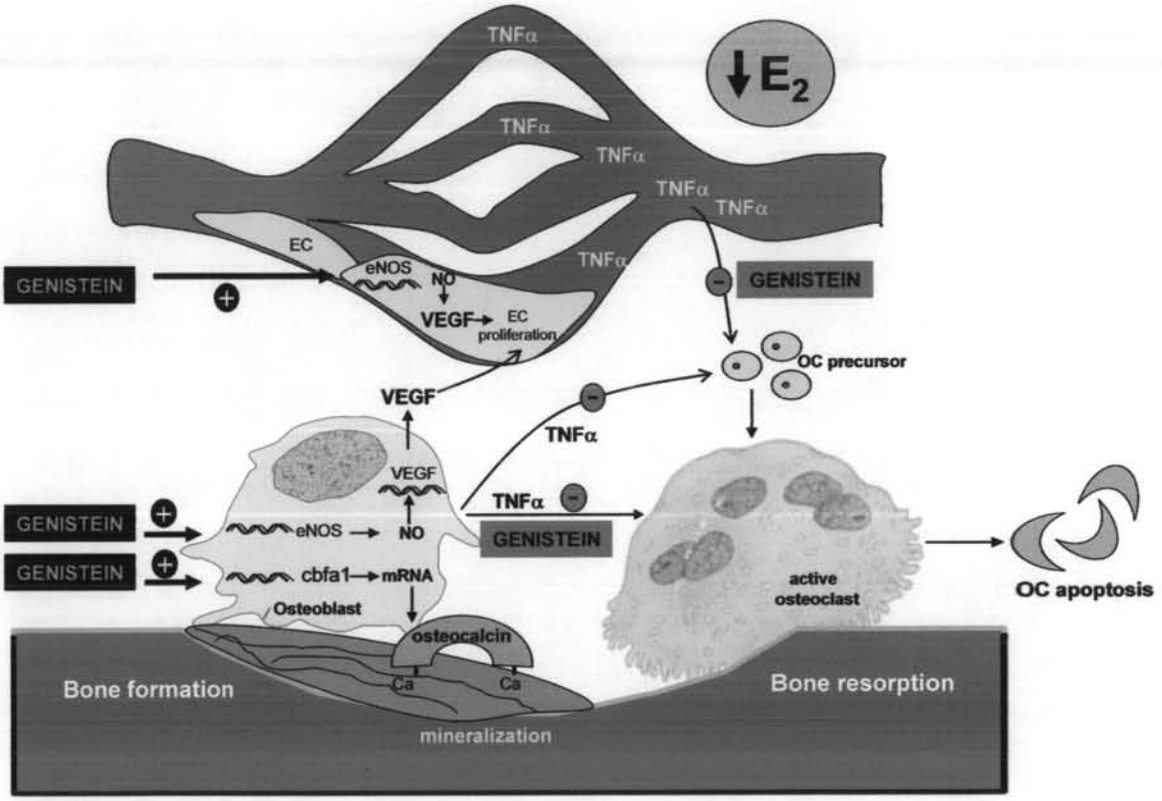

Figure 5.3 The mechanisms (proposed) for the preventive effect of genistein on bone microvasculature and remodeling are as followings: During the early phase (1-wk - 3-wk) of E_2 depletion: a) The increase in the common factor, TNF–α which is mostly referred as an indicator linked between E_2 -depletion and inflammatory response was identified. b) The increases in biochemical markers of "transient" state, osteocalcin and alkaline phosphatase were existed. c) However, the decrease in serum VEGF expression together with the decrease in bone capillary density was found. e) Bone loss was then indicated significantly after at 6-wk ovariectomy.