CHAPTER V

DISCUSSION AND CONCLUSION

Pueraria mirifica, a phytoestrogen containing herb, has been used as a traditional medicine and exhibits comparable effects to estrogen in aging people. It is well established that estrogen replacement therapy (ERT) can lower the incidence of cardiovascular disease and osteoporosis in postmenopausal women. This research was aimed to investigate the protective effects of a *P. mirifica* preparation on vascular functions and bone turnover in ovariectomized rats model. Contractile and relaxation response of isolated thoracic aorta were determined by isolated organ bath technique. Pathologic examination of the vascular tissue, NO production and correlated lipids profile in blood were also evaluated. Biochemical markers of bone turnover including alkaline phosphatase and NO production in blood were used as indicators of bone remodeling status. Bone mass and pathologic examination of femoral bones were used as indicators of bone structure. In addition, estrogen and *P. mirifica* exert their protective effects on vascular and bone via a nitric oxide dependent mechanism are also discussed.

As suggested in recent studies, the ovariectomized rat is a standard model to study the development of vascular dysfunction (Moien-Afshari et al., 2003) and osteoporosis associated with endogenous estrogen deficiency (Coxman et al., 1996; Yang et al., 2005).

1. Effects of *Pueraria mirifica* on body weight gains in ovariectomized rats

The body weight gain of untreated ovariectomized rats was significant higher than that of the non-ovariectomized rats. The average of the body weight gain of the ovariectomized rats receiving *P. mirifica* or estradiol valerate were significant lesser than the untreated ovariectomized group and non-ovariectomized group. Previous studies demonstrated that estrogens play an important role and exhibit many effects on lipid metabolisms and lipid accumulation in body compartments (Loose-Mitchell and Stancel, 2001). Estrogen deficit animals are found to be increase in body weight, fat deposition and feed intake. It was reported previously that estrogen caused a retard in growth rate of rats and mice associated with feed consumption decreasing (Gibson et al., 1967; Heywood and Wadsworth, 1980; Hart, 1990; Biegel et al., 1998). So far, the effects of estrogen on body fat are not clearly understood. Supplementation of phytoestrogens or exogenous estrogens has been reported to improve lipid metabolism and alleviate increasing of body weight and fat deposition. Beside these, some phytoestrogens containing in *P. mirifica* such as genistein and daidzein were found to suppress feed intake and body weight gains in rats (Magee, 1963; Toda et al., 1999) by decreasing of cortisol synthesis. Cortisol is found to be responsible for feed appetite stimulation (Mesiano et al., 1999).

2. Effects of *Pueraria mirifica* on vascular functions and pathogenicities of thoracic aortas in ovariectomized rats

Endothelial dysfunction is one of the earliest pathological expressions occurring after organ ischemia and balance between vasorelaxation and vasoconstriction can be disturbed. Ovariectomy causes endothelial dysfunction is associated with estrogen deficit (Moien-Afshari et al., 2003). Since abnormalities in endothelial-dependent arterial relaxation develop early in the course of atherogenesis,

vascular responses to an endothelium dependent vasodilator, acetylcholine and endothelium-independent vasodilator, NO donors were compared. In this study, treatment of ovariectomized rat with estrogen or *P. mirifica* resulted in improvement of the cholinergic response, endothelium-dependent relaxation, of the aorta. Similar results were demonstrated also in ovariectomized rabbits (Auttapongpaiboon, 2002; Wattanapitayakul et al., 2005). Interestingly, it was observed in this study that ovariectomy resulted in attenuation of the maximum contractile response of the thoracic aorta induced by high concentration of noradrenaline. Adrenergic stimulation can induce both contractility and relaxation. Stimulation of α1-adrenoceptors leads to contraction by inhibition of adenylyl cyclase through inhibitory G-proteins. Ligand occupation of ß2-adrenoceptors activates adenylyl cyclase via stimulatory G-proteins, increase cAMP and induces relaxation. Stimulation of α2-adrenoceptors in the rat superior mesenteric artery leads to the release of NO and causes NO-mediated relaxation (Bockman et al., 1996). The adrenergic responses in some tissues are modulated by the hormonal environment. Under estrogen predominance, ß2-adrenoceptors-mediated cAMP productions are reduced and contraction prevails, whereas progesterone-predominance favors relaxation (Gsell et al., 2000). In addition, down regulation of α2-adrenergic receptor in estrogen treated ovariectomized rat was found to be related to an increase in vascular contraction response to noradrenaline (El-Mas and Abdel-Rahman, 2004). Accordingly, NA causes an endothelium-dependent contraction mediated by α1-adrenoceptors and that contraction is due to the endothelium-derived contracting factor (EDCF), thromboxane A_2 (Usui et al., 1987), and the involvement of COX metabolites (arachidonic acids) in the NA-induced contraction is also endothelial dependent (Wang et al., 2005). Accordingly, it was recently reported that estrogen treated ovariectomized rat demonstrated an increase in expression of cyclooxygenase-II, thromboxane synthase and thromboxane receptor. A recent study found also that increased vasopressin-induced contraction via constrictor prostanoids, $PGF_{2\alpha}$ and TXA_2 was exhibited in estrogen-treated

ovariectomized rats (Li and Stallone, 2005). Taken together, the data obtained from measurements of vascular functions indicated that treatment with *P. mirifica* or estrogen can preserve endothelial functions and restore balance between vasorelaxation and vasoconstriction in ovariectomized rats.

The changes in vascular functions were also in accordant with the structural changes of vessel demonstrated by the pathological examination. In addition, the improvement of the vascular response of endothelium dependent was well correlated with the increasing of blood NO production found in *P. mirifica* or estrogen treated groups. These results demonstrated also that estrogens or *P. mirifica* exerted significant protective effects on endothelial functions. Since endothelial dysfunction manifested as decreased bioactive NO level and contributes significantly to subsequent functional and cellular injury, treatment with estrogen or *P. mirifica* can also restore NO production and improve the endothelium-dependent vasorelaxation. Our results supported many studies reported recently that NO production by endothelial cells is significantly decreased in postmenopausal women and in ovariectomized animals, and that estrogen significantly enhances NO production from endothelial cells (Rahimian et al., 1997; Auttapongpaiboon, 2002; Catania et al., 2002). In the present study, in ovariectomized rats, NO production is markedly decreased compared with non-ovariectomized rats and restored to normal level by treatment with *P. mirifica* or estrogen. These results indicated that *P. mirifica* significantly preserved in vivo basal NO production in ovariectomized rats. The exact mechanism by which *P. mirifica* may exert its basal NO restoration effect can not be determined in this study. However, it is conceivable that NO production is likely achieved through an up-regulation of NO producing enzymes, nitric oxide synthase (NOS) or prevention of NO destruction by reactive oxygen species. It was previously reported that estrogen increases NO production via a traditional genomic pathway that directly enhances NOS activity (Kim et al., 1999) and via a prevention of NO destruction by reactive oxygen species (Hwang et al., 2003). Several studies supported the stimulatory effects of

17ß-estradiol on several isoforms of nitric oxide synthase including inducible nitric oxide synthase (iNOS), upregulate the transcription of constitutive nitric oxide synthase (cNOS) in endothelial cells (Holm et al., 1997; Binko and Majewski, 1998).

Phytoestrogens found in *P. mirifica* including isoflavones and coumestrols restore the endothelial functions by improving of NOS enzyme activity and increase resistant to LDL-cholesterol oxidation (Setchell, 1998). It was studied in ovariectomized animals and found that anti-atherogenic effects of phytoestrogens and estrogens are due to their antioxidative activities (Lissin and Cooke, 2000). It was demonstrated recently that estrogen inhibits the mitochondrial production of free oxygen molecules (Calif, 2005). Previous studies have shown that excessive amount of free radicals in the body, through oxidative stress process can damage endothelial cell and lead to atherosclerosis. Estrogens prevent the oxidative modification of LDL-cholesterol to oxidized LDL-cholesterol ($_{OXD}$LDL-C), the key component in endothelial injuries. The $_{OXD}$LDL-cholesterol plays an important role in endothelial injuries and initiates the migrations of monocytes and T lymphocytes into subendothelial spaces. These are the causes of foam cell formation, calcification and myointimal proliferation into vascular lumen that lead to atherosclerosis (Arnal et al., 2004). In the present study, total cholesterol especially, the LDL-cholesterol of untreated ovariectomized rats was found to be higher than those in normal control group. Whereas, there were no significantly differences of lipid parameters between *P. mirifica* treated and untreated ovariectomized rats, triglyceride level was significantly greater in the group of ovariectomized rats treated with estrogen in comparison to all other groups. The influence of estrogen on high triglyceride level was previously reported in the same experimental model. It was found that castration of male rat reduced triglyceride while neutered female is without effect. In addition, they reported further that estrogen administration to ovariectomized rats resulted in increase of blood triglycerides levels (Hamosh and Hamosh, 1975). Eventhough, there were tendency of triglyceride lowering and HDL-C increasing effect of *P. mirifica* as

demonstrated in its treated group, these results were not significant different compared to the ovariectomized nontreated group. The effects of *P. mirifica* on lipid profiles in ovariectomized rats presented in this study are similar to the previous report studied in ovariectomized rabbits (Auttapongpaiboon, 2002). The increases in lipid parameters in ovariectomized rats are due to the loss of lipid metabolism control in which endogenous estrogens play an important role. To compare with the estrogen treated group, treatment with *P. mirifica* in ovariectomized rats showed its favorable effect on lipid profiles, especially, the triglyceride lowering effect. Increase blood triglyceride level is in part a result of increased VLDL-TG secretion, an effect mediated by estrogen (Walsh et al., 1991). A clinical report showed that oral estrogen increase triglyceride levels in some patients. However, it was discussed that in most patients this effect was considered to be not a problem because of the favorable effects on HDL-C and LDL-C of estrogens (Walsh et al., 1991; Wakatsuki et al., 1998; Wakatsuki et al., 2001). Since hypertriglyceridemia is also associated with atherosclerosis, even in the absence of hypercholesterolemia. It can also lead to pancreatitis in excessive concentrations. Thus, the TG lowering effect of phytoestrogens in *P. mirifica* tablet exhibit more beneficial effects on lipid metabolism control comparing with the conventional estrogen replacement.

3. Effects of *Pueraria mirifica* on bone mass and pathogenicities of femoral bones in ovariectomized rats

Alkaline phosphatase (ALP) was used as biochemical markers of bone turnover in this study. The primary importance of measuring ALP is to check the possibility of bone disease or liver disease. It has been used as indicators of bone remodeling status. In the first decade after the onset of menopause, there is an increase in level of osteocalcin, bone specific alkaline phosphatase (bALP) reflecting the increase in bone turnover (Riis, 1991). These levels can be reduced after treatment with hormone

replacement therapy (Johansen et al., 1988). The present study provides also the evidence of increased ALP level in ovariectomized rats indicating the increase in bone turnover occurred also in the early phase of estrogen deficiency. Treatment with *P. mirifica* or estrogen resulted in reduction in ALP in blood shows the beneficial effects of estrogen or *P. mirifica* on bone. However, according to the method used in this study, the ALP measured in blood was the total amount of ALP release from various tissues into the blood. Thus, another measurements including NO production, relative bone weight, relative ash weight, calcium content as well as the pathological examination of femurs were also used to evaluate the bone sparing effect of *P. mirifica* and estrogen replacement therapy in this study.

Ovariectomy resulted in decrease of relative bone weight, ash weight and a tendency of low calcium content in femur bone. These results were corresponded with a previous reports studied in ovariectomized rats (Uchiyama et al., 2002; Hao et al., 2005). According to the microscopic examination, femoral bones of the ovariectomized rats revealed bone degeneration and necrosis. Treatment with *P. mirifica* or estrogen in ovariectomized rats showed the lower degree of bone degenerative changes. It has been accepted that endogenous estrogens exhibit bone protecting effects. Estrogen depletion in post menopausal women can lead to bone loss. Endogenous estrogens control bone turnover rate by increase ratio of bone formation and decrease bone resorption (Loose-Mitchell and Stancel, 2001). Treatments of 17ß-estradiol or phytoestrogens such as isoflavones in postmenopausal women were reported to elevate bone mass and prevent pathological bone fractures (Gass and Dawson-Hughes, 2006). Accordingly, *Curcuma comosa*, the phytoestrogens containing herb can protect against bone loss associated with estrogen deficiency (Amornratanayut, 2006). The effects of estrogens and phytoestrogens on bone are theoretically supposed to be the direct effects on the bone cells, mainly consist of osteoblasts and osteoclasts, which contain both type of estrogen receptors (Onoe et al., 1997). The decrease of bone resorption is due to the lowering activity of

osteoclasts. Estrogen treatment resulted in decrease of osteoclasts-stimulating cytokines production (interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrotic factor-alpha (TNF-α) etc.) and increase of bone anti-resorptive factor production (insulin-like growth factor-1 (IGF-1), bone morphogenic protein-6 (BMP-6), transforming growth factor-beta (TGF-ß) etc.). In addition, the increasing bone formation is primarily due to the mechanisms concerning to an increased osteoblasts activities such as the increase of osteoblasts differentiation and proliferation, the production of osteoclasts-inhibiting factors (osteoprotegrin (OPG) etc.) from osteoblasts (Loose-Mitchell and Stancel, 2001).

Recently, it has been suggested that the physiological effects of estrogen and mechanical stress on both cardiovascular function and bone turnover are exerted, at least in part via elevation of nitric oxide synthesis. Postmenopausal women have reduced serum NO levels and both short- and long-term estrogen replacement therapy elevate these levels (Wimalawansa, 2000a). There is clear evidence that estrogen can induce endothelial NOS in a variety of cells, including those of bone (Fox et al., 1998; Samuels et al., 2001). Endothelial NOS and inducible NOS were found in osteoblast-like cells associated with calcify cartilage spicules in the distal femoral metaphysia (Hukkanen et al., 2003). Previous studies of Wimalawansa et al. (2000b) in ovariectomized rats showed the involvement of NO on skeletal homeostasis. NO counteracts the bone loss associated with ovariectomy. According to recent studies, treatment with low-dose and middle dose of nitroglycerine, NO donor, prevented ovariectomy induced bone loss. Nitroglycerine reversed the ovariectomy induced bone loss in rats related with increasing of bone mineral density (Hao et al., 2005).

According to the results from this study, the increase of NO production found in *P. mirifica* and estrogen treatment in the ovariectomized rats may exert their protective effect on bone and vascular through enhanced antioxidant activity in endothelial cell. It was reported recently that decreased oxygen free molecules production resulted in increased NO levels indicating the antioxidant activity of

phytoestrogens (Hwang et al., 2003). The phytoestrogens in *P. mirifica* reduced plasma total cholesterol, triglyceride and LDL-cholesterol significantly in hypercholesterolemic rabbit and increase the oxidation resistance of LDL-cholesterol (Ratanachamnong et al., 2000).

In conclusion, the results of this present study demonstrated the benefit of lower body weight gains obtained from the treatment with *P. mirifica* in ovariectomized rats. The improvement of endothelium-dependent vascular relaxation in ovariectomized rats was due to the beneficial effects on lipid profiles and endothelial cells preservation. Improvement of endothelial dysfunction which was correlated with an increase of NO production in blood was considered to be the results of the antioxidant effects of *P. mirifica* in comparison with estrogen. These are involved in atherosclerosis prevention and restoration of vascular smooth muscle co-ordination in arterial wall and balance in adrenergic response of vascular contraction. In addition, the preventive effects of bone loss associated with estrogen deficiency of *P. mirifica* are considered to be the antioxidant properties or the direct effects of its phytoestrogens on bone cells.

The results in the present studies provided the evidences that *P. mirifica* may exert estrogen-like effects. The data supports the beneficial effects and knowledge in using of *P. mirifica* in Thai traditional medicine. The efficacy of using *P. mirifica* as an alternative hormone replacement therapy (HRT) in postmenopausal women is also demonstrated. In order to clarify the exact mechanisms of its protective effects on vascular and bone in aging women, further studies of *P. mirifica* effects on estrogen receptors in various tissues as well as its antioxidant properties should be examined.