ROLES OF ESTROGEN ON SENSORY NERVE INNERVATION OF BONE IN OSTEOPOROSIS-INDUCED FEMALE RATS

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บทบาทของฮอร์โมนเอสโตรเจนต่อเส้นประสาทรับความรู้สึกของกระดูกในหนูขาวเพศเมียที่ถูกเหนี่ยวนำให้เกิดภาวะกระดูกพรุน

นางสาว พิมพ์วิภา เอื้อศิลามงคล

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พิมพ์วิภา เอื้อศิลามงคล : บทบาทของฮอร์โมนเอสโตรเจนต่อเส้นประสาทรับความรู้สึกของกระดูกใน
หนูขาวเพศเมียที่ถูกเหนี่ยวนำให้เกิดภาวะกระดูกพรุน (ROLES OF ESTROGEN ON SENSORY
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จุดประสงค์ของงานวิจัยในครั้งนี้เพื่อศึกษาถึงลักษณะของเส้นประสาทชนิดรับความรู้สึกที่อยู่ในกระดูก
ของหนูขาวเพศเมียที่ถูกเหนี่ยวนำให้เกิดโรคกระดูกพรุนจากภาวะพร่องฮอร์โมนเอสโตรเจนและผลของการ
ให้ฮอร์โมนเอสโตรเจนทดแทน หนูขาวสายพันธุ์วิสตาร์เพศเมียอายุ 6 เดือน จำนวน 60 ตัว แบ่งออกเป็น 5
กลุ่มๆละ 12 ตัว กลุ่มที่ 1-4 ได้รับการผ่าตัดเอารังไข่ออกทั้งสองข้างและได้รับการฉีดสารที่ใช้ทำละลาย หรือ
ฮอร์โมนเอสโตรเจนเข้าไปทดแทนที่ปริมาณ 1, 5 และ 15 ไมโครกรัมต่อกิโลกรัมน้ำหนักตัว เข้าใต้ผิวหนังทุก
วัน ตามลำดับ กลุ่มที่ 5 เป็นกลุ่มควบคุมที่ได้รับการผ่าตัดเช่นเดียวกันแต่รังไข่ทั้งสองข้างไม่ได้ถูกตัดออกไป
หลังจากได้รับการทดสอบเป็นเวลาติดต่อกัน 90 วัน โดยทุกวันมีการวัดปริมาณอาหารที่หนูขาวกินและชั่ง
น้ำหนักตัว เมื่อสิ้นสุดการทดสอบหนูขาวทั้งหมดถูกทำการุณยฆาตโดยการเจาะเลือดออกจากหัวใจ ทำการเก็บ
ตัวอย่างเลือดเพื่อนำมาวัดระดับของแคลเซียมและฟอสฟอรัสในซีรั่ม เลือดบางส่วนถูกแยกเก็บซีรั่มเพื่อนำมา
หาระดับอัลคาไลน์ฟอสฟาเตสจากกระดูก ทำการแยกเก็บมดลูก และชั่งน้ำหนักทันที กระดูกต้นขาด้านซ้ายถูก
แยกเก็บและรักษาไว้ที่อุณหภูมิเยือกแข็งแล้วนำมาหาค่าความหนาแน่นของมวลกระดูกและค่าแคลเซียมที่อยู่
ในกระดูก ส่วนกระดูกต้นขาด้านขวาถูกนำมาเตรียมศึกษาลักษณะของเส้นประสาทชนิดรับความรู้สึก โดย
วิธีอิมมูโนฮิสโตเคมมิสทรี จากผลการศึกษาพบหนูที่ตัดรังไข่และไม่ได้รับฮอร์โมนทดแทนมีอัตราการเพิ่ม
ของน้ำหนักตัวเฉลี่ยในแต่ละวันมากกว่าหนูที่มีรังไข่ โดยที่มีอัตราการกินอาหารเฉลี่ยไม่แตกต่างกัน ($p<0.05$)
และผลดังกล่าวลดลงเมื่อได้รับฮอร์โมนเอสโตรเจนทดแทนที่ 1, 5 และ15 ไมโครกรัมต่อกิโลกรัมน้ำหนักตัว
การศึกษาความหนาแน่นทั้งหมดของมวลกระดูกต้นขาพบว่าหนูที่ตัดรังไข่และไม่ได้รับฮอร์โมนทดแทนมีค่า
มวลกระดูกทั้งหมดลดลงโดยที่ไม่มีการเปลี่ยนแปลงของระดับแคลเซียมในกระดูก แต่พบว่าระดับอัลคาไลน์
ฟอสฟาเตสที่จำเพาะต่อกระดูกซึ่งเป็นค่าที่บ่งชี้ถึงกระบวนการผลัดเปลี่ยนเนื้อกระดูกมีค่าสูงสุดในหนูกลุ่มนี้
ผลการทดลองแสดงให้เห็นว่ามีการเสียหายของเนื้อกระดูกเกิดขึ้นภายหลังจากที่ได้รับการตัดรังไข่เป็นเวลา 90
วัน และจากการแยกวัดความหนาแน่นของมวลกระดูกในแต่ละส่วนของกระดูกต้นขาของหนูที่ไม่มีรังไข่
พบว่าบริเวณที่ปลายกระดูกด้านล่างมีค่าต่ำที่สุด ผลการศึกษาโดยวิธีอิมมูโนฮิสโตเคมมิสทรีโดยการย้อมด้วย
แอนติบอดีต่อตัวบ่งชี้ของระบบประสาท(พีจีพี 9.5) และแอนติบอดีต่อสารสื่อประสาทที่สร้างจากระบบ
ประสาทรับสัมผัส แคลซิโทนินยีนรีเรทเทตเปปไทด์ (ซีจีอาร์พี) หรือ ซับสแตนด์ พี ผลการทดลองแสดงให้
เห็นว่ามีการลดลงของการปรากฏของปฏิกิริยาทางอิมมูนของทั้งแอนติบอดีสามตัวในส่วนของกระดูกต้นขา
ด้านปลายล่างซึ่งเป็นบริเวณที่มีการลดลงของมวลกระดูกของหนูที่ไม่มีรังไข่ แต่ในส่วนของกระดูกต้นขาด้าน
ปลายบนนั้นถึงแม้ว่ามีการลดลงของการปรากฏของปฏิกิริยาทางอิมมูนของพีจีพี 9.5 หรือซับสแตนด์ พี แต่
กลับมีการเพิ่มมากขึ้นของปฏิกิริยาทางอิมมูนซีจีอาร์พีโดยที่บริเวณนี้มวลกระดูกของหนูที่ไม่มีรังไข่และไม่ได้
รับฮอร์โมนทดแทนไม่มีความแตกต่างจากหนูที่มีรังไข่ ผลการเปลี่ยนแปลงดังกล่าวของทั้งสองบริเวณ
สามารถป้องกันได้ด้วยการให้เอสโตรเจนทดแทนที่ 1 ไมโครกรัมต่อกิโลกรัมน้ำหนักตัว ผลจากการศึกษาครั้ง
นี้แสดงให้เห็นถึงความสำคัญของระบบประสาทชนิดที่มีสารสื่อประสาทชนิดซีจีอาร์พีว่ามีความสำคัญต่อการ
ป้องกันการเสียหายของมวลกระดูก

สาขาวิชา................ศรีรวิทยา…….…......ลายมือชื่อนิสิต..
ปีการศึกษา..................2551.......................ลายมือชื่ออาจารย์ที่ปรึกษาหลัก...
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม...

4889111620   : MAJOR PHYSIOLOGY
KEY WORD   : ESTROGEN / SENSORY NERVE INNERVATION /
OSTEOPOROSIS / OVARIECTOMIZED RATS / CALCITONON GENE-
RELATED PEPTIDE / SUBSTANCE-P

       PIMWIPA UEASILAMONGKOL: ROLES OF ESTROGEN ON SENSORY NERVE INNERVATION OF BONE IN OSTEOPOROSIS– INDUCED FEMALE RATS. THESIS PRINCIPAL ADVISOR: ASSOC. PROF. SUTTHASINEE POONYACHOTI, D.V.M., Ph.D. THESIS CO-ADVISOR: ASST. PROF. NARATTAPHOL CHAROENPHANDHU, MD, Ph.D. 72 pp

       The aim of this study was to investigate the presence of sensory nerve innervation in bone of osteoporosis-induced female rats and the effect of estrogen supplementation. Sixty female Wistar rats aged 6 months were randomly assigned into 5 groups. Group 1-4, which were OVX-, E_2 1μg, E_2 5 μg or E_2 15 μg, underwent bilateral ovariectomy and received daily subcutaneous injection of 10% DMSO in propelyne glycol the drug vehicle, 1, 5 or 15 μg/kg BW of 17-β-estradiol, respectively. Group 5 was sham operated received the same treatment as ovariectomy group but ovaries were not removed. All of them were treated for ninety consecutive days. During the treatment, daily feed intake and body weight were measured. At the end of the treatment, all rats were anesthetized and sacrificed by cardiac puncture. Blood sample was collected to analyze for calcium and phosphorus concentration in serum. Some part of blood sample was allowed to clot, and serum was collect and kept at -20 ^{o}C for bone-specific alkaline phosphatase (AP) measurement. Uterine weight was determined immediately after removal. The left femur was kept at -20 ^{o}C for measurement of bone mass density (BMD) and calcium content using dual-energy x-ray absorptiometry (DEXA) and atomic absorption spectrophotometry, respectively. The right femur was prepared for immunohistochemistry study (IHC). We showed that body weight gain with no alteration of feed intake in OVX group was increased compared with sham group ($p < 0.05$). The changes were prevent by daily estrogen injection at dose of 1, 5 or 15 μg/kg BW ($p < 0.05$). Ovariectomy for 90 days without estrogen supplementation in the present study of pubertal rats significantly reduced the total bone mass density but not bone calcium content. The bone turnover marker, both total and bone specific AP were highest in OVX group. The evidence suggested that bone loss was evident after 3 months ovariectomy. The part of femoral bone of the OVX group that predominantly showed lowest BMD was the distal part. The IHC was also performed by using the neuronal and sensory neurotransmitter marker antibodies, anti-PGP 9.5 and anti-calcitonin gene related peptides (anti-CGRP) and anti-substance P antibody, respectively to stain on the semi-thin section of femoral bone, followed with an appropriate secondary antibody conjugated with fluorochrome, FITC or Texas red. The IHC results revealed a dramatic decrease of PGP-9.5, CGRP and SP-immunoreactivity (ir) in the distal part of femur of OVX group. The up-regulation of CGRP- ir but not SP-ir was indicated in the proximal part of femur which did not show evident of bone loss in the OVX group. All of these changes prevented by daily estrogen injection (1 μg/kg BW). The results of this study indicated the significance of nerve containing CGRP in the regulation of bone metabolism to protect against bone loss.

Field of study…….Physiology…..Student's Signature...…………………...............

Academic year…......2008………..Principal advisor's Signature...………………..

             Co-advisor's Signature…...……..………………

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LIST OF ABBREVIATIONS

AP   Alkaline phosphatase

BSA   Bovine serum albumin

BW   Body weight

Bone-AP   Bone specific alkaline phosphatase

Con   Control

CGRP   Calcitonin gene related peptide

CGRP-ir   Calcitonin gene related peptide immunoreactivity

DEXA   Dual energy x-ray absorptiometry

DFI   Daily food intake

DMSO   Dimethyl sulfoxide

DWG   Daily weight gain

E_2 17β-estradiol

ER   Estrogen receptor

ER-α   Estrogen receptor alpha

ER-β   Estrogen receptor beta

hr   Hour

μg   Microgram

M   Molar

mg   Milligram

min   Minute

ml   Milliliter

OVX   Ovariectomy

PGP 9.5   Protein gene product 9.5

PGP-ir   Protein gene product 9.5 immunoreactivity

PBS   Phosphate- Buffered Saline

ROI   Region of interest

S.E.M.   Standard error of mean

SP   Substance P

SP-ir   Substance P immunoreactivity

UW   Uterine weight

CHAPTER I

INTRODUCTION

Osteoporosis is a bone disease in which the amount of bone mass is decreased and the structural integrity of bone architecture is impaired. Cortical bone becomes more porous and thinner. This makes bone weaker and more likely to fracture. However, bone loss is greater in post menopausal women than in men of the same age. Estrogen deficiency was first recognized in the postmenopausal osteoporosis by Albright and colleagues in 1941. Their observations provided the rationale for the widely use of estrogen hormone replacement therapy (HRT) in prevention or alleviation of the untreatable osteoporosis. In order to successfully prevent bone loss and avoid unpleasant effects of chronic HRT such as breast cancer or venous thrombosis, a number of alternative of disease managements and the use of non-sex steroid treatments especially exercise, anti-bone resorption drugs (Biophosphonates; Fosamax® and Actonel®) and Calcitonin (Miacalcin® nasal spray) have been suggested (Miller, 2001). No single medication is suitable for everyone, each has its own risks and benefits. However, the mechanisms by which the skeletal effects of sex steroids are mediated are incompletely understood and are the subject of enormous research effort.

Basically, estrogen plays an important role not only in the reproductive tissue but also in non-reproductive tissues such as nerves and bone by acting directly or indirectly on its receptors and related receptors. For its role on bone homeostasis, estrogen influences pubertal growth, regulates bone maturation, and maintains bone mass by regulating the balance between bone resorption and bone formation. Research during the last decade has revealed that estrogen regulates bone homeostasis through unexpected regulatory effects on the immune system and on oxidative stress and through direct effects on bone cells.

In addition, there are other factors which control bone remodeling cycle such as parathyroid hormone, thyroid hormone, growth hormone, glucocorticoids, vitamin D_3 and many neuropeptides including the calcitonin-gene related peptides (CGRP),

vasoactive intestinal peptides (VIP) and substance P. Several groups have reported interesting effects of molecules known primarily to be present in the nervous system on bone resorptive and bone forming cells. The effects of these substances are in general as profound as those caused by well known osteotropic hormones, cytokines and growth factors. Indeed, bone cells express receptors for many of the neuronal messengers present in these skeletal nerve fibers. Since activation of such receptors leads to profound effects on the activity of both osteoblasts and osteoclasts, the existence of neuroosteogenic, or neuro-immuno-osteogenic interactions has therefore been suggested (Lerner, 2002), in analogy with the more well known neuro-immune or neuro-immune-endocrine system.

The importance of the nervous system in bone development and metabolism is also confirmed by several clinical observations as well as experimental findings. Surgical and chemical denervation in animals leads to a skeletal phenotype, not only in loaded parts of the skeleton, but also in unloaded bones such as ear bones. Therefore, this suggests that the skeletal pathology cannot be solely explained by disuse. In addition, during bone healing, sensory nerve grows into the fracture site (Hukkanen et al., 1993). Medson and co-workers (2000) additionally reported the time-dependent growth of sensory nerve fiber which is marked by the expression of calcitonin-gene related peptides or substance P (SP) with PGP9.5, a neuronal marker and GAP 43, marker of regenerating nerve, into a bone conduction chamber model. The development of sensory innervations in long bone was investigated in rat tibia in fetuses and in neonatants. These results have shown that the pattern of sensory nerve fiber appearance corresponds with the development and repairing of bone (Gajda et al., 2005; Medson et al., 2000).

Generally, an intensive network of autonomic and sensory nerve fibers innervates bone not only in the periosteum (Krunger et al., 1989) but also within bone marrow, cortical bone and epiphyseal plates, where they may play an important part on the regulation of bone remodeling cycle. Although many skeletal nerve fibers are associated with the blood vessels, several blood vessel-unrelated nerves and free nerve endings at bone cells have also been demonstrated (Irie et al., 2002). In particular, CGRP, which is the major neuropeptide co-released with substance P at the sensory nerve ending, induces potent vasodilation in bone and also has direct and indirect effects on development and activity of osteoclasts (Cornish et al., 1999).

CGRP was indicated to play a local role in bone metabolism through a mechanism that is in part different from that of calcitonin. Estrogen has been shown to increase the production and release of CGRP in sensory neurons by enhancing nerve growth factor–mediated CGRP production (Gangula et al., 2000; Lanlua et al., 2001). Ovariectomized rat showed decreases of CGRP mRNA and tissues CGRP levels in dorsal root ganglion neurons and gastric tissue, respectively, thereby attenuating gastric mucosal repairing. However, these results were completely reversed by 4 weeks of administration of estradiol and isoflavone, suggesting that estrogen acts on tissue repairing via the regulation of neurons containing CGRP (Shimozawa et al., 2007). Thus, estrogen can affect bone cells directly or indirectly via the sensory nerve endings which play an important role in regulating bone remodeling cycle. However, there is no evidence of the effect of estrogen deficiency on the modulation of CGRP- and SP-expression which are predominantly localized at the sensory nerve innervation in bone.

Taken together, sensory nerve containing CGRP or substance P which innervates bone can approve to a major role in regulating bone remodeling similar to estrogen but the mechanism of the sensory nerve innervations in estrogen deficiency is unclear. Therefore, this study aimed to investigate the effect of estrogen on sensory nerve innervations of bone in the ovariectomy-induced osteoporosis in rats which is the model of postmenopausal osteoporosis.

Research question

Does the absence of estrogen have any effect on the sensory nerve innervation of bone in osteoporotic rat model?

Hypothesis

As estrogen has a regulatory effect on innervation of sensory nerve in bone where there is a co-expression of CGRP or substance P with PGP-9.5, there should be changes in the sensory nerve innervation in osteoporotic bone of ovariectomized rats.

Research objectives

  1. To investigate CGRP-, SP- and PGP 9.5- expressed nerve innervation in bone and its relation to a decrease in bone density in the ovariectomized rats.

  2. To investigate the reverse effect of estrogen supplement on change in the sensory neural innervation in bone and decrease of bone density in the ovariectomized rats

CHAPTER II

LITERATURE REVIEWS

Bone

Anatomy of bone

Bone is a connective tissue that protects internal organs and bone marrow, act as a body structure and also a reservoir of minerals especially calcium and phosphorus. Bone can be classified based on shape and type of structure (Martini, 2004)

Bone shapes (Fig 2-1)

1. **Long bones** are long and slender; they are normally found in limbs of our body such as arms, forearm, thigh and leg.

2. **Short bones** are small and are located in wrist and ankle.

3. **Flat bones** are thin and flat, for an examples skull bone, scapula, ribs and sternum.

4. **Irregular bones** have a mixed shape with short, flat, ridge or notch, for example, vertebrae and pelvic.

5. **Sesamoid bones** are small and flat. They are found associated with tendon and appear near a joint i.e. patella.

Bone tissues are classified into 2 types, which are

1. **Compact or cortical bone**, a dense, hard bone tissue mostly found in a shaft of long bone and the surface of flat bone.

2. **Cancellous or trabecular bone**, a spongy-like bone tissue, locating at the end of long bone and the inner part of flat bone.
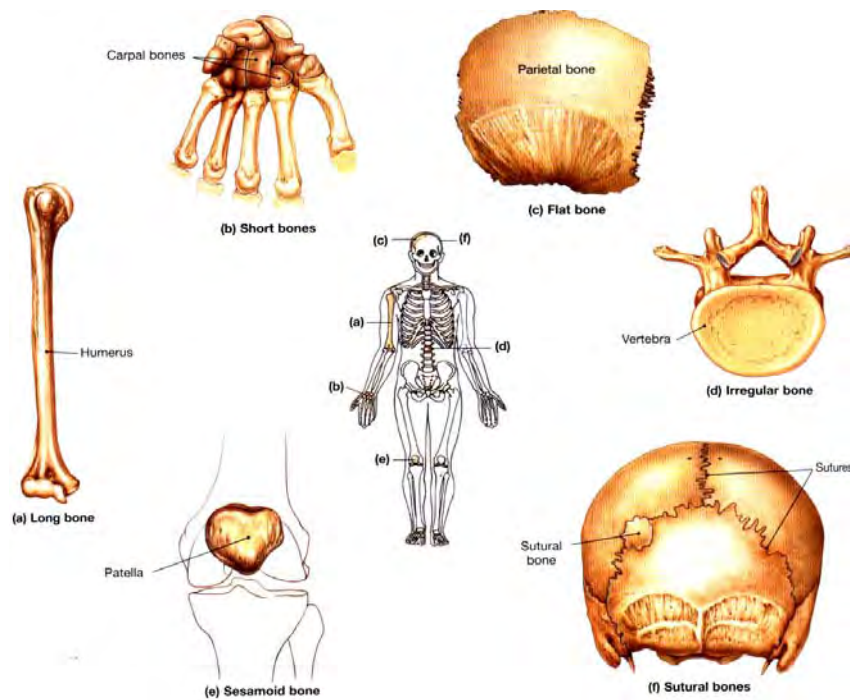
Figure 2-1 Shapes of bone (Martini, 2004)

Any bone in our body is consisted of these 2 types of bone tissue. For long bone, it can be divided into 3 parts (Fig. 2-2);

1. **Epiphysis** found at both ends of long bone, contain mainly the cancellous bone tissue.

2. **Metaphysis** is next to the epiphysis. This part is narrow and made of mainly of cancellous bone tissue, but with some of cortical bones tissue present as well.

3. **Diaphysis** is a section which connects both sides of a long bone. Compact bone is mostly located in this part (Martini, 2004).

Bone architecture and histology

Bone is composed of four layers (Fig. 2-3 and 2-4), the outer is enveloped with a connective tissue, *periosteum*, which is enriched with blood vessels and nerve supply. Continuing from the periosteum is *compact bone*, dense and hard bone tissue.

In this layer, blood vessel that penetrates into bone is surrounded with bone tissue and together they form a system called *osteon* or *Haversian system* which connects with other system with *Volkmann's canal* (Fig. 2-4 and 2-5). Volkmann's canal is a hollow canal which is the route for nerve and blood vessel to pass through. Next to compact bone is a spongy bone tissue, *cancellous bone*. In this layer, bone tissue forms a network which blood vessel is not surrounded by bone tissue and does not form osteon as in compact bone. The inner surface is *endosteum* which lines bone marrow cavity.
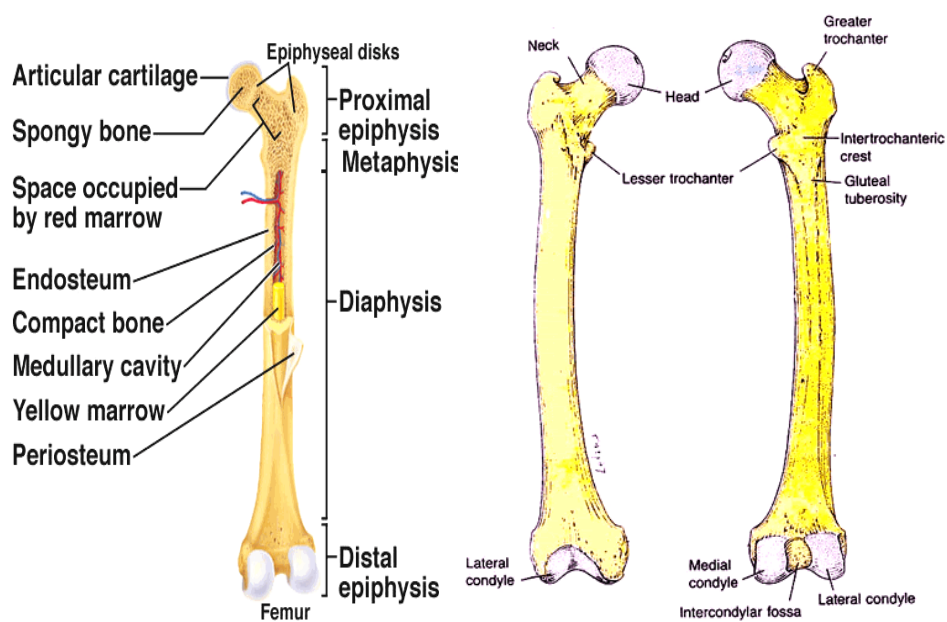
Figure 2-2 Structure and anatomy of femoral long bone (Martini, 2004)
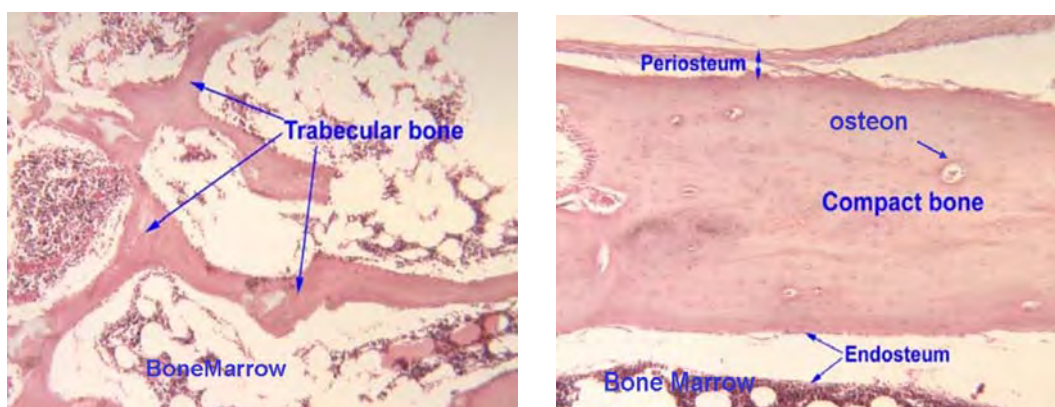
Figure 2-3 Microscopic picture of a longitudinal section of bone (*left*) cancellous (*right*) compact bone (Modified from Martini, 2004)
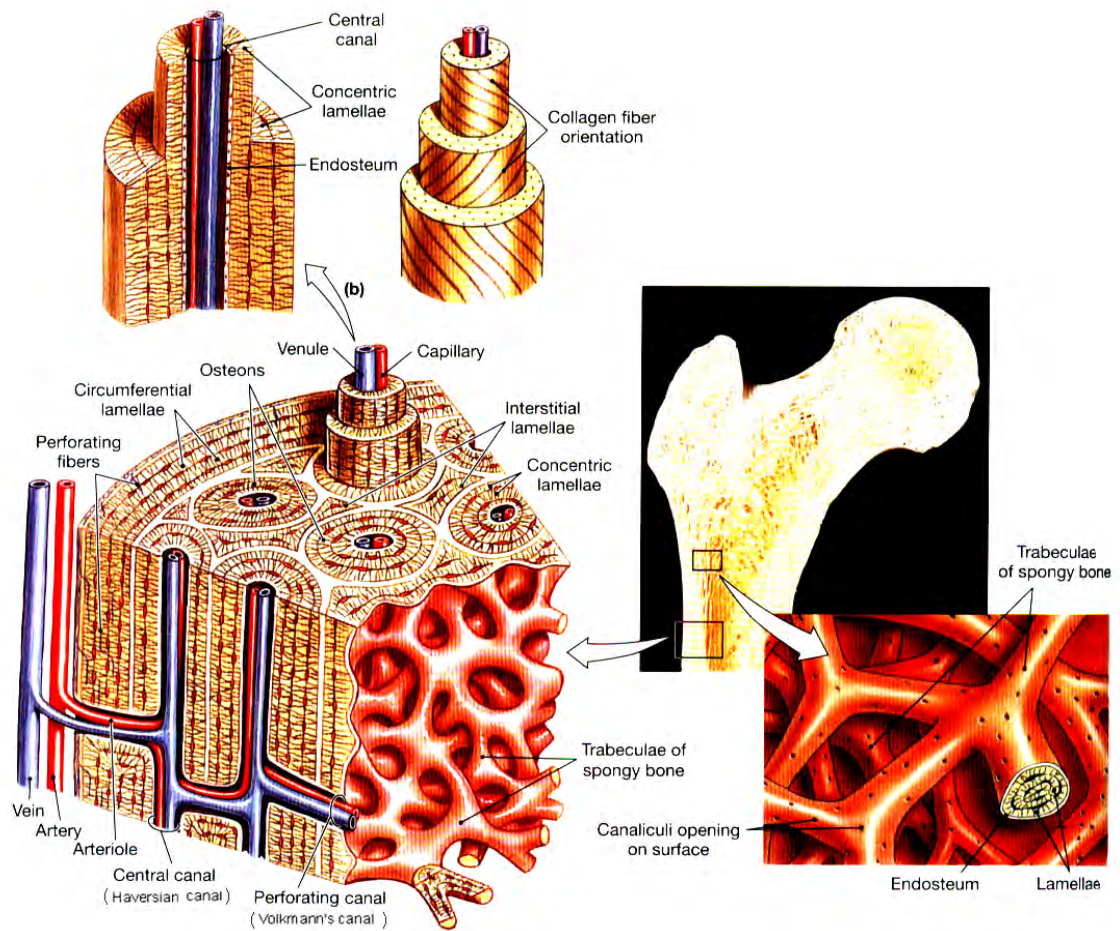
Figure 2-4 Bone architecture (Modified from Martini, 2004)
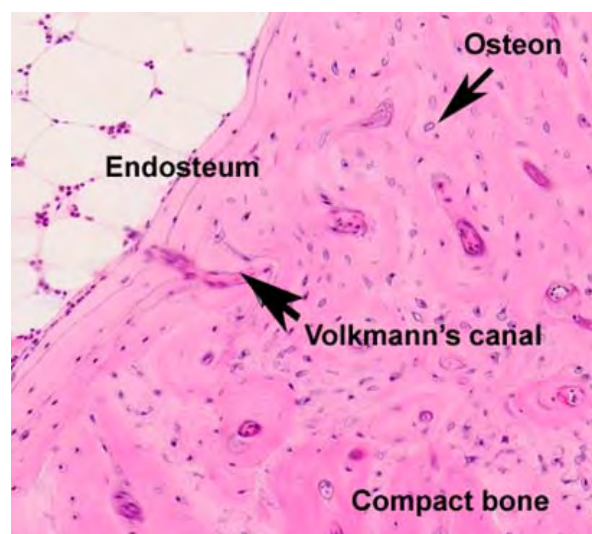
Figure 2-5 Microscopic of a longitudinal section of compact bone

(Modified from Martini, 2004)

Composition of bone

Bone matrix is an intercellular substance of bone tissue which consists of *organic substance* and *inorganic bone salts*. For the organic substance, it contains bone cells called *osteocytes*. Each osteocyte is located in a pocket called *lacunae* and surrounded with collagen fibers and ground substances. As describe previously, bone is a reservoir of minerals in our body especially for calcium and phosphorus, which are deposited around collagen type I, so that they form an inorganic part of bone matrix (Martini, 2004).

Blood and nerve supply in bone (Fig 2-6)

Similar to other organs in the body, bone is supplied with blood and nerves. Blood vessel penetrates from outer to inner, periosteum to endosteum. Nerves, mostly sensory nerves, enter bone along with the blood vessels which are most dense in the periosteum, less dense in bone marrow and less in mineralized bone (Mach et al., 2002). There are 3 types of blood vessels in long bone as follow (Martini, 2004, Johnson, 2004); **(1) Nutrient artery and vein** supply diaphysis and penetrate bone tissue from the periosteum. They also supply the shaft of femur. The large artery diversifies into small vessels which invade into the osteon or *Haversian system*. **(2) Metaphyseal and epiphyseal vessel** supply areas of epiphysis and metaphysis of long bone. **(3) Periosteal vessel** is a small vessel, supplying the periosteum and superficial osteon of shaft.
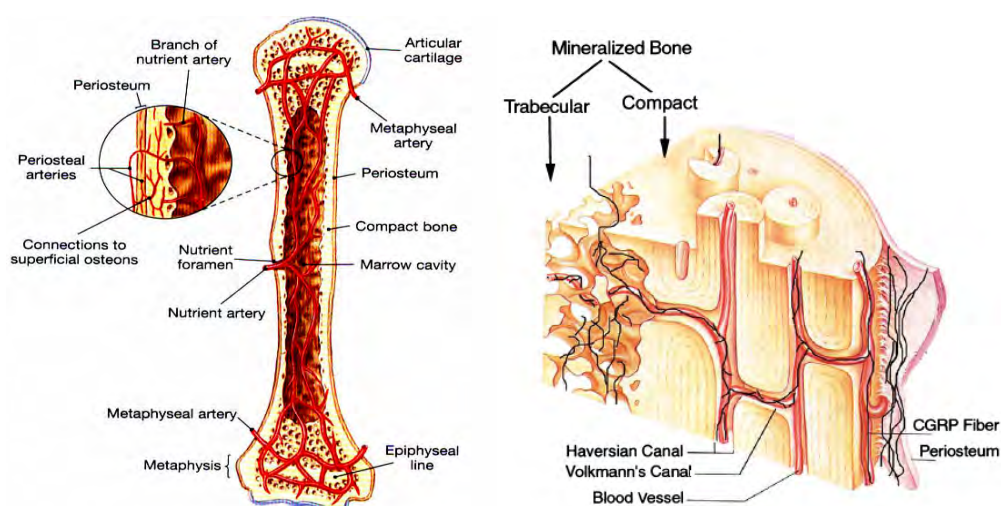
Figure 2-6   Architecture of blood (left) and nerve supply (right) in long bone

(Mach et al., 2002)

Bone cells (Fig. 2-7)

Mature bone is consisted of 3 types of bone cells (Kacsoh, 2000, Martini, 2004);

(1) Osteoblast is a bone forming cell that derives from pluripotent mesenchymal stem cell which can also differentiate into chondrocytes, adipocytes, myoblasts and fibroblasts. Osteoblast produces protein, especially the type I collagen and other organic substances of the matrix; the mixture is called *osteoid*. Osteoids increase the strength of new tissue with the deposition of calcium and phosphorus and they become part of the bone matrix.

(2) Osteocyte. When the calcification process starts, type I collagen is calcified and osteoblasts are trapped. Then, osteoblast is converted into osteocytes. Osteocyte locates in a pocket called *lacuna* with each osteocyte connecting together by a calnaliculi network. However, osteoblasts at bone surface do not convert to osteocytes but become bone lining cells.

(3) Osteoclast is a large multinucleated giant cell, which differentiates from monocyte-macrophage lineage (Roodman, 1999). It has multiple vacuoles, lysosomes and highly acidophilic cytoplasm. It resorbs bone tissue by releasing proteolytic enzymes and hydrochloric acid, the process of which is called bone resorption.
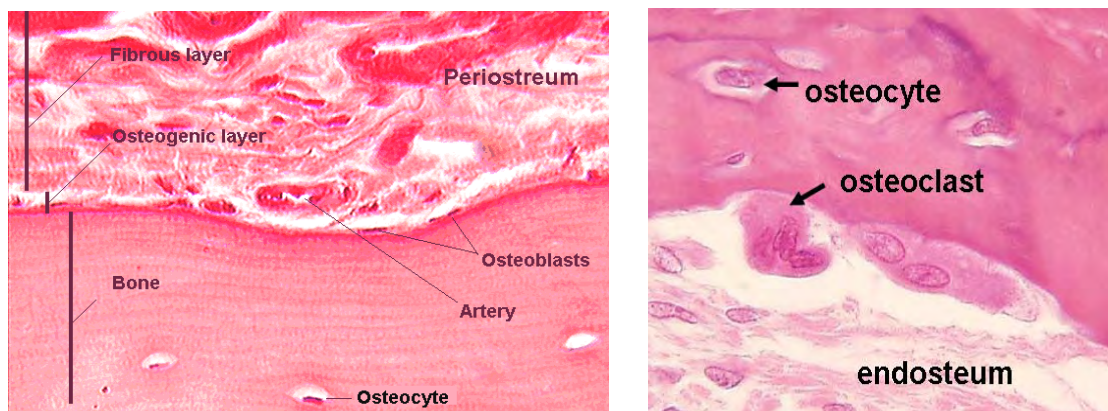
Figure 2-7 Histology of three types of bone cells, osteoblast, osteocyte and osteoclast as precent in compact bone (Modified from Martini, 1992)

Bone development

The growth of bone is consisted of 2 processes; (1) *ossification* which is the production of bone matrix and, (2) *calcification* or mineralization which is a process of mineral deposition.

Ossification (Fig. 2-8)

Ossification is divided into 2 types; (1) intramembranous ossification and (2) endochondral ossification.

Intramembranous ossification starts with the differentiation of osteoblast from mesenchymal stem cell and active. Osteoblast produces and secretes collagen type I around itself. After that is a process of calcification by calcium salt. Location where ossification begins is called an ossification center, and bone grows, outward in small pieces called spicules. Following this process, blood vessel comes into the new bone tissue. Some of spicules fuse together and blood vessel is trapped within. Cancellous bone tissue is generated by intramembranous ossification, whereas, for compact bone, there is a remodeling process around the trapped blood vessel, which produced osteon system (Martini, 2004).

Endochondral ossification begins with hyaline cartilage, which is formed at the origin of embryo development. Firstly, chondrocyte at the center of shaft increases in size, and its lacunae expand, and the matrix is reduced and calcified. After that, blood vessel around the perichondrium that covers the hyaline cartilage, penetrate into the cartilage and bring the osteoblast inside the cartilage where a bone collar begins. Moreover, osteogenic cells and capillaries also invade bone tissue and start to form *"Primary ossification center"*. A new bone is formed at both sides of the cartilage and whole diaphysis is filled with cancellous bone.

Via the blood vessel, osteoclasts enter the cartilage and start to resorp a cancellous bone tissue at the center of diaphysis to generate marrow cavity. Thus, this process leads to the increased diameter and length of bone. Capillaries and osteoblasts still invade new bone and create *"Secondary ossification center"*. An epiphysis is also filled with cancellous bone tissue, with some of the original cartilage remaining where it is exposed to the joint cavity, so it is called articular cartilage. A narrow cartilage at the metaphysis called *epiphyseal cartilage* or *epiphyseal plate* separates the epiphysis from the diaphysis. Epiphyseal plate is responsible for increasing bone in length

especially at puberty. Growth hormone and thyroid hormone stimulate epiphyseal plate enlargement leading to increase in body height (Martini, 2004; Ornoy, 1995).

Most bones develop and grow by utilizing both types of ossification, for instance, long bone grows longitudinally by endochondral ossification and grows in diameter by intramembranous ossification.
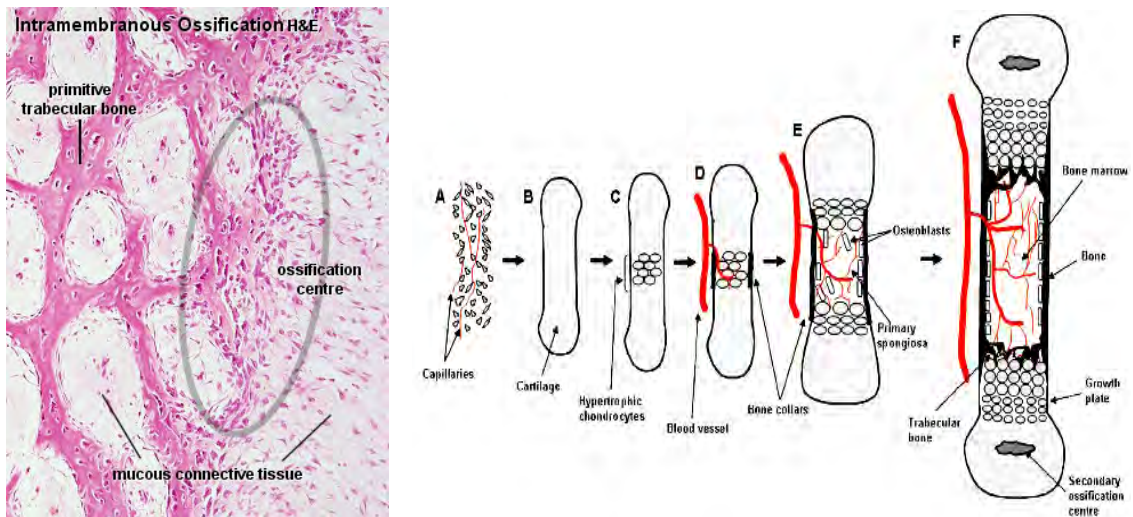
Figure 2-8   Bone growth; intramembranous ossification (*left*) and endochondral ossification (*right*) (Martini, 2004; Ornoy, 1995).
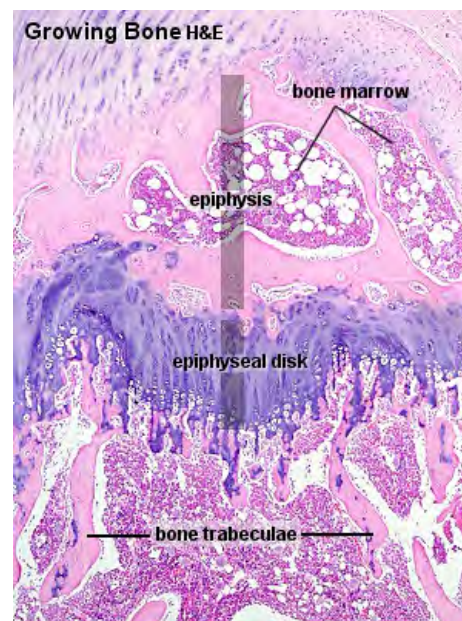
Figure2-9 Histology of epiphyseal plate (Martini, 2004)

Calcification

In this process, calcium and phosphorus which come from blood become bound at a specific site to collagen type I to form $Ca_{10}(PO_4)_6(OH)_2$ or hydroxyapatite. Because of this process, osteoblasts are trapped by the calcified collagen before developing into osteocytes which locate in the lacunae. Osteocytes are connected with one another by canaliculi (Hadley, 1992).

Physiology of bone

Calcium and phosphorus metabolism

Calcium is very important for muscle contraction, blood coagulation and nerve function. The plasma, calcium level is approximately 2.5 mmol/L. Calcium binding with protein such as albumin or globulin is a nondiffusible form. The diffusible form is free ion or calcium complexed with anions such as HCO_3^- and citrate (Ganong, 1995 A, Hadley, 1992). Ninety-nine percent of calcium is in bone. Bone calcium is present as an exchangeable form in the fluid of bone matrix and as a non-exchangeable form, hydroxyapatite a crystals-like form that is only slowly exchangeable. These two forms of calcium are independent and play roles in regulation of calcium homeostasis in bone. About 500 mmol of Ca^{2+} per day is moved in and out of the exchangeable pool in bone, while the non-exchangeable form is involved with bone remodeling cycle. All of calcium is filtrated in the kidney, with about 90-98%, being resorbed, 60% at the proximal tubules and the rest at ascending limb of Henle loop and distal tubules. Dietary, calcium is absorbed at the small intestinal (Ganong, 1995A).

Phosphorus is an important part of ATP, cyclic AMP and other compounds in the body. Total phosphorus in body is about 16.1-25.8 mmol with 85-90% in bone in serum concentration is about 12 mg/dL. Phosphorus is absorbed in the small intestine especially the duodenum. The intestinal phosphorus absorption depends on the dietary intake and can occur by both active transport and passive diffusion. Phosphorus in the serum is filtrated by kidney which reabsorbs about 85-90% at the proximal tubule by active transport (Ganong, 1995A).

Because calcium is very important in many functions in the body, they must be maintained within a narrower range than phosphorus. There are three major hormones that regulate calcium metabolism; parathyroid hormone (PTH), calcitonin hormone and 1, 25-dihydroxyvitamin D_3 (Hadley, 1992).

1. Parathyroid hormone is synthesized in the parathyroid gland. The secretion of parathyroid hormone is regulated by plasma calcium level. Low concentration of calcium in plasma stimulates parathyroid hormone release. The effects of PTH are both direct and indirect on three major organs, kidney, small intestine and bone. Briefly, PTH stimulates the reabsorption of calcium and excretion of phosphate in the kidney. However, it indirectly via vitamin D_3 stimulates the intestinal absorption of calcium. PTH has a direct effect on bone cells. PTH stimulates proliferation and differentiation of osteoclasts which lead to increase in bone resorption and inhibition of osteoblasts. Thus, resulting in an increase in calcium concentration (Hadley, 1992).

2. Calcitonin hormone is synthesised in C-cells of thyroid gland. High concentration of calcium stimulates secretion of calcitonin hormone. Effects of calcitonin are to increase the absorption of calcium from food, promote calcification in bone, inhibit activity of osteoclasts in vitro and increase calcium excretion in urine (Hadley, 1992, Ganong, 1995A).

3. 1, 25-Dihydroxyvitamin D_3 or vitamin D_3 is produced in the skin of human by the stimulation of 7-dehydrocholesteral by sunlight. Then converted to cholecalciferol in the liver and synthesized 1, $25(OH)_2$ D_3 in the kidney. Effects of vitamin D_3 on calcium regulation are stimulation of calcium absorption in the small intestine reabsorption calcium in the kidney and demineralization of bone (Hadley, 1992).

Bone Metabolism

Bone remodeling cycle

As a reservoir of calcium in our body, bone has to remodel throughout life by a combination of bone formation and bone resorption which is called *bone remodeling cycle*.   There are 4 phases in bone remodeling cycle and the first is called *(1) activation phase*, which begins with osteoclast, assembles, differentiates and starts excavating cavity at bone surface. After that, osteoclast disappears in the *(2) reversal phase,* and osteoblasts are then recruited principally to the site that was resorbed previously. Osteoblasts fill the cavity with a new bone in the *(3) formation phase*. *(4) Mineralization phase* is the last phase of this cycle when new bone is mineralized with calcium and phosphate (Kanis, 1996).
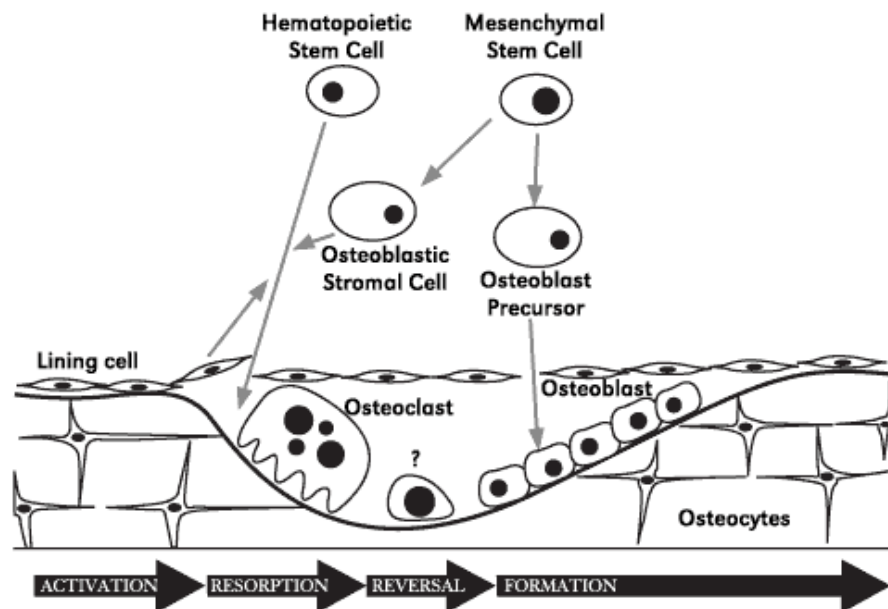
Figure 2-10 Bone remodeling cycle (Kanis, 1996)

Even though hormones from endocrine glands come from blood, several local factors are sometimes released by bone cells or other neighboring cells and have effects on bone remodeling cycle. For an example, interleukin 11, interleukin 6 and macrophage-colony stimulating factor (M-CSF) produced by bone marrow stromal cells and osteoblast have an effects on differentiation pathway of osteoclasts. In addition, mature osteoclasts can produce cytokines such as interleukin 1, which also stimulates osteoclast activity (Roodman, 1999). Osteoblast activity is stimulated by interleukin 1 and inhibited by tumor necrosis factor (TNF) (Hughes, 2000).

Aging bone and abnormal metabolism of bone

Bone becomes thinner and weaker with an increase in age. In aging people, osteoblast is inactive, but not osteoclast, leading to diminished density or osteopenia. The loss is not the same in all parts of bone, i.e. epiphysis, vertebrae and jaw loss are more than others leading to elderly having increased risk of fracture and losing their height and teeth (Martini, 2004).

Because of many factors can control bone metabolism, if there are any dysfunctions of these, bone will be affected. Disorder of mineralization in vitamin D insufficiency is a cause of "osteomalacia" (in adult) or "ricket" (in children) where by bone becomes weak and deformed. Hormone impairment is also related to abnormal bone metabolism such as hypo- and hyperparathyroidism which lead to hypo- and hyper-calcemia, respectively. Moreover, sex hormones, especially is estrogen, is suggested as a major key regulator of bone metabolism. Therefore imbalance or deficiency of estrogen is the main cause of bone density loss or "osteoporosis" in postmenopausal women (Martini, 2004, Hadley, 1992).

Osteoporosis

Osteoporosis is a disease in which the architecture of bone is affected i.e, becoming porous, fragile, thinner, leading to increased risk of fracture. Osteoporosis can be classified into 2 classes; primary and secondary. The term "primary osteoporosis" is used for a condition in which bone loses its density and strength without associated with diseases. An example of the causes of this class is postmenopausal or senile osteoporosis. On the other hand, the term of "secondary osteoporosis" is used for an osteoporosis which is related with disease or drug that can cause bone density decrease such as Cushing's syndrome, hyperparathyroidism or drug in the group of corticosteroids or heparin.

Several factors are also associated with osteoporosis, for example genetics, nutrition or smoking. The osteoporosis can be diagnosed by measuring bone mineral density (BMD) or bone mass measurement using the dual energy X-ray absorptiometry (DXA or DEXA). In addition to the detection of abnormal BMD, the diagnosis of osteoporosis requires investigations into potentially modifiable

underlying causes; this may be done with biochemical markers test and X-rays. The biochemical markers of bone formation and resorption in urine, serum and plasma are also use to predict the incidence of bone loss or osteoporosis (Swaminathan, 2001). For example, bone alkaline phosphatase is an enzyme that represents the function of bone forming cell. When bone remodeling is increased such as in osteoporosis, the level of bone AP in serum will be increase.

Osteoporosis is a common disease in postmenousal women. The main cause of this disease is lacking of estrogen hormone because ovarian function is impaired. To remedy the lacking of estrogen, hormone replacement therapy (HRT) can solve this problem. However, estrogen acts via its receptor, α-subtype and β-subtype, which distributed widely in the body such as in the breast, cardiovascular system, central nervous system, liver and bone. Even though HRT can prevent bone loss but it also causes side effects in other organs such as increased risks of stroke, venous thrombosis or breast and endometrial cancer (Rossouw et al., 2002). For this reason, other estrogenic agents with fewer side effects but selective to estrogen receptor especially the selective estrogen receptor modulator (SERM) are needed as alternative treatment strategies. Many non-pharmacological methods (i.e. diet or exercise management) or non-sex steroid treatments such as anti-resorptive bisphosphonates (Alendronate[®], Etidronate[®], Risedronate[®], Ibandronate[®]) therapies combined with calcium plus vitamin D, and calcitonin (Miacalcin[®]) are also useful for prevention or alleviation of the untreatable osteoporosis (Miller, 2001).

Estrogen

As a female sex hormone, estrogen is a steroid hormone that is produced in the ovaries. Theca cells secrete androgen hormone which are conversed by aromatase enzyme in the granulosa cells into estrogen (Gruber, 2002). The first estrogen subtype is 17β-estradiol (E_2) which is the most potent of estrogens (Ganong, 1995B). Effect of estrogen is mediated by estrogen receptors, α-subtype and β-subtype. Estrogen receptors not only locate at the reproductive organs but are also found in other organs such as blood vessels, liver, nervous system and bone. The major function of estrogen is to regulate the reproductive system of female such as development of the reproductive organs and the secondary sexual characteristics.

In bone, estrogen plays a major role in the regulation of bone remodeling cycle. Osteoblast and osteoclast express estrogen receptors so they can be direct target of estrogen. It was reported that estrogen could inhibit bone resorption and stimulate osteoblast function (Tobias et al., 1999). Estrogen deficiency causes an imbalance of this cycle. In 1997, Robinson and co-workers reported that estrogen had an effect on differentiation of human osteoblastic cells and it controls osteoclast activity via estrogen receptor-α (Nakamura et al., 2007). Lack of estrogen is responsible for the imbalance of this delicate equilibrium (Manolagas and Jillka, 1992). High levels of IL-6 have been associated with ostoclastogenesis and estreogen can inhibit the production of IL-6 (Manolagas, et al., 2002). Interleukin-1 and M-CSF which are produced by stromal cells and osteoblasts were inhibited by estrogen (Venken et al., 2008).
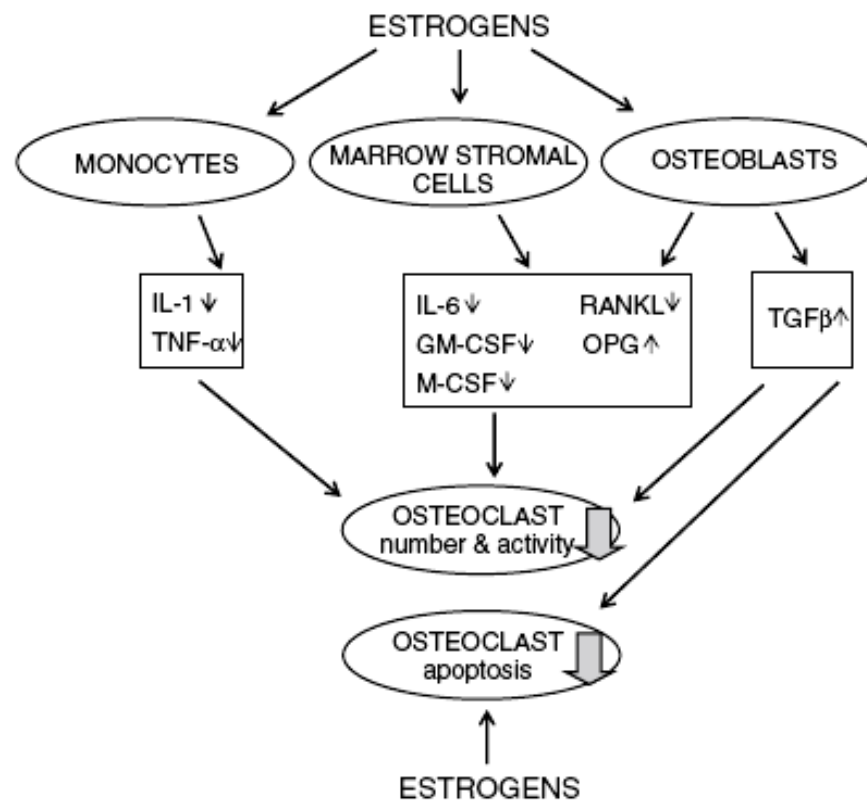
Figure 2-11 Effect of estrogen on bone (Venken et al., 2008)

Sensory nerve and its role on the bone regulation

Intensive network of sensory nerve and autonomic nervous system enter bone from periosteum the same as blood vessels which supply all parts of bone and have an important role in the regulation of bone formation and resorption. The importance of the nervous system in bone metabolism is also confirmed by several clinical observations as well as experimental findings. Surgical and chemical denervation in animals leads to a skeletal phenotype, not only in the loaded parts of the skeleton, but also in unloaded bones such as ear bones. For example, surgical and pharmacological sympathectomy in Mongolian gerbils resulted in enhancement of osteoclast numbers and resorptive surface in ear bone (Sherman and Chole, 1996).

Additionally, sensory nerve has an important role in bone repair. Sensory nerve grew into bone conduction chamber which was correlated with the growth of bone tissue in a time dependent manner (Madsen, 2000). Sensory nerve was re-innervated into fracture site during healing process in rat tibial bone and this may mediate or deliver essential factors for formation of new bone tissue (Li, 2001). Furthermore, sensory nerve was observed during bone development and found that it appeared to correspond with the development of bone (Gajda, 2005). Therefore, sensory nerve may be important for bone development and normal healing processes.

The sensory nerve which can be identified by the co-expression of peptides, calcitonin gene-related peptide (CGRP) or substance P (SP) with the PGP-9.5 neural proteins, grows into a fracture site during bone healing (Hukkanen et al., 1993). Surgical denervation of sensory nerve was found to reduce CGRP and SP expression in nerve fibers in the rat hind foot and resulted in reduced growth of metatarsal bones, a phenomenon not observed in rats immobilized by tenotomy (Edoff et al., 1997). The evidence suggest that these sensory neuropeptides may have osteogenic effect on bone cells.

Calcitonin gene-related peptide (CGRP)

Calcitonin gene-related peptide (CGRP), similar to calcitonin, amylin and adrenomedullin belongs to a superfamily of peptide (Wimalawansa, 1996). CGRP-I or CGRP-alpha is a 37-amino acid peptide generated by alternative tissue specific splicing of the primary transcript of the calcitonin gene. Another closely related

member is CGRP-II or CGRP-beta, which is different from human CGRP-I only in 3 amino acids (Irie et al, 2002) but it no osteogenic effect (Hirt and Bernard, 1997).

CGRP is primarily located in the small, periphery, myelinated and unmyelinated nerve fibers close to the blood vessels (Brain and Grant, 2004). CGRP is a potent vasodilatator. Administration of human CGRP in rats and dogs induces a dose-dependent peripheral vasodilatation via its receptor, CGRP1, and this effect is inhibited by pretreatment with its receptor antagonist (Shen et al, 2001). This effect is also observed in human forearm blood flow (Vanmolkot et al, 2006). Interestingly, level of CGRP in the plasma was lower in ovariectomized rats than in sham groups. After administration of α-CGRP, skin temperature was increased and this result paralleled with the dilation of skin vascular (Noguchi, 2002).

Not only cardiovascular effect, CGRP-I or α-CGRP has a major role on regulation of bone metabolism. In 1997, Valentijn and co-workers (1997) showed that α-CGRP stimulated the production of the insulin-like growth factor-I (IGF-I) and inhibited cytokine tumor necrosis factor or by osteoblasts, suggesting that CGRP may have effects on bone cells. Using ovariectomized rats as a model of high bone turnover rate, they found that α-CGRP inhibited bone resorption but not bone formation. Alpha-CGRP increased proliferation of human osteoblast-like cells, while inhibiting osteoclast precursor development and decreasing committed precursor fusion to form multinucleated cells (Villa et al, 2000, Cornish, 2001).

Substance P

Substance P (SP) is an 11-amino acid neurotransmitter belonging to tachykinins family and synthesized in the dorsal root ganglion. SP is also found in sensory nerve ending and has effect on bone remodeling similar to CGRP the co-localizing neuropeptide. SP has the highest affinity for NK-1 receptors (Goto et al., 2001). In 1997, Togari and co-workers reported that osteoblasts did not express mRNA of SP receptors but Goto and co-workers (1998) reported that SP receptors on osteoblasts could be found but only in minute amount. In 2007, Goto and co-workers reported that NK-1 receptor was expressed only in late-staged osteoblast differentiation. SP was found to stimulate the function of osteoblast in a time- and dose-dependent manner.

Controversially, SP also has an effect on osteoclasts. Osteoclasts have been found to express SP receptors, NK-1 receptor. The SP causes an acute rise of intracellular calcium in osteoclasts and stimulates the pit forming activity of rabbit osteoclasts (Goto et al., 1998, Mori et al., 1999). SP also stimulated osteoclast formation via synovial fibroblastic cells, this effect was inhibited by the antagonist of NK-1 receptors (Matayoshi et al., 2005) confirming that SP receptors in osteoclasts are function.

Estrogen and sensory nerve containing-CGRP and -SP

Even though estrogen is a hormone of the female reproductive system, it also has effects on other systems in our body, especially the nervous system. Several lines of evidence indicate that estrogen is benefit to central nervous system such as in Alzheimer's disease (AD) and anxiety. Estrogen replacement therapy (ERT) in postmenopausal women is associated with delayed onset and reduced risk of AD and anxiety suggesting its involvement in the postmenopausal-induced nervous system disorders (Yaffe et al., 1998).

In 1997, Papka and co-workers studied the presence of estrogen receptor in ganglia which supply axon to uterus, particularly the sixth lumbar (L6) and the first sacral (S1), dorsal root ganglia (DGR) and pelvic parasympathetic ganglia (PG) in intact and ovariectomized rats. Their results suggested that a certain population of neurons can serve as a binding site of estrogen. Scoville et al. (1997) found that estrogen up-regulated neurofilament gene expression appeared to occur in all DRG neurons. Moreover, estrogen deficiency can diminish the density of nerve innervation in bone (Burt-Pichat et al., 2005).

Both CGRP and SP are synthesized at DRG and estrogen may play an important part in their synthesis. During pregnancy, the high estrogen level stimulated synthesis of CGRP and SP in L6-S1 and DRG in rats (Mowa et al., 2003A, Mowa et al., 2003B). In addition to the central nervous system, estrogen increased the innervation of sensory nerves containing-CGRP at the mammary gland (Blacklock and Smith, 2004).

Thus, estrogen can affect bone cell directly or indirectly via sensory nerve ending which plays an important role in regulating bone remodeling cycle. However, there is no evidence of the effect of estrogen deficiency on the modulation of CGRP- and SP-expression which predominantly localize at sensory nerve innervation in bone.

CHAPTER III

MATERIALS AND METHODS

Animal care and preparation

Female Wistar rats weighing 170-200 gm (aged 2 months) at the beginning of the experiment were obtained from the National Laboratory Animal Center, Mahidol University (NLAC-MU) Thailand. All rats were housed in pair per cage under 12 hours light/dark cycles at room temperature ($25\pm2^{o}C$). Water and standard rat chow were supplied *ad libitum*.

After 4 months of animal care (rat aged 24 months), in order to confirm the puberty, all rats were determined for estrous cycle by vaginal smear according to the method of Marcondes et al. (2002). Clean plastic pipette was filled with 10 μl of normal saline (NaCl 0.9%) and inserted, not deeply, into the rat vagina. Lavaged vaginal fluid was then smeared on glass slide and sample was stained and observed under a light microscope with 10x and 40x objective lens. A proestrous smear consists of a predominance of nucleated epithelial cells, an estrous smear primarily consists of anucleated cornified cells, a metestrus smear consists of the same proportion amount of leukocytes, cornified and nucleated epithelial cells and a diestrous smear primarily consists of a predominance of leukocytes.

Operation protocol performed at diestrous period. All rats were anesthetized with Isoflurane® by inhalation. In ovariectomized groups, both ovaries were removed. In sham group, rats were received same protocol as ovariectomized rat but both ovaries were left intact. After the operation, all rats were allowed to recovery for 2 days before the experiment started.

Experimental protocol

To investigate effect of OVX and estrogen on CGRP, SP and PGP 9.5-expressed nerve innervation in bone and its relation to a decrease of bone density in the ovariectomized rats. Sixty female rats were divided into 5 groups as follows;

1. Sham operated control group (Sham)

2. Ovariectomized rats with 10%DMSO in propylene glycol, subcutaneously. (Vehicle)

3. Ovariectomized rats with 17β-estradiol supplementation daily at 1 μg/kg, subcutaneously (OVX + E_2 1 μg/kg)

4. Ovariectomized rats with 17β-estradiol supplementation daily at 5 μg/kg, subcutaneously (OVX + E_2 5 μg/kg)

5. Ovariectomized rats with 17β-estradiol supplementation daily at 15 μg/kg, subcutaneously (OVX + E_2 15 μg/kg)
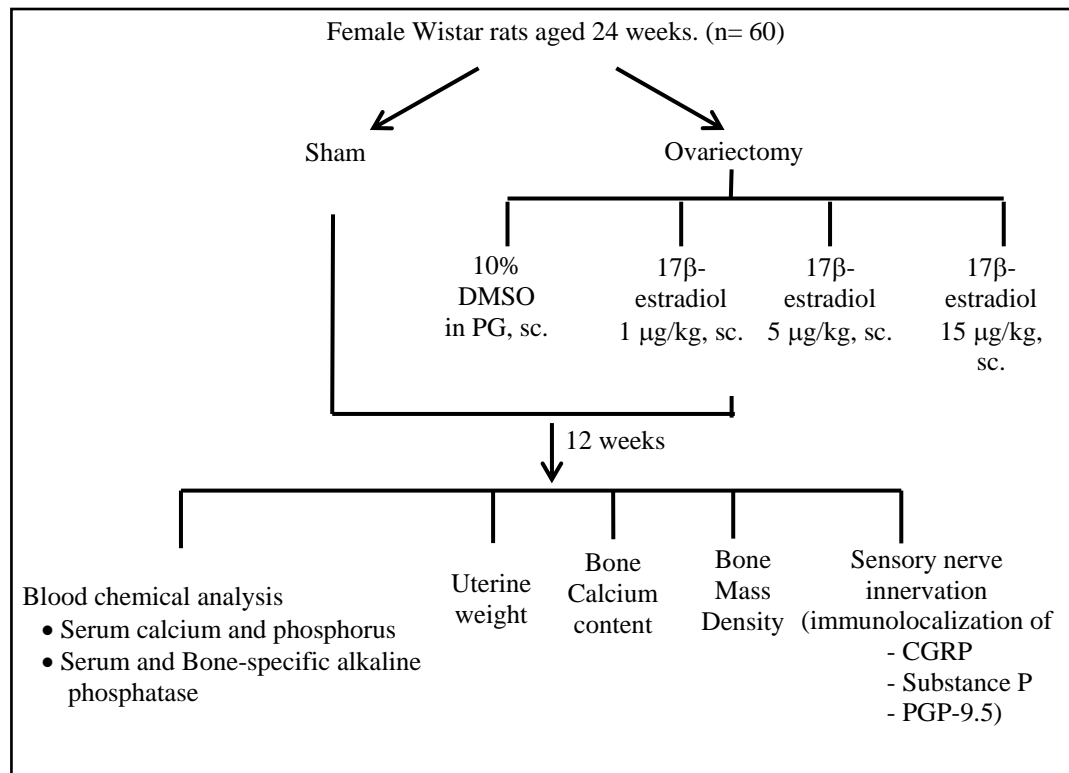
Figture.3-1 Diagram of experimental protocol
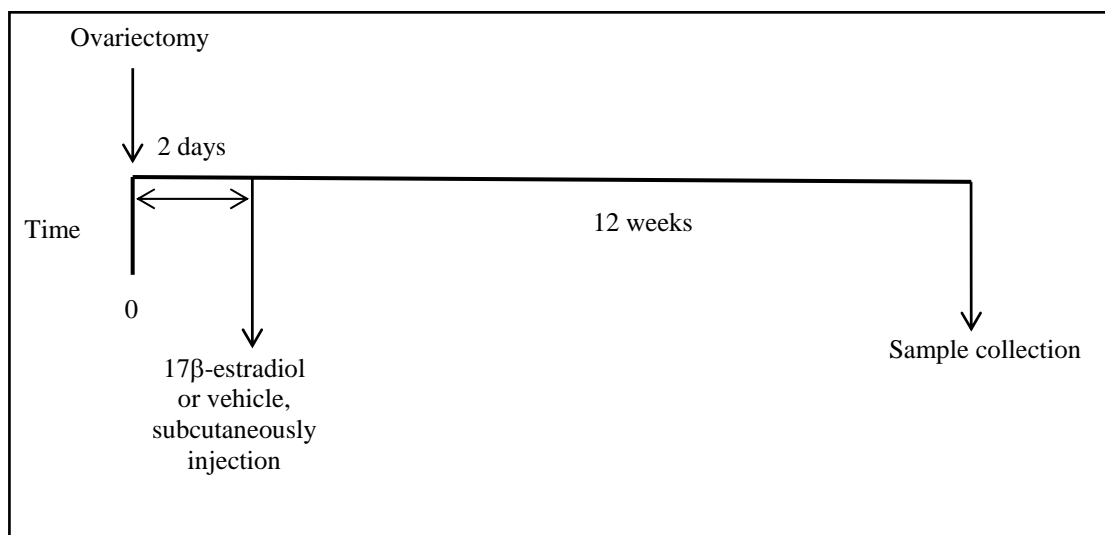
Figure 3-2 Diagram of time line used in the study

Methods

The measurement of body weight, food intake and uterine weight

To investigate effect of estrogen on body weight changes, body weight (BW) and volume of food intake were measured daily. Uterine weight was also determined immediately after sacrifice to indicate deficiency of sex hormones after removal of ovaries.

The average daily weight gain (DWG), the average daily feed intake (DFI) and the percentage of uterine-to-body weight ratio (%UW/BW) were calculated as follows:

$$\text{DWG (g/d)} \quad = \quad \text{Final BW (g) - initial BW (g)/total days}$$

$$\text{DFI (g/d)} \quad = \quad \text{Summation of feed intake (g)/total days}$$

$$\text{\%UW/BW} \quad = \quad [\text{UW (g)/BW (g)}]\times 100$$

Estrogen supplementation

All rats in ovariectomized groups with estrogen supplementation were received daily subcutaneously injection with 17β-estradiol 2 days after the operation at doses of 1, 5 or 15 μg/kg BW into the dorsal region of the neck. In ovariectomized group with vehicle, rats were injected with 10% DMSO in propylene glycol (PG) at the volume of 0.2 ml.

Sample collection

After 3 months of the experiment, all rats were deeply anesthetized with Isoflurane® by inhalation on the day of sacrificed. Blood sample was collected by cardiac puncture and allowed to clot. Serum was subsequently collected after centrifugation at 3000 rpm at $4^{o}C$ for 10 minutes and then kept at $-20^{o}C$ until analyzed. Left femur and lumbar L1-L6 were kept frozen at $-20^{o}C$ for further analysis of bone mineral density and right femur immediately fixed with 4% paraformaldehyde for immunohistochemical process.

Bone mineral density measurement

To investigate effect of estrogen on bone density, left femur bone density was measured by using dual-energy x-ray absorptiometry (DEXA) bone densitometer (Lunar PIXImus Corp, Madison, WI, USA) and the Small Animal PIXImus Software version 2 2.10 was proceeded for bone density calculation. Two x-ray beams with different energy levels were absorbed, and absorption by soft tissue is subtracted out to give the density of bone. Values for whole femoral bone mineral density (BMD, g/cm^{2}), femoral bone mineral content (BMC, g/cm), bone area (cm^{2}), total tissue mass (calculated by the software, g), total area, fat content (g) and lean content (total tissue mass minus fat content, g) were obtained.

Since ratio of cortical and cancellous bone tissue is different in each part of femur bone, and may be differently affected by hormone, sub-regions of femur were measured separately (Fig.3-3). Total femur bone density (TBD) and 4 sub-regions (Fig. 3-3) which are proximal (1/4 of total bone length from the beginning of femur), distal (1/4 of total bone length from the end of femur), shaft (2/4 of total bone length

the middle of total length) and head of femur (area in the box lining from grater trochanter to lesser trochanter) were measured by using ROI (region of interest) mode which is provided by this instrument. Measurements of sub-region bone density were expressed in total and percentage of the total bone density. Density of vertebrae at levels 1-6 was also measured with DEXA and expressed as the total vertebrae BMD.
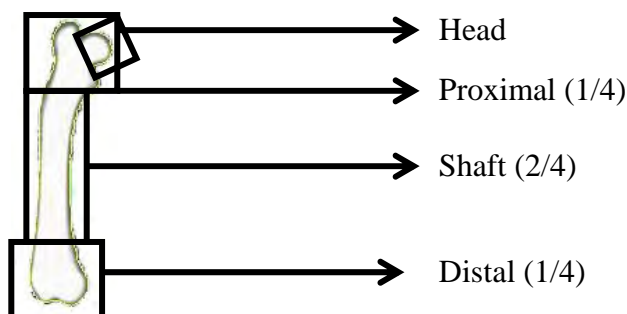
Figture 3-3 Diagram of four subregions of femoral bone

Bone calcium measurement

To determine bone mineral content and metabolism, calcium content in dried femur was measured using the same femoral bone as for BMD study. Immediately after the BMD study, left femur was cleaned off tissue and blood with 0.1 M phosphate buffer saline, and then weighed, so referred to a *sample weight*. The clean bone was dried at 80 °C for at least 48 hours in a small oven. On the following day, the dry bone was weighed again and referred to *dry bone weight*. Subsequently, bone was burnt to ash overnight in covered crucibles at 700 °C in the oven. The bone ash was cooled down and weighed (*ash weight*). For the evaluation of calcium composition, the bone ash sample weight of 0.001 gram was diluted in 3N HCl with diluting fluid ($SrCl_2 \cdot 6H_2O$ and 70% $HClO_4$). The diluted bone ash was immediately analyzed with atomic absorption spectrophotometry. Total calcium and total calcium per dry bone weight were calculated as follows:

Total calcium (mmol) = [[Calcium (mmol/L)]×[volume of diluting fluid]×[diluting factor]×[ash weight] /1000]]/sample weight (g)

Total calcium/dry bone weight (mmol/g) = Total calcium (mmol)/dry bone weight (g)

Bone specific alkaline phosphatase

To determine bone formation, bone specific alkaline phosphatase (BAP) marker in serum was determined. Frozen serum was thawed to room temperature and centrifuged at 3000 rpm for 10 minutes. Sixty microlitres of serum was divided into 2 microtubes of each sample, for analyses of total alkaline phosphatase and BAP, respectively.

Using lectin precipitation protocol (Jackson et al, 1996), diluted lectin (Sigma-Audrich Inc., St. Louise, MO, USA) at the concentration of 5 mg/ml in 0.1M PBS at volume 30 μl was added into serum at the ratio of 1:1. The serum containing lectin was inubated at 37 °C for 30 minutes in order to precipitate BAP. After centrifugation at 1500 g for 20 minutes, supernatant was collected. Both serum and serum after precipitation with lectin were sent to the laboratory to measure alkaline phosphatase. Bone specific alkaline phosphatase was calculated by subtraction of an AP in supernatant of lectin-precipitated serum from total AP in a whole serum.

Immunohistochemistry

To investigate the presence of sensory nerve innervation, immunohistochemistry was performed. Using sham group as a normal condition, OVX with vehicle treated group as estrogen deficiency condition and estrogen at the effected dosed of 1 μg/kg BW, right femur was fixed with 4% paraformaldehyde for 2 weeks, then washed with washing solution of phosphate-buffered saline (0.1 M PBS; pH=7.4). In order to decalcify, bone was immersed in 10% EDTA for 10-15 days. Decalcified bone was subsequently immersed overnight in sucrose 30% for cryoprotection and rinsed with 0.1 M PBS (pH=7.4) before mounting in O.C.T$^{®}$ (Sakura Finetechnical Co., Ltd., Tokyo, Japan) medium. Bone specimens were longitudinally sectioned at thickness of 14 μm on a cryostat, and were placed on gelatin-coated slides and allowed to dry well in room air. The sections were subsequently processed for immunohistochemistry beginning with treatment in 5% normal goat serum, 0.1% triton-x in 0.1 M PBS to block non-specific binding for 60 minutes. The sections were then incubated with primary antibodies (Table 3-1), either of rabbit polyclonal anti-human CGRP (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), goat polyclonal anti-human SP (1:50; Santa Cruz Biotechnology

Inc., Santa Cruz, CA, USA) or rabbit polyclonal anti-human PGP 9.5 (1:200; Chemoscience Pte Ltd., Singapore) in diluent consisting of 2% bovine serum albumin with 0.1% triton-x in 0.1 M PBS overnight at 4 °C.

Sections were then washed out the excess of primary antibody in 0.1 M PBS for 5 minutes, 3 times. After washing, the sections were exposed to appropriate secondary antibodies (Table 3-1) which is donkey anti-rabbit IgG conjugated with FIT-C (1:400; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or donkey anti-goat IgG conjugated with Texas-Red (1:400; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in the dark for 1 hour at room temperature. Finally, sections were washed out the excess secondary antibody with washing solution and mounted under anti-fading solution Vectashield® (Vector Laboratories, Inc., Burligame, CA, USA). The positive results in the sections were visualized under the fluorescence microscope with appropriate wavelength. The sections were photographed with Nikon optiphoto microscope. At least twenty sections covering the middle section of each bone tissue were investigated. The positive control of SP-, CGRP- and PGP 9.5-immunoreactive were also performed in 4%parafomaldehyde fixed dorsal root ganglia isolated from sham rats. In addition, the negative control, section was added with blocking solution instead primary antibody followed by both secondary antibodies in the same section. The results of immunoreactivity were presented as descriptive results.

Table 3-1 Description of antibodies used for immunohistochemistry in the present study (*CGRP* calcitonin gene related peptide, *SP* substance P, *PGP9.5* protein gene produce 9.5, *FITC* fluorescein isothiocyanate, *TR* Texas-red)

Antigen	Host	Dilution	Source
CGRP	Rabbit	1:50	Santa Cruz Biotechnology Inc., Santa Cruz, CA
SP	Goat	1:50	Santa Cruz Biotechnology Inc., Santa Cruz, CA
PGP 9.5	Rabbit	1:200	Chemoscience Pte Ltd., Singapore
FITC	Donkey	1:400	Santa Cruz Biotechnology Inc., Santa Cruz, CA
TR	Donkey	1:400	Santa Cruz Biotechnology Inc., Santa Cruz, CA

Data analysis

All data except the immunohistochemistry were presented as mean and standard error of mean (S.E.M). For comparison among all groups of animals, one way analysis of variance (one-way ANOVA) was used, and followed by student Newman Keus multiple comparison test.

CHAPTER IV

RESULTS

Effects of OVX and estrogen supplementation on growth

Body weight gain represents the average of the changing weights of all rats throughout the experiment. Average body weight gain of rats in OVX group (0.43 ± 0.05 g/d, n=11 rats) was highest (*p<0.001*). In OVX with estrogen supplementation group (E_2 1 μg group, 0.10 ± 0.05 g/d, n=10 rats), body weight gain revealed no significant difference from sham group (*p>0.05*), however higher doses of estrogen at 5 or 15 μg/kg BW respectively decreased the average body weight gain by -0.03 ± 0.04 g/d (n=12 rats), *p<0.001* and -0.11 ± 0.04 g/d (n= 12 rats), *p<0.05* (Fig. 4-1).
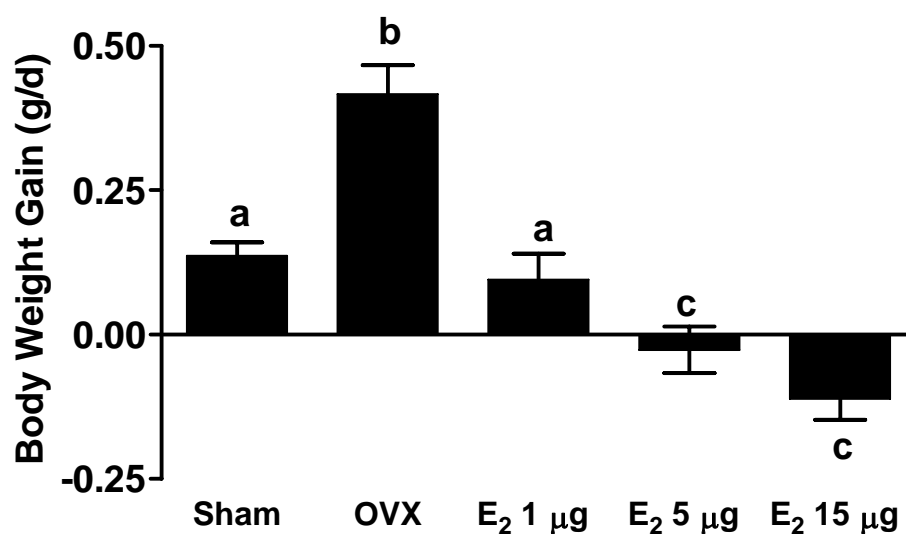
Figure 4-1   Effects of OVX and estrogen supplementation on average body weight gain of female Wistar rats. The histograms represent mean ± S.E.M. Bars with different letters are significantly different from one another (*P < 0.05*).

Effects of OVX and estrogen supplementation on food intake

Daily feed intake, which is an average volume of food that all rats consumed everyday, were found to similar in all group (sham = 9.83 ± 0.19 g/d; OVX = 10.54 ± 0.11 g/d; E_2 1 μg = 10.30 ± 0.30 g/d; E_2 5 μg = 10.23 ± 0.19 g/d; E_2 15 μg = 10.27 ± 0.14 g/d, *p>0.05*, n=10-12 rats) (Fig 4-2).
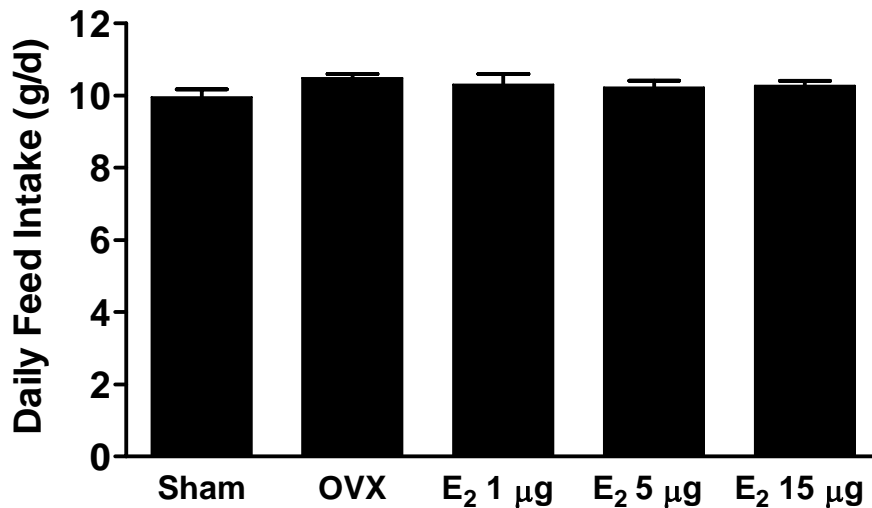
Figure 4-2 Effects of OVX and estrogen supplementation on daily feed intake of female Wistar rats. The histograms represent mean ± S.E.M. Bars were not denoted with any sign, since there were no significantly different from one another ($P > 0.05$).

Effects of OVX and estrogen supplementation on uterine weight

Since uterus is known as a target organ for estrogen hormone, the effects of estrogen depletion and supplementation on uterine weight was examined. In the present study, the ratio of uterine weight to body weight of all rats was measured immediately after sacrificed. In this study, in order to rule out the bias of the age-dependent uterine growth, uterine weight was represented as percent uterine weight per body weight (%uterine wt/BW). Uterine weight in OVX group was significantly decreased when compared with sham group (0.16 ± 0.01 % of BW, n=11 rats, *p<0.001*). However, this effect can be reversed by daily subcutaneously injection with

17β-estradiol, either of 1, 5 or 10 μg in a dose-dependent manner (OVX = 0.04 ± 0.002 % of BW; E_2 1 μg = 0.12 ± 0.01 % of BW; E_2 5 μg = 0.13 ± 0.01 % of BW; E_2 15 μg = 0.14 ± 0.01 % of BW, *p>0.001*, n=10-12 rats) (Fig 4-3).
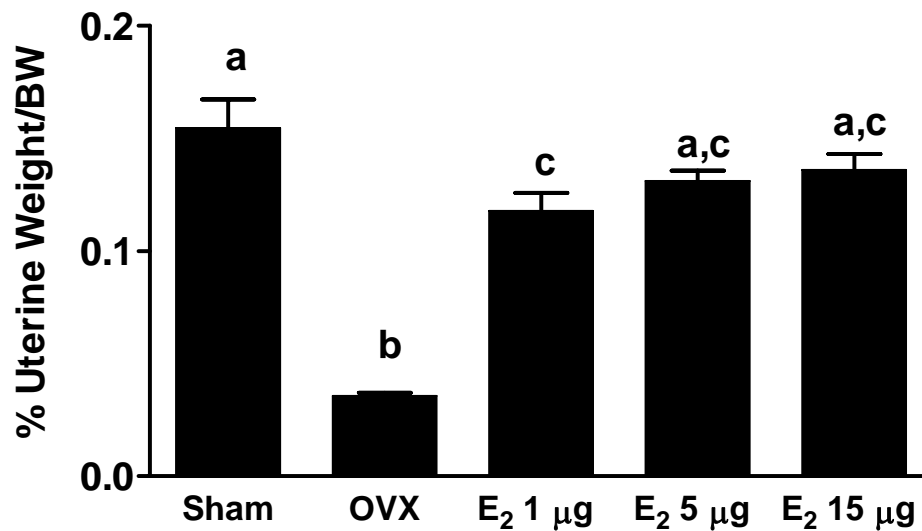

Figure 4-3 Effects of OVX and estrogen supplementation on uterine weight of female Wistar rats. The histograms represent mean ± S.E.M. Bars with different letters are significantly different from one another ($P < 0.05$).

Effects of OVX and estrogen supplementation on serum calcium and phosphorus

Calcium and phosphorus level in serum were also performed in order to examine the regulation of sex steroid on calcium and phosphorus metabolism. In this experiment, ovariectomized rat with or without estrogen supplementation revealed the similar levels of serum calcium to those of normal sham groups (sham = 10.84 ± 0.27 mg/dl vs. OVX = 10.66 ± 0.33 mg/dl; E_2 1 μg = 11.09 ± 0.22 mg/dl; E_2 5 μg = 11.38 ± 0.23 mg/dl; E_2 15 μg = 10.83 ± 0.18 mg/dl, *p>0.05*) (Fig.4-4 A).

Even though, serum phosphorus level of OVX was lower than those of sham group, the statistical analysis with by student's unpaired t-test showed no significant different at p = 0.056 (sham = 5.63 ± 0.38 mg/dl vs. OVX = 4.72 ± 0.43 mg/dl, Fig.4-4 B). In addition, estrogen supplementation had no effect on the serum phosphorus level when compared to sham or OVX group (E_2 1 μg = 4.69 ± 0.33 mg/dl; E_2 5 μg = 5.07 ± 0.33 mg/dl; E_2 15 μg = 5.33 ± 0.31 mg/dl, *p>0.05*) (Fig.4-4 B).

A

B
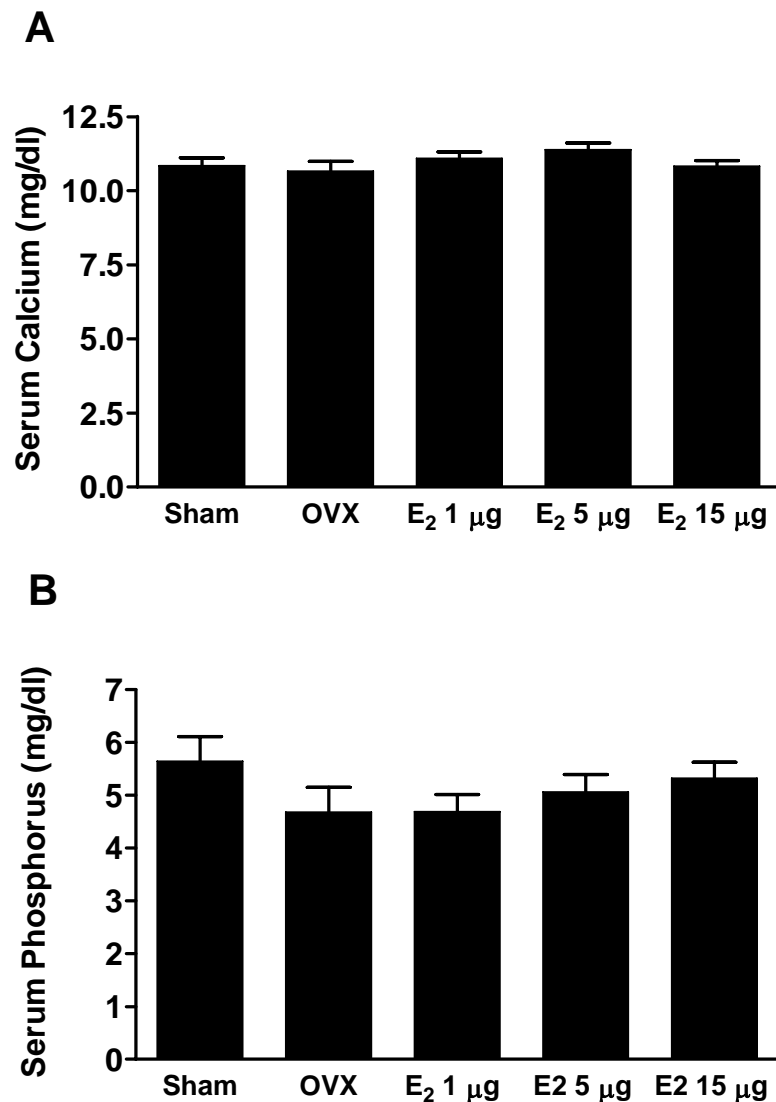
Figure 4-4 Effects of OVX and estrogen supplementation on A) calcium level in serum and B) phosphorus level in serum of female Wistar rats. The histograms represent mean ± S.E.M. Bars were not denoted with any sign, since there were no significantly different from one another ($P > 0.05$).

Effects of OVX and estrogen supplementation on serum and bone specific alkaline phosphatase

Bone-specific alkaline phosphatase is a marker of bone forming cell, osteoblast, which is released when osteoblasts are active. In the present study, change in the levels of total alkaline phosphatase (AP) paralleled with change in the bone specific alkaline phosphatase (bone-AP). Ovariectomized rat tended to have a higher

level but the significantly different from sham rats (Table 4-1 and Fig.4-5). Daily supplementation by subcutaneously injection with 5 μg/kg BW but not lower dose or higher dose of 17β-estradiol significantly decreased both total AP and bone-AP when compared with OVX group (Table 4-1 and Fig. 4-5).

A

B
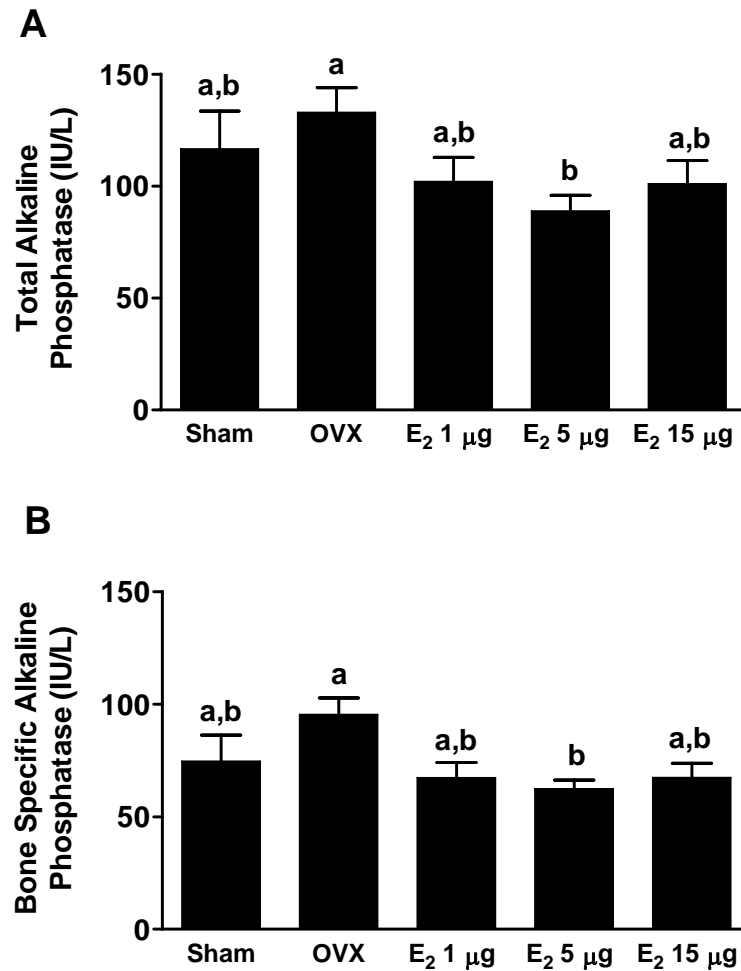
Figure 4-5 Effects of OVX and estrogen supplementation on A) total alkaline phosphatase and B) bone specific alkaline phosphatase of female Wistar rats. The histograms represent mean ± S.E.M. Bars with different letters are significantly different from one another ($P < 0.05$).

Table 4-1   Summary of the average levels (mean ± SEM) of serum total- and bone-specific alkaline phosphatase female Wistar rats

	Sham (n = 10 rats)	OVX (n = 11 rats)	E_2 1 µg (n =12 rats)	E_2 5 µg (n = 12 rats)	E_2 15 µg (n = 12 rats)
Total AP (IU/L)	116.60 ± 15.23[a,b]	132.9 ± 10.94[a]	102.0 ± 10.77[a,b]	88.83 ± 7.0[b]	101.0 ± 10.35[a,b]
Bone AP (IU/L)	74.63 ± 11.65[a,b]	95.38 ± 7.35[a]	67.36 ± 6.68[a,b]	62.37 ± 3.91[b]	67.43 ± 6.37[a,b]

Data represent mean ± S.E.M. Different letters in the same row are significantly different from one another ($P < 0.05$), ANOVA followed by Newman-Keuls Multiple Comparison test.

Effects of OVX and estrogen supplementation on bone mineral density

Lumbar vertebrae bone mineral density

Density of lumbar vertebrae level 1-6 was measured and we found that there is no significantly different in any groups of this experiment (p>0.005) (Fig. 4-6 and Table 4-2).
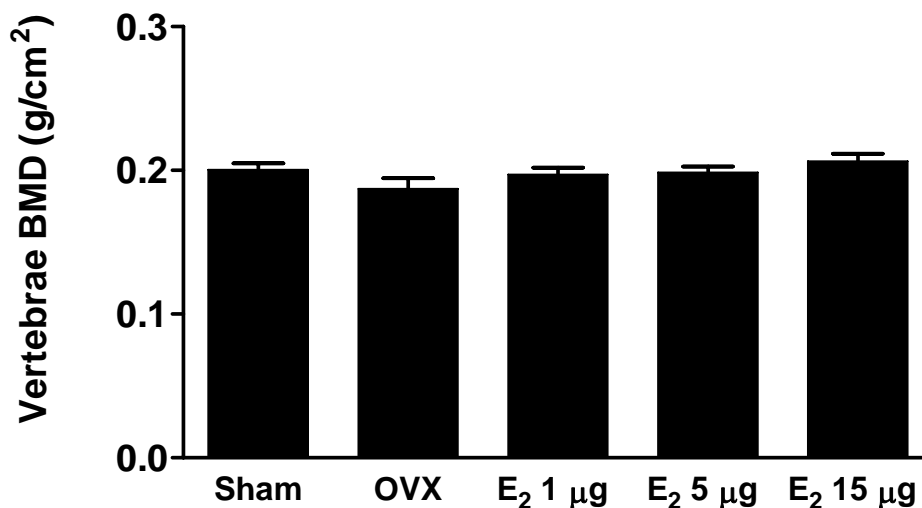
Figure 4-6 Effects of OVX and estrogen supplementation on the 1st-6th-lumbar vertebrae BMD of female Wistar rats. The histograms represent mean ± S.E.M. Bars were not denoted with any sign, since there were no significantly different from one another ($P > 0.05$).

Femur bone mineral density

In the present study, the total bone mineral content (BMC) and bone mineral density (BMD) of whole femur were examined by DEXA. The total BMC and BMD of femur in OVX group were significantly lower than those of sham group (p<0.05) (Fig. 4-7 and Table 4-2). Ovariectomized rats which were received estrogen supplementation at either dose of 1, 5 or 15 μg/kg BW reversed the decreased BMD to the level of sham group (Fig. 4-7 and Table 4-2).

Focus on the BMD of each part of femur, which is proximal, shaft, distal and head part of femur. In the shaft and proximal part excluding head of femur, the number of BMD were not altered by bilateral ovariectomy supplemented with or

without estrogen (Table 4-2). Even though, the ratio of shaft BMD to total bone volume was highest in this study. In addition, ovariectomized rat treated with 5 µg/kg BW daily showed an increase of this number however it was lower than ovx rats (Fig. 4-8B and Table 4-2). In contrast, related number of femoral head BMD to total bone volume did not significantly reveal differences in all experimental and control rat, whereas the measured BMD of femoral head were highest in E_2 1 µg and 15 µg group (Fig. 4-8D and Table 4-2).

At distal part of femur, OVX group significantly (p<0.05) decreased density when compared with the sham group. Estrogen supplementation starting at dose of 1 µg/kg BW to 15 µg/kg BW significantly increased both total BMD and % BMD of total volume of distal femur to those of sham group (Fig 4-8C and Table 4-2).

The total bone length was additionally examined in the present study. There were significantly differences among the experimental rats by ANOVA (p=0.048, n=10-12 rats) (Table 4-2). The average femur length of OVX rat and OVX rat supplemented with estrogen 1 or 15 mg/Kg BW were longer than the femur of sham and E_2 5 µg rats. Even though, the longest femur length was belong to E_2 15 µg group.
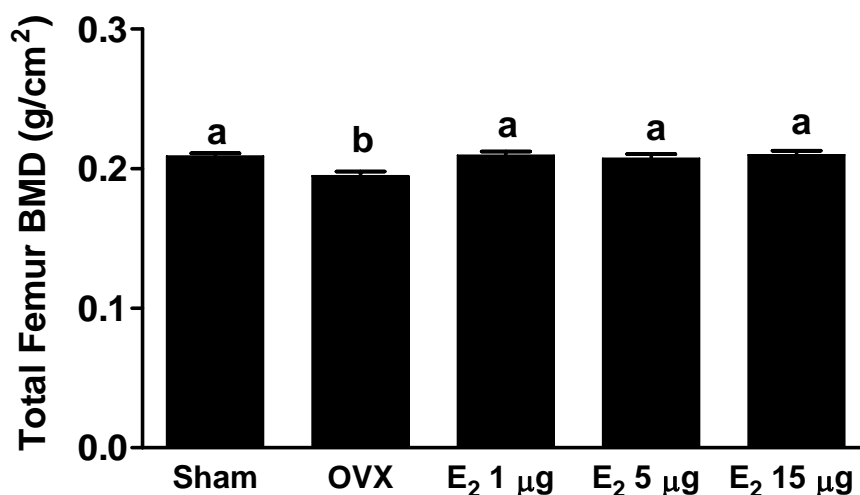
Figure 4-7   Effects of OVX and estrogen supplementation on total femoral BMD of female Wistar rats. The histograms represent mean ± S.E.M. Bars with different letters are significantly different from one another (*P* <0.05).

Table 4-2   Summary of the effects of OVX and estrogen supplementation on lumbar vertebrae and femoral bone mineral density and length of female Wistar rats

Groups / Bone	Sham (n=11 rats)	Vehicle (n=10 rats)	E$_2$ 1μg (n=12 rats)	E$_2$ 5 μg (n=12 rats)	E$_2$ 15 μg (n=12 rats)
Vertebrae (L1-L6)					
BMD (g/cm^2)	0.2004±0.004[a]	0.1873±0.007[a]	0.1972±0.005[a]	0.1986±0.004[a]	0.2061±0.006[a]
Total Femur					
BMD (g/cm^2)	0.2091±0.002[a]	0.1949±0.003[b]	0.2096±0.003[a]	0.2074±0.003[a]	0.2102±0.003[a]
BMC (g/cm)	0.4405±0.008[a]	0.4361±0.013[b]	0.4543±0.011[a]	0.4450±0.009[a]	0.4604±0.010[a]
Length (mm)	3.556±0.018[a]	3.600±0.033[a,b]	3.600±0.025[a,b]	3.533±0.014[a]	3.617±0.021[b]
Proximal					
BMD (g/cm^2)	0.03559±0.002[a]	0.03487±0.002[a]	0.03538±0.003[a]	0.03685±0.002[a]	0.03491±0.003[a]
% of BV/TV	95.51±0.56	94.16±0.46	95.45±1.3	94.33±0.35	94.85±0.35
Shaft					
BMD (g/cm^2)	0.1994 ± 0.003[a]	0.1923 ± 0.003[a]	0.2015 ± 0.003[a]	0.2006 ± 0.003[a]	0.2031 ± 0.003[a]
% of BV/TV	95.33±0.60[a]	98.72±0.60[b]	96.13±0.51[a]	96.79±0.80[a,b]	96.66±0.50[a]
Distal					
BMD (g/cm^2)	0.2376± 0.002[a]	0.2125± 0.005[b]	0.2349±0.003[a]	0.2329 ± 0.004[a]	0.2355±0.004[a]
% of BV/TV	113.70±0.75[a]	109.70±10.72[b]	112.10±0.86[a]	112.20±1.00[a]	112.00±0.70[a]
Head					
BMD (g/cm^2)	0.1681±0.003[a]	0.1578±0.003[a]	0.1712±0.002[b]	0.1664±0.003[a,b]	0.1712±0.002[b]
% of BV/TV	80.60±0.606[a]	81.23±0.40[a]	81.74±0.80[a]	80.25±0.90[a]	81.53±1.01[a]

Data represent mean ± S.E.M. Different letters in the same row are significantly different from one another ($P < 0.05$), ANOVA followed by Newman-Keuls Multiple Comparison test.

Figure 4-8 Effects of OVX and estrogen supplementation on percentage ratio of bone mineral density of A) proximal part, B) shaft part, C) distal part of femur and (D) head of femur. The histograms represent mean $\pm$ S.E.M. Bars with different letters are significantly different from one another ($P < 0.05$).

Effects of OVX and estrogen supplementation on calcium content in femur

To study the bone mineral content which is related to the bone density, bone ash weight is presented as total mineral content in femur bone. In the present study, bone ash weight of OVX group was significantly lower than sham group ($p < 0.05$) (Table 4-3 and Fig.4-9). The decreased ash weight of dried femoral bone induced by ovariectomy was restored by subcutaneous supplementation with all 3doses of 17β-estradiol in this study (Table 4-3 and Fig 4-9). Even though the total bone ash weight of all estrogen treated groups tended to lower than those of sham group (Table 4-3 and Fig.4-9).

In addition, the amount of calcium in femur ash using atomic absorption spectrophotometry was measured. Even though, the calcium content of E_2 15 μg showed the highest level, there were no significant difference among the groups ($p > 0.05$) (Table 4-3 and Fig.4-10).
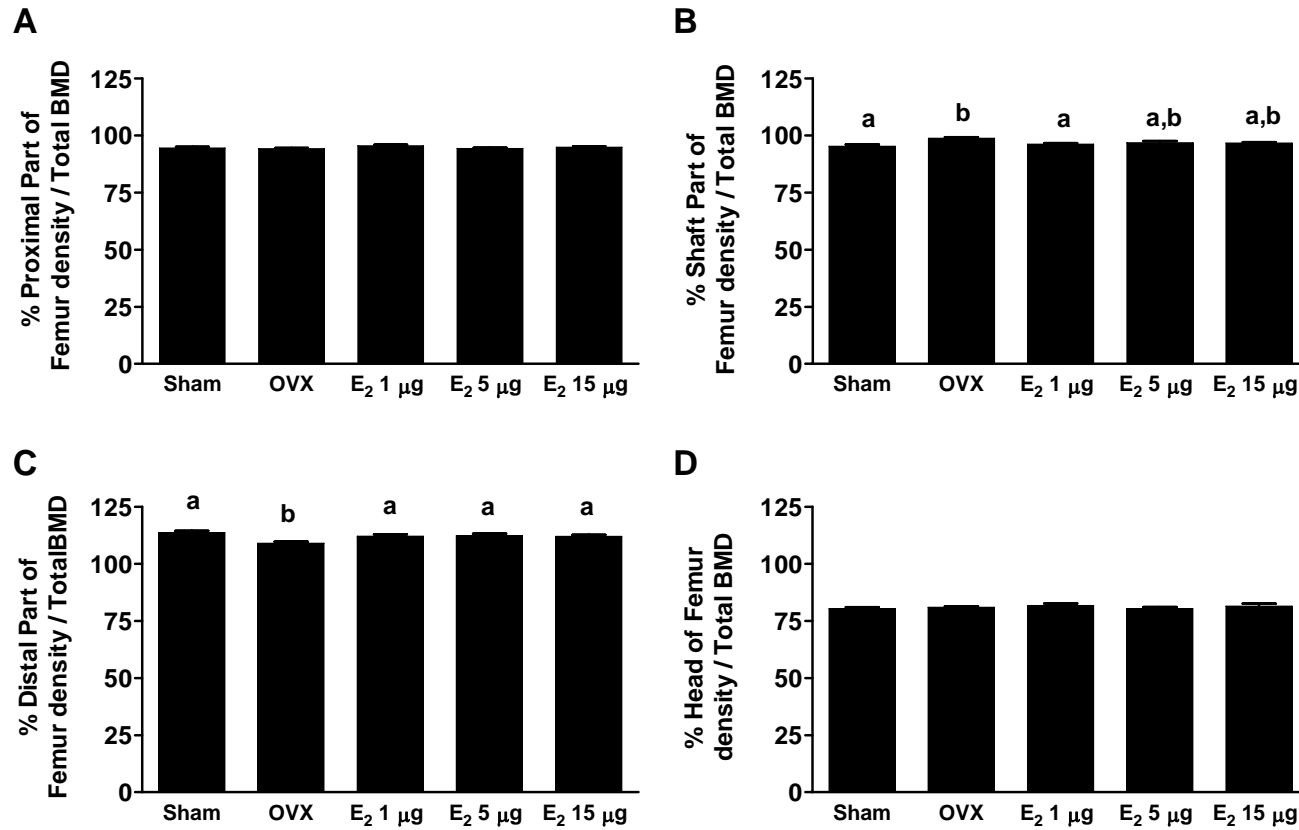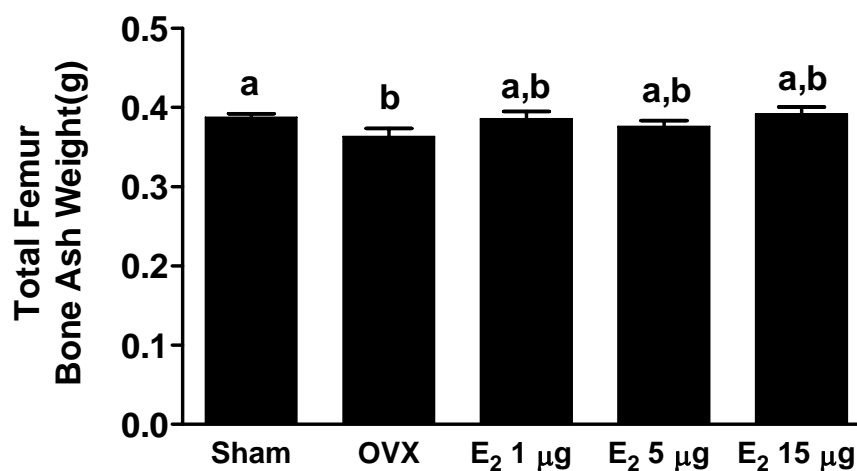

Figure 4-9 Effect of OVX and estrogen supplementation on total femur bone ash weight. The histograms represent mean ± S.E.M. Bars with different letters are significantly different from one another ($P < 0.05$).

Table 4-3 Summary of the effects of OVX and estrogen supplementation on femoral bone calcium content of female Wistar rats

	Sham (n =10 rats)	OVX (n = 11 rats)	E$_2$ 1 µg (n =12 rats)	E$_2$ 5 µg (n =12 rats)	E$_2$ 15 µg (n = 12 rats)
Total femur bone ash weight (g)	116.60 ± 15.23[a,b]	132.9 ± 10.94[a]	102.0 ± 10.77[a,b]	88.83 ± 7.0[b]	101.0 ± 10.35[a,b]
Total calcium content (mmol)	0.5878 ± 0.348[a]	0.5575± 0.048[a]	0.6125 ± 0.028[a]	0.5876 ± 0.028[a]	0.6567 ± 0.044[a]
Total calcium content per bone dry weight (mmol/g)	0.9678 ± 0.054[a]	0.9350 ± 0.090[a]	1.005 ± 0.057[a]	0.9867 ± 0.057[a]	1.0600 ± 0.085[a]

Data represented mean ± S.E.M. Different letters in the same row are significantly different from one another ($P < 0.05$), ANOVA followed by Newman-Keuls Multiple Comparison test.

A

B
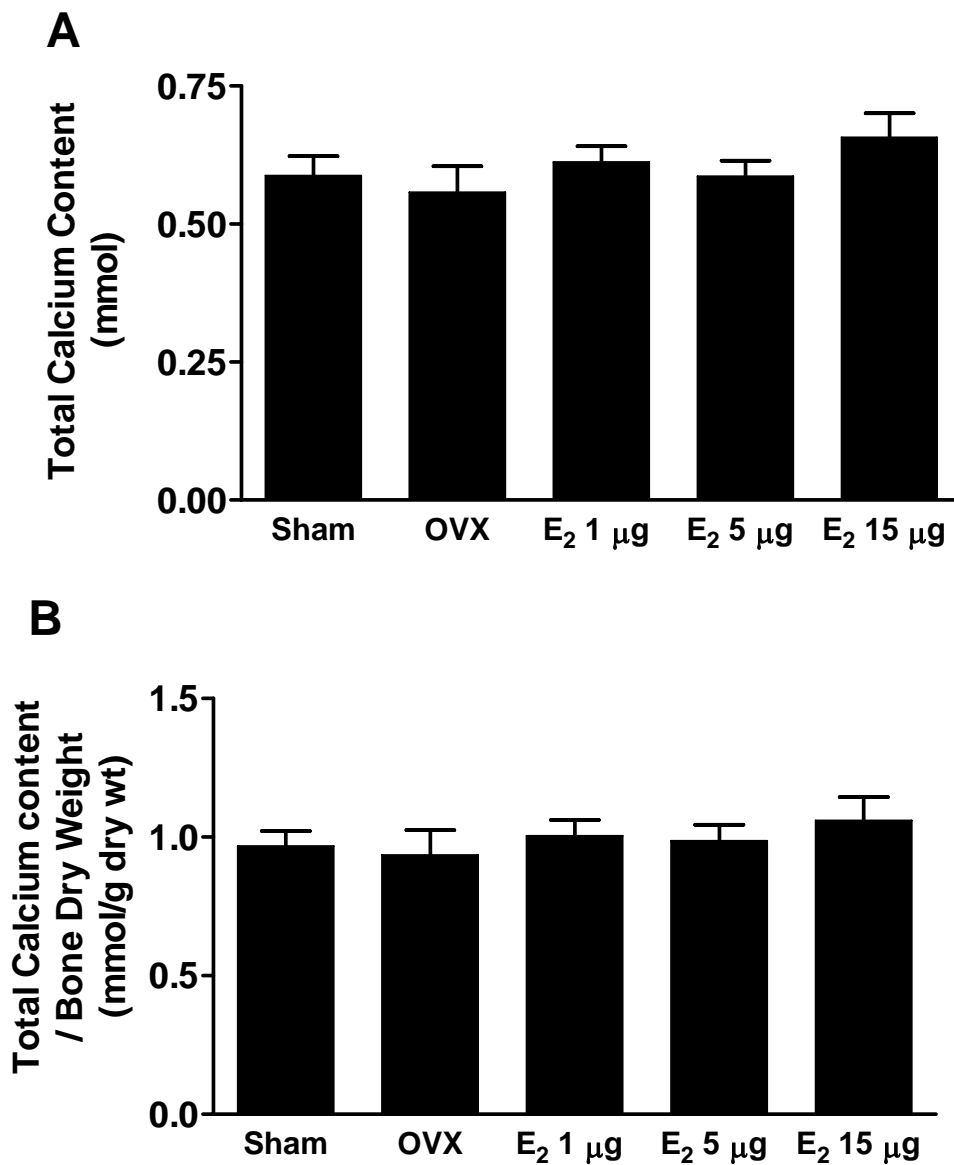
Figure 4-10 Effects of OVX and estrogen supplementation on A) total calcium content and B) total calcium content bone per femur dry weight of female Wistar rats. The histograms represent mean ± S.E.M. Bars were not denoted with any sign, since there were no significantly different from one another ($P > 0.05$).

Effects of OVX and estrogen supplementation on PGP-9.5, CGRP and substance P expression

To investigate the presence of sensory nerve in bone in various conditions, thus immunohistochemistry protocol was used. Neuropeptides, CGRP and SP were examined with PGP-9.5 the neural protein marker to represent the sensory nerve that innervates 4 regions of interest in the femur which are proximal, shaft, distal, head and epiphyseal plate in 14 micron thick decalcified bone sections.

To test the specificity and affinity of antibody used in this study, fixed tissue section of the dorsal root ganglia (DRG) from sham group (n =3 rats) was used as a positive control. The immunoreactivity of PGP 9.5 (PGP-ir) was found in the cytosol of all neural bodies of DRG (Fig. 4-11A). In consistency with PGP-ir, the adjacent section pre-incubated with anti-CGRP or anti-substance P antibody also revealed the positive immunoreactivity in the same neurons (Fig. 4-11B and C). In addition, the adjacent tissue section pre-incubated with only the secondary antibodies used in the study showed no immunoreactivity. The immunohistochemistry results of DRG indicate the high specificity and affinity of antibody used in the present study.

To study roles of estrogen on the sensory nerve containing in femur, the same immunohistochemistry protocol as above was used. Decalcified femur bone section samples from at least three of sham, OVX+vehicle or OVX+E2 μg/kg BW 17β-estradiol rats were respectively represented as normal, absence and the lowest effective concentration of estrogen requirement. The effective concentration of 17β-estradiol +OVX rats were chosen based on the study of bone mineral density, which this concentration showed the most effective dose in inhibiting the decrease BMD induced by OVX.
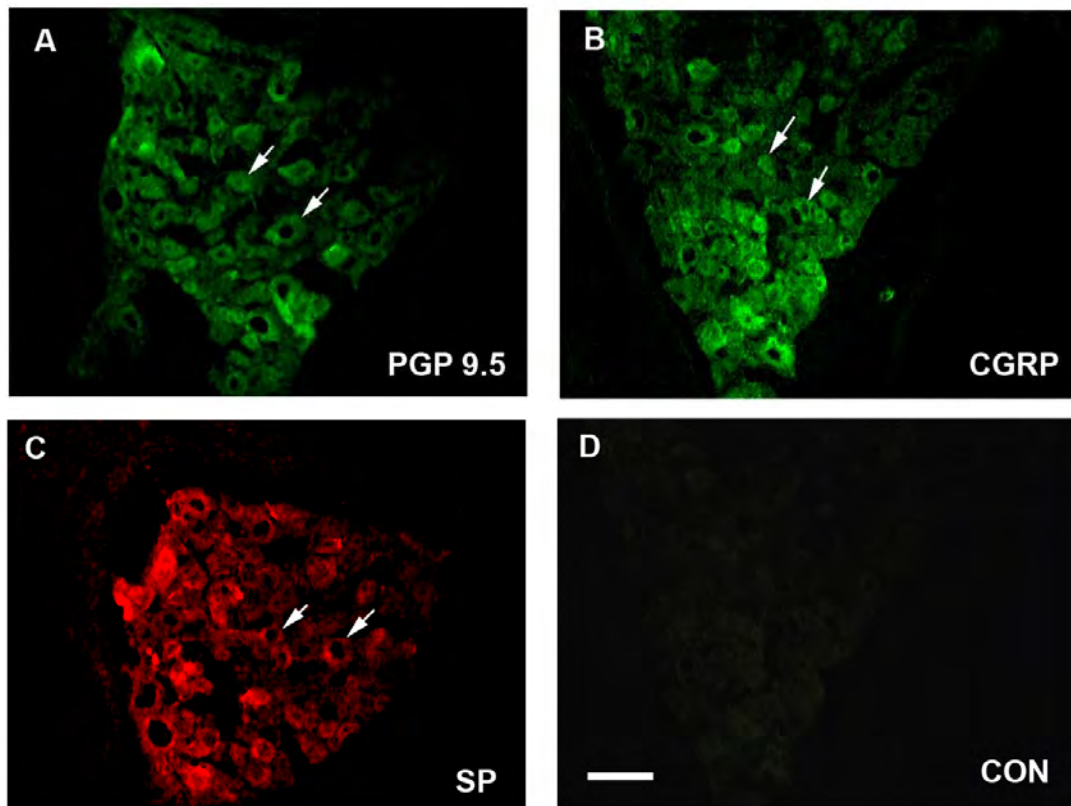
Figure 4-11 Photomicrograph illustrates localization of A) PGP-9.5 nerve marker, and sensory nerve neuropeptides, B) calcitonin gene related peptide (CGRP) and (C) substance P (SP), in dorsal root ganglia sections from sham group (n= 3 rats). The omission control (con) was also shown in panel (D). Small arrow indicates the immunoreactivity of neural bodies. Bar scale 400 μM.

Immunohistochemistry of proximal part of femur (Fig. 4-12)

There is no immunoreactivity of CGRP, PGP 9.5 and SP in the articular cartilage area of all proximal or epiphyseal part of femoral bone sections. In sham group, abundance of PGP-ir nerve fibers was localized in the bone cartilage. Even though, OVX with or without estrogen supplementation revealed the same finding, the number of immunoreactive fibers were less than sham group. For the immunoreactivity of CGRP, finding of CGRP immunoreactive (CGRP-ir) showed the parallel pattern to PGP-ir in all experimented rat. However, more CGRP-ir fibers especially at the endosteum of epiphyseal trabeculae bone were observed in the tissue section of estrogen treated- and untreated-OVX rats. SP-ir nerve fibers were also revealed at the same location but generally less than PGP-ir and CGRP-ir. However, positive nerve fibers immunoreactivity of anti-SP antibody seemed to be decreased in OVX with vehicle group and reversed when supplementation with 1 μg/kg BW of 17β-estradiol.

Figure 4-12 Photomicrograph illustrates localization of (*panel A, D, G*) PGP-9.5 nerve marker, sensory nerve neuropeptides (*panel B, E, H*) calcitonin gene related peptide (CGRP) and (*panel C, F, I*) substance P (SP) in proximal femur of sham group (A-C), ovariectomized rat (OVX; D-F) and 1 μg/kg BW 17β-estradiol supplemented OVX rat (G-I) (E$_2$ 1 μg; n= 3 rats). Large arrow and small arrows indicates immunoreactivity at the surface of endosteum and nerve fiber localized in bone cartilage and trabeculae, respectively. The omission control (not shown) revealed only the nonspecific immunorective in the bone marrow cavity. Bar scale 400 μM. *cart = bone cartilage; tb =epiphyseal trabeculae bone; bm = bone marrow.*
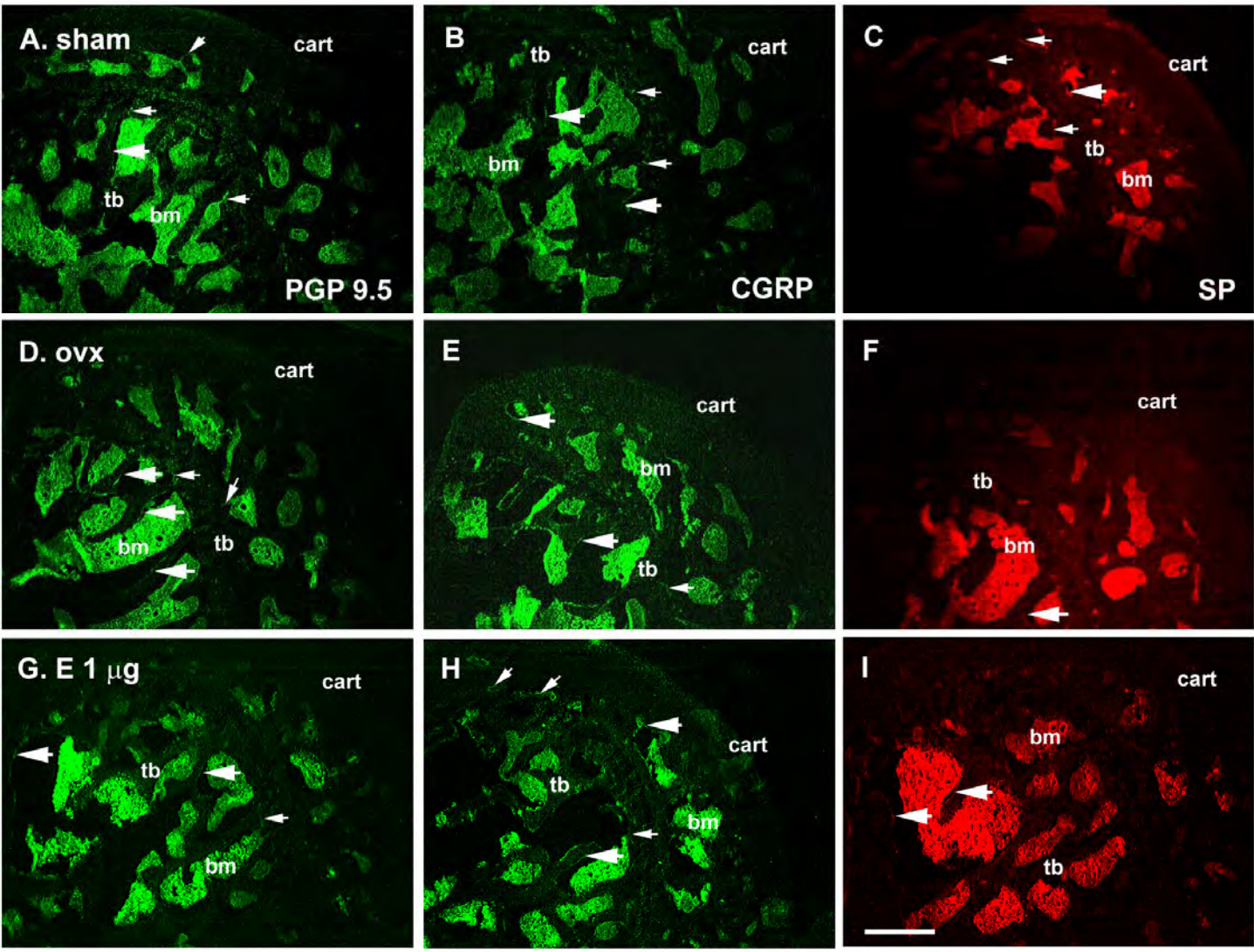
Immunohistochemistry of shaft part of femur (Fig. 4-13)

In this region which extremely cortical bone tissue, the present study revealed PGP-9.5-, CGRP- and SP-ir nerve fibers at the periosteum of estrogen treated- and untreated- OVX but not sham group. In addition, the E_2 1 μg group seemed to show more immunoreactive fibers of these three markers.

Inside the cortical bone, there were many Volkmann's canal systems where blood vessel and nerve were found. As described earlier in chapter II, sensory nerves also enter bone with blood vessel, thus in this region, may find immunoreactive at either blood vessel or nerve fibers. However, colocalization of CGRP-ir or SP-ir with PGP-9.5-ir in the adjacent section may help to locate nerve fiber. In this study, many of CGRP- PGP 9.5 and SP-ir nerve fibers in the cortical bone matrix of all rat groups were distributed in the similar pattern. In OVX with vehicle group, SP-ir nerve fiber tended to be less than sham and E_2 1 μg group. At the inner side of endosteum, there were a few SP-ir nerve fibers seen in OVX but not in sham or E_2 1 μg group.

Figure 4-13 Photomicrograph illustrates localization of (*panel A, D, G*) PGP-9.5 nerve marker, sensory nerve neuropeptides (*panel B, E, H*) calcitonin gene related peptide (CGRP) and (*panel C, F, I*) substance P (SP) in shaft of femur of sham group (A-C), ovariectomized rat (OVX; D-F) and 1 μg/kg BW 17β-estradiol supplemented OVX rat (E_2 1 μg; n= 3 rats). Large arrow and small arrows indicates immunoreactivity at volkmann's canal and nerve fiber at perisoteum, respectively. The omission control (not shown) revealed the nonspecific immunorective in the bone marrow. Bar scale 400 μM. *cort = cortica; bm = bone marrow; endo = endosteum; perio = periosteum; vc = volkmann's canal.*
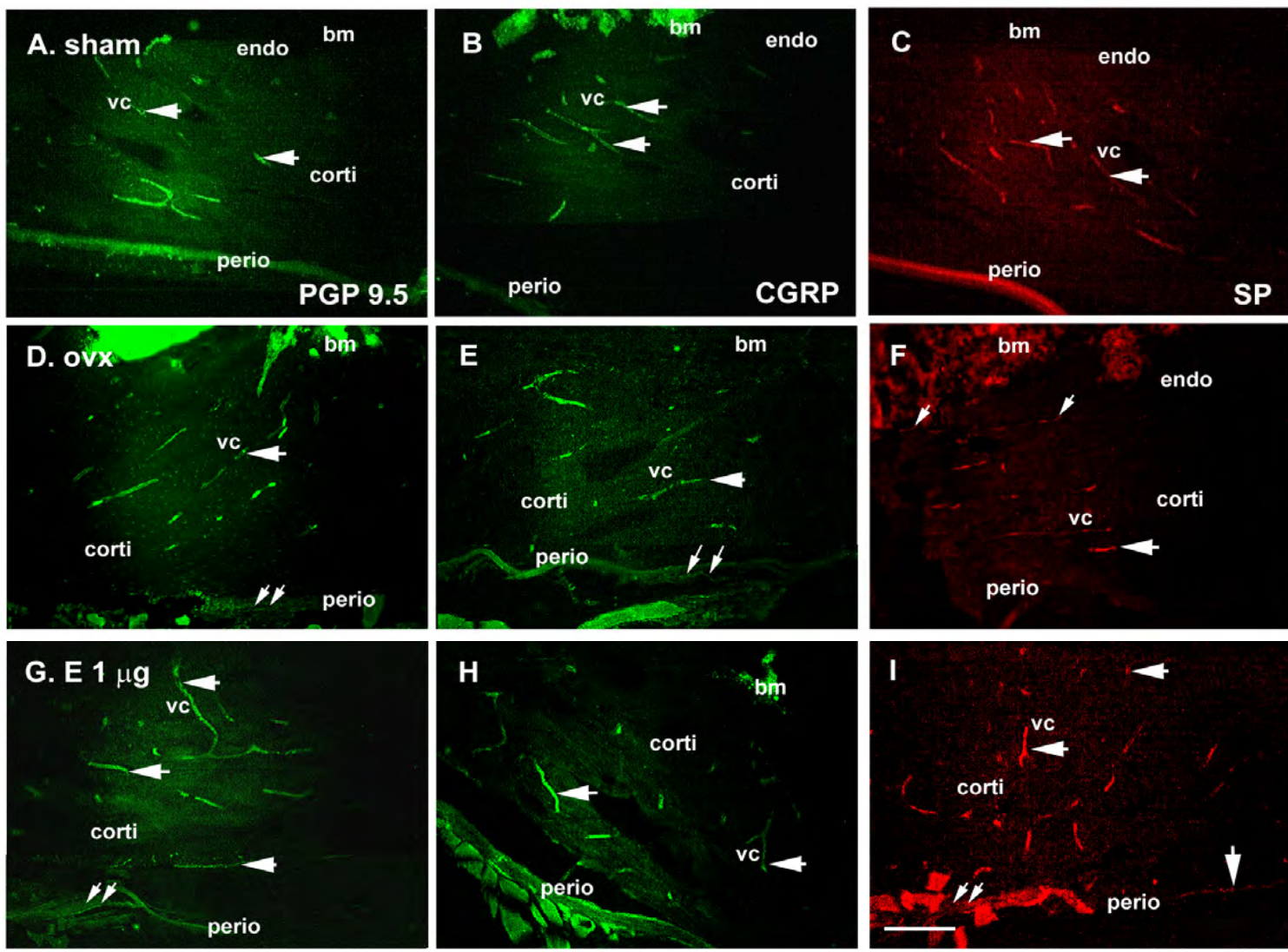
Immunohistochemistry of distal part of femur (Fig. 4-14)

Similar to the proximal part of femur, bone tissue of this region is mostly composed of cartilage and cancellous bone type. At the articular cartilage region, CGRP- and PGP 9.5-ir fibers were present in all experimented groups including OVX group. Only a few SP-ir nerve fibers were seen sham group but not in OVX treated or untreated with estrogen. Focus on the immunostaining of at epiphyseal trabeculae, many PGP-9.5 and CGRP- but a few of SP-positive fiber were revealed at the endosteum where the osteoclast resided, and at epiphyseal bone. In the OVX treated with vehicle, the number of these three marker antibodies was decreased and weak. The changes were reversed by estrogen supplementation, even though the number of SP-immunoreactivity remained less than sham group.

Figure 4-14   Photomicrograph illustrates localization of (*panel A, D, G*) PGP-9.5 nerve marker, sensory nerve neuropeptides (*panel B, E, H*) calcitonin gene related peptide (CGRP) and (*panel C, F, I*) substance P (SP) in distal femur of sham group (A-C), ovariectomized rat (OVX; D-F) and 1 µg/kg BW 17β-estradiol supplemented OVX rat (E$_2$ 1 µg; n= 3 rats). Large arrow and small arrows indicates immunoreactivity at the surface of endosteum and nerve fiber localized in bone cartilage and trabeculae, respectively. The omission control (not shown) revealed only the nonspecific immunorective in the bone marrow cavity. Bar scale 400 µM. *cart = bone cartilage; tb = trabeculae bone; bm = bone marrow.*
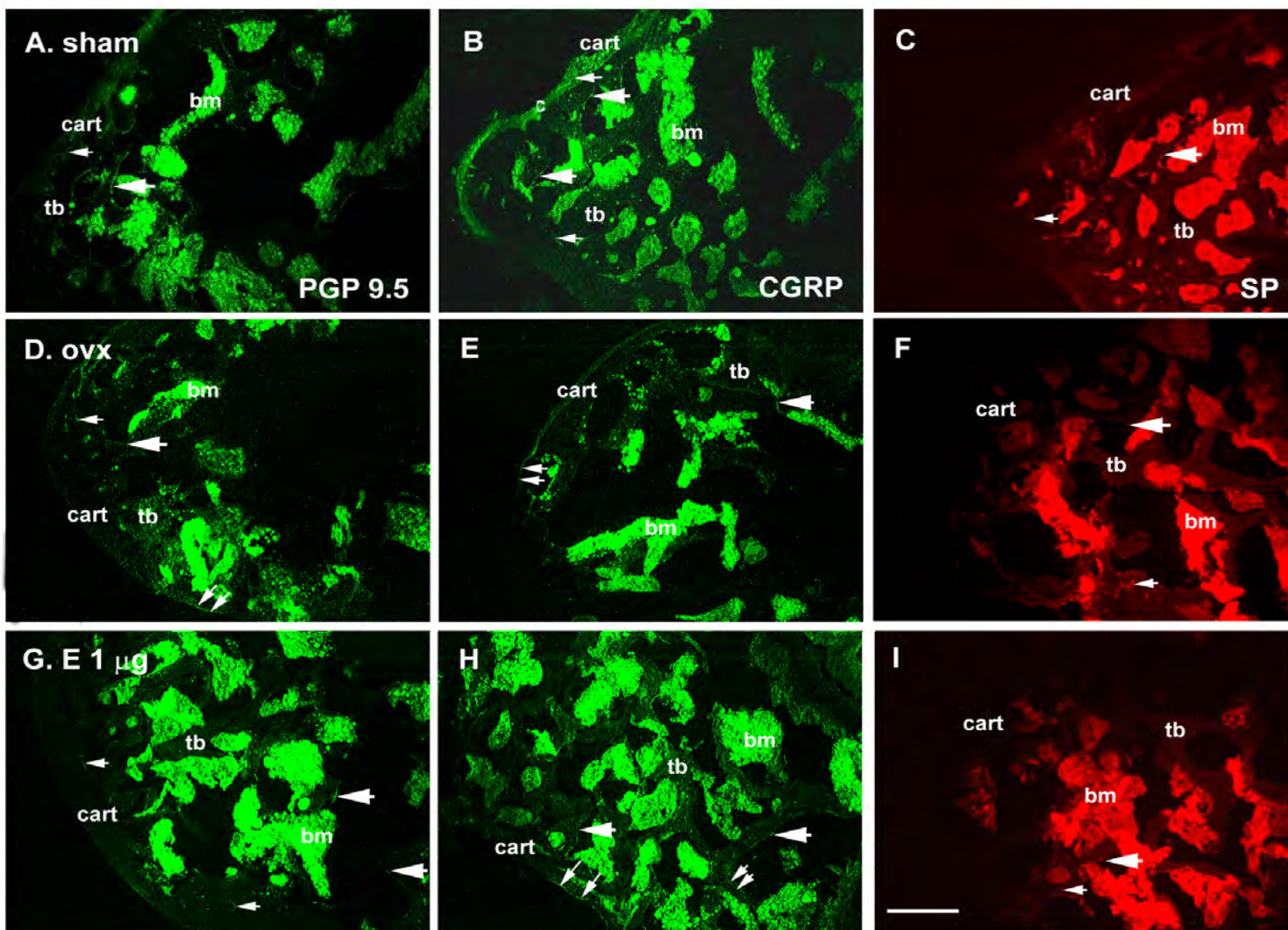
*Immunoreactive in epiphyseal plate region of femur* (Fig. 4-15)

Consideration of the morphological characteristics of epiphyseal plate or growth plate of all rat groups, femoral bone section of OVX showed the most widened epiphyseal plate. Above of plate is an epiphyseal trabeculae region, when many CGRP-ir and PGP-9.5-ir but rarely SP-ir immunoreactivity were found at the endosteum lining at the trabeculae bone cavity in sham group. In contrast, the distribution PGP- and CGRP-ir but not SP-ir nerve fibers were increase but relocated to the region below the plate (metaphysis) in the OVX treated with vehicle or estrogen group.

Figure 4-15   Photomicrograph illustrates localization of (*panel A, D, G*) PGP-9.5 nerve marker, sensory nerve neuropeptides (*panel B, E, H*) calcitonin gene related peptide (CGRP) and (*panel C, F, I*) substance P (SP) in epiphyseal plate of sham group (A-C), ovariectomized rat (OVX; D-F) and 1 μg/kg BW 17β-estradiol supplemented OVX rat (E_2 1 μg; n= 3 rats). Large arrow and small arrows indicates immunoreactivity at the surface of endosteum and nerve fiber localized in epiphyseal plate and trabeculae, respectively. The omission control (not shown) revealed only the nonspecific immunorective in the bone marrow cavity. Bar scale 400 μM. *tb = trabeculae bone; bm = bone marrow; perio = periosteum; epi = epiphyseal plate.*
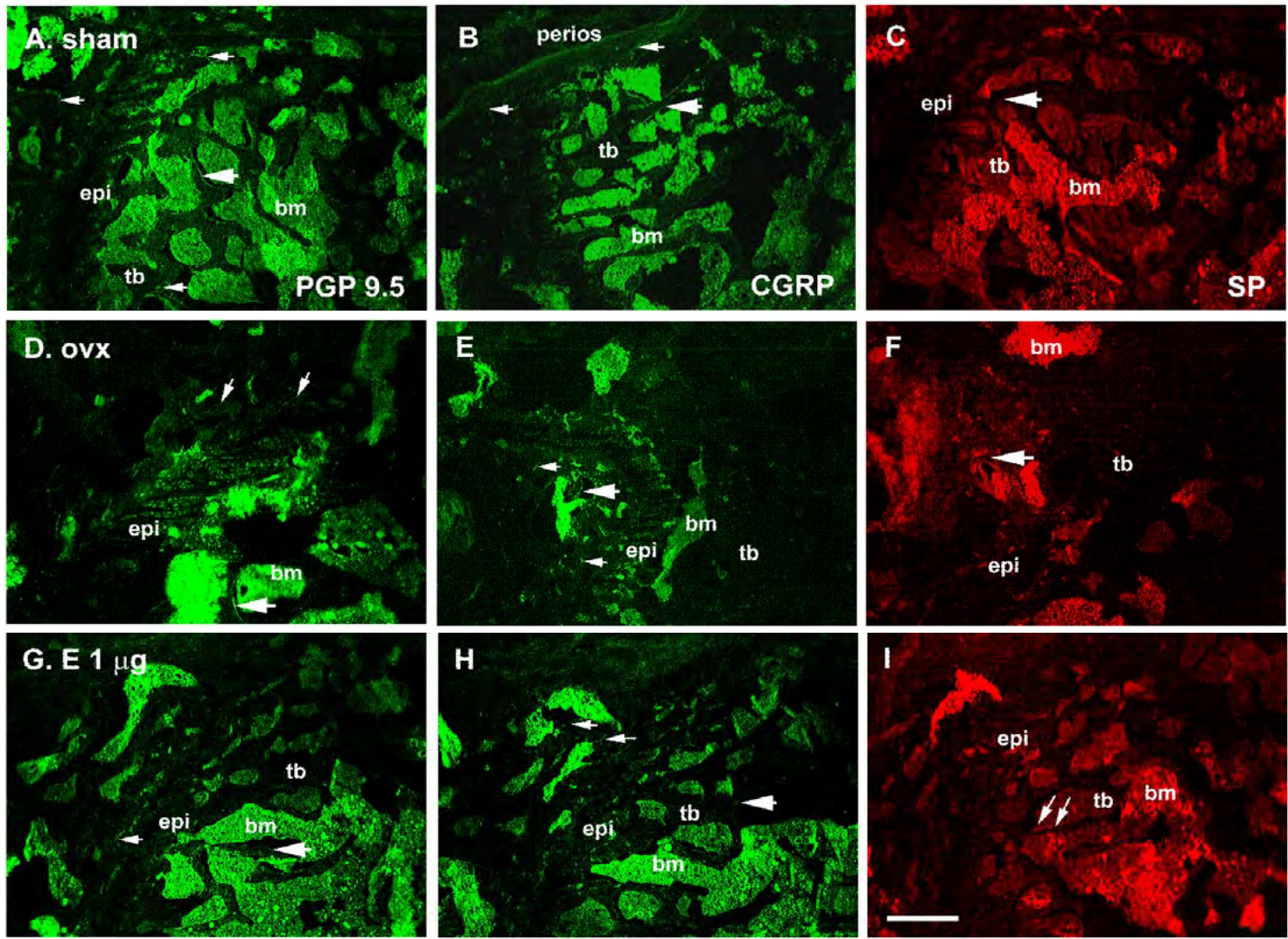
CHAPTER V

Discussion and Conclusion

This study aimed to investigate the presence of CGRP-, SP- and PGP 9.5-expressed nerve innervation in bone and its relation to a decrease of bone density in the ovariectomized rats and the reverse effects of estrogen supplementation. In the past, the nervous system, the central nervous system (CNS) and peripheral nervous system received (PNS) a little thought regarding their dissociation the bone loss. The previous studies of a number of neuropeptide receptors expressed on bone cells in the *in vivo* murine transgenic and pharmacologic models recently demonstrated that complex regulatory machinery controlling bone remodeling received inputs from the CNS and PNS (Elefteriou, 2008). Most studies on the regulation of bone remodeling by the nervous system emphasized on the relation between bone loss and metabolic disorders related to feed intake because of its predominant mechanism and function. However, only some researches revealed the findings of normal sympathetic and sensory nerve fibers distribution in the periosteum and bone using the immunohistochemical study in young animal (Hill et al., 1991; Konttinen et al, 1996). Burt-Pichart et al (2005) recently reported a dramatic decrease in nerve innervation density related to bone loss in the trabecular bone of tibia of ovariectomized rats. However, the profile of neurotransmitters contained in nerve such as CGRP and substance P, which have both local effect on bone cells and convey the signal to CNS, had never been reported in OVX-induced osteoporosis. Moreover, most of immunohistochemistry (IHC) studies emphasized the trabeculae bone such as tibia and vertebrae but not the femur which contains more cortical bone because trabeculae bone is the first site affected by sex hormone depletion. The present study firstly demonstrated that femoral bone with the low BMD in OVX group a different pattern of nerve innervation containing CGRP and substance P from sham group. The alteration induced by OVX was also reversed by estrogen supplementation.

Animal model

Basically, osteoporosis in postmenopausal women started with the deficiency of estrogen, follow by imbalance of bone remodeling cycle leading to diminished

bone mass density. Overall structural bone becomes thinner, more fragile and has higher risk of fracture. Therefore, to study a mechanism of osteoporosis and effect of estrogen supplementation, ovariectomized rat (OVX) is considered to be a good animal model. OVX rats have served as an animal model for investigation of the effects of estrogen deficiency on the bone mass, structure and turnover at various skeletal sites (Frost and Jee, 1992; Kalu, 1991). Bone loss in OVX rats is usually immediate, earlier and more substantial in areas of cancellous bone than cortical bone. Soon after the OVX procedure, more than 200% increase in bone resorption and formation rates, and consequently a detectable bone loss appeared on the 14th day. The total calcium and BMD were decreased in OVX but is reversed by estrogen treatment.

However, there are still no reference values or standard sites available for BMD measurements in rats. Therefore, previous studies have performed measurement at different sites, such as vertebrae, proximal-distal femur, femur shaft, proximal tibia or tibia shaft. Bagi et al. (1997) suggested that the proximal femur was the most appropriate site, and bone loss was first detected at 6 weeks after OVX procedure. Additionally, Fukuda and Iida (2004) had investigated the density of long bone in hind limbs in female rats of various age groups. They reported that the age related decreases in total BMD began at 15 months in femur and tibia and yet cancellous bone BMD in female rats does not reach a highest valve until the age of 6 months. Indeed, cancellous bone tissue is a major affected site of the imbalance of bone remodeling cycle because it has more surface than the cortical bone tissue. Therefore, it has a higher process of bone turnover rate and has more chance to be resorbed by active osteoclasts (Ornoy, 1995). It seemed more appropriate to simulate the incidence of the osteoporosis in postmenopausal women with mature bone. Thus, in the present study, instead of using young growing rats as in previous studies, I decided to use OVX female rats aged 6 months as a postmenopausal osteoporosis model to investigate the alteration of CGRP-, SP- and PGP 9.5-expressed nerve innervation in femur bone in various conditions.

Effects of OVX and estrogen supplementation on body weight gain and food intake

Body weight gain and food intake were determined daily. This study reported that body weight gain of OVX group was higher than in sham group, however

estrogen supplementation at dose of 1 µg/kg BW restored normal weight gain. Moreover, higher doses of estrogen (5 and 15 µg/kg BW) further decreased the body weight gain. It was not surprising since estrogenic effects have been suggested to regulate fat metabolism by decreasing lipogenesis but increasing lipolysis and fat deposition. Basically, adipose tissue expresses both of the classical estrogen receptors, α- subtype and β-subtype (Cooke and Naaz, 2004). Indeed, the body weight gain in postmenopausal women are mediated via ER-α, not ER-β, because propylpyrazoletriol (selective ER-α agonist) but not diarylpropionitrile (selective ER-β agonist) reduced the weight gain in both postmenopausal woman and ovariectomized murine model (Roesch, 2006; Wegorzewska et al, 2008). In the present study, rats in OVX group had the highest body weight gain and estrogen supplementation could reverse this effect in a dose dependent manner. In contrast to body weight changes, food intake was not significantly different in any group in the present study. However, others reported that estrogen deficit animals were found to have increases in body weight, fat deposition and feed intake (Roesch, 2006; Wegorzewska et al, 2008). Administration with estrogen caused retarded growth rate associated with the decrease in food consumption in growing mice and rats (Hart, 1990; Biegel et al, 1998). The explanation for the different finding on feed intake of the present study from other research groups could be that estrogen deficiency led to increased fat accumulation. Fat tissue has been indicated to produce and release leptin, adipose hormone, which has effects to decrease food intake at the same area as estrogen does in the hypothalamus (Henry and Clarke, 2008; Gao and Horvath, 2008). Most studies that reported the increase in food intake by OVX was done in the young animal study model (Roesch, 2006; Santollo et al., 2007). It is possible that the animals in the present study was older than those in previous study, and may have the different ER subtype and function at the brain area that regulate food intake from younger animals.

Effects of OVX and estrogen supplementation on uterine weight

In this study, bilateral ovaries of all rats except those in sham group were removed in order to induce estrogen deficiency. However, estrogen level in serum was not measured in our experiment. Therefore, in order to confirm the successful of ovariectomy and estrogen deficiency, the present study used the uterine weight, the

growth and function of which are regulated by estrogen via the ER-α, as the indicator of estrogen levels in the blood (Barton et al., 1998; Mödder et al., 2004). Dramatic decrease in uterine weight after ovariectomy was always reported elsewhere. Similar to other studies, uterine weight expressed as percent uterine weight per body weight of OVX group treated with vehicle was lowest in the present study. This change was inhibited by estrogen supplementation even at the lowest dose of 17β-estradiol (1 μg/kg BW) used in this study. This finding confirms that the ovariectomy procedure to induce estrogen depletion was successfully.

Additionally, all doses of estrogen supplementation that were used in this study were sufficient, for maintaining the reproductive system.

Effects of OVX and estrogen supplementation on serum calcium and phosphorus

Calcium and phosphorus are the major compositions of bone matrix. Since osteoporosis is the disease that results in bone loss, it is possible that calcium and phosphorus should be released into the extracellular fluid. Indeed, calcium and phosphorus are very important to the body function, so the major regulatory system which is consisted of three hormones, parathyroid hormone, vitamin D_3 and calcitonin, and their target organs (small intestine, kidney and bone) keeps these two ions within a level of narrow range (Hadley, 1992). The results showed that calcium and phosphorus level in serum was in the normal value suggesting that bone loss found in OVX group were caused by estrogen deficiency. In addition, some other groups suggested that even though the serum calcium was always kept within the normal range, the urine calcium was increased in aging ovariectomized rat with bone loss (Gaumet et al, 1997). This is happened because when calcium was released into the serum by bone resorption, the increased serum calcium inhibit PTH release leading to increase of calcium excretion from renal tubule to urine (Hadley, 1992). Therefore, level of urinary calcium together with serum calcium should be considered as indicator of bone metabolism imbalance.

Effects of OVX and estrogen supplementation on serum and bone specific alkaline phosphatase

Alkaline phosphatases are a group of enzymes found primarily in liver (isoenzyme ALP-1) and bone (isoenzyme ALP-2). Bone specific alkaline phosphatase

(bone-AP) is an enzyme that is produced and released by osteoblast. Serum bone-AP level is a highly specific and sensitive indicator of bone turnover. Bone-AP is significantly increased on average in healthy and osteoporotic postmenopausal women (Takahashi et al., 1997). These AP levels can be reduced after treatment with hormone replacement therapy (Johansen et al., 1988). Since the present study demonstrated that both total-AP and bone-AP were highest in OVX group and lowest in the OVX with estrogen (E_2 5 μg/kg BW) supplementation group. In fact, total AP has been recommended that it may not be suitable for marker of osteoblast because of other organ such as liver also produced AP. According to these two parameters we suggested that bone loss was evidence in OVX group, however it was prevented by estrogen supplementation.

Effects of OVX and estrogen supplementation on bone mineral density

In addition to the bone-AP osteoporosis marker, the classical noninvasive method used for diagnosis of osteoporosis is the measurement of bone mineral density. The present study applied the method of Dual Energy X-ray Absorptiometry (DEXA) to determine bone mass density of femur bone and vertebrae bone. Beside the measurement of total BMD, BMD of each region of femoral bone i.e. proximal, shaft part, distal part and head of femur was also investigated. The present study showed that total femoral BMD was decreased in OVX group and was restored by estrogen supplementation. The evidence was consistent with many other reports. But when one focused on each part of the femur, only the distal part but not proximal or head of femur, in OVX showed the decreased BMD. Instead of decreased BMD, the femur shaft of OVX group was actually increased when compared with sham group, however the effects was reversed by estrogen supplementation. The evidence suggested opposite roles of estrogen on the regulation of trabeculae and cortical bone. Interestingly, when considered the BMD of femur in a part, the proximal part of femur revealed the lowest BMD than the distal and shaft of femur. This may be explained by the composition of this part is more cartilage than distal and shaft Sievänen et al., 1994). Since the proximal part contains more chondrocytes and blood vessels than the distal part of femur, the loss of bone mass density was occurred later than the distal part.

In addition to the femur, a lumbar vertebra BMD was measured because osteoporotic vertebral fractures are a major health problem in the world. I also focused on lumbar level 1-6 because this part is predominantly cancellous bone, which has higher rate of bone turnover than cortical bone (Ornoy, 1995). The present study showed no significant difference in lumbar vertebrae BMD among groups. Even though, there was tendency to decrease in OVX group, the change was significant. The finding of this study especially of the vertebrae and the shaft part of femur BMD was not consistent with other reports which indicated the decrease bone density in the lumbar vertebrae of OVX rats. The controversy results may be because of the protocol of bone mineral density measurement which other research group usually perform in intact lumbar vertebrae. Therefore, only the 4th lumbar is able to measure. In the present study, I separated the lumbar vertebrae out of the rat body and measure all part of excised lumbar vertebrae. It is possible that the peak bone of different part of lumbar vertebrae may not be occurred at the same period of time, so the total of L1-L4 BMD is in average. The explanation was supported by the research group who used the mature rats and considered the same region of lumbar assessment as my study, Jiang and co-workers (1997). They revealed the BMD of L1-L4 vertebrae and femoral bone in sham and OVX to increase rapidly until 3 month post-OVX then stabilized or decreased thereafter. But in the distal femoral metaphysis, consisting mostly of trabeculae bone decreased at all observation periods at 3, 6, and 9 months postsurgery. They also indicated that bone mass of rat cortical bone is not matured until 7.5 months of age. Since we examined the BMD at 3 month post-OVX that rat age was 9 months, it was possible that the decrease in femur shaft BMD was just established. It would be more appropriate to examine BMD at 9 months post surgery or consider rats at peak bone mass as a model of mature rat and to perform OVX. In the present study, the mechanism of estrogen on the regulation of bone density appeared to stimulate or inhibit function of osteoblast and osteoclast (Tobias and Compston, 1999; Robinson et al, 1997; Nakamura et al., 2007), roles of estrogen on the other regulatory system including on bone nerve have not been investigated.

Effects of OVX and estrogen supplementation on bone ash weight and calcium content in femur.

Consistent with the total BMD results, total femur bone ash weight representing total mineral in femur of OVX group was lower than sham group and

this effect could be reversed by estrogen supplementation. However, level of calcium content in femur ash was not significantly different from sham group but calcium content in the 15 μg/kg BW estrogen supplementation group seemed to be the highest level. These results were contradictory to that of Gaumet and co-wokers in 1997 who studied the influence of OVX and estradiol treatment on calcium homeostasis in aging rat. They reported that OVX decreased bone dry weight and ash weight that was related with diminished bone calcium content. Even though, my results agree with that of Deyhim and co-workers (2003), who studied effects of estrogen depletion and isoflavone supplementation on bone metabolism. They found that neither OVX rat nor isoflavone treated rat shared significant difference in calcium of femur bone. The explanation for my different findings was the same as for bone mineral density.

Effects of OVX and estrogen supplementation on PGP-9.5, CGRP and substance P expression

Immunohistochemistry method was used for the investigation of the presence of PGP 9.5, CGRP and SP expression on sensory nerve innervation in femur bone in various conditions. Because the ratio of cortical and cancellous bone tissues is different in each part of bone, I considered the IHC on specific region i.e. proximal, shaft, distal part and epiphyseal plate of the femur. Moreover, since estrogen supplementation at dose of 1 μg/kg BW reversed the effect of OVX on bone density, I used femur bone from this group to represent as an estrogen supplementation condition.

In proximal part, PGP 9.5 immunoreactivity (PGP 9.5-ir) found in all groups. It seemed to be abundant in sham group but decreased in OVX group and reversed by estrogen supplementation. Even though, the proximal femur bone density did not show any differences among rat groups, the results agree with the study of Burt-Pichat and co-workers in 2004. They showed the dramatic decrease of neuronal markers neurofilament 200 during bone loss associated with estrogen withdrawal. The present study first demonstrated that CGRP-ir increased in OVX group, and these results was reversed by estrogen supplementation. In addition to the increase CGRP-ir, decrease of SP-ir was revealed in OVX group. The alteration of CGRP-ir and SP-ir pattern seemed to be opposite. And this change may help to protect bone loss associated with estrogen deficit. CGRP has a major role on regulating bone metabolism. It stimulates

the production of the insulin like growth factor-I (IGF-I) and inhibits cytokine tumor necrosis factor (Valentijn, 1997). Moreover, CGRP also inhibited bone resorption and increased osteoblast proliferation in human osteoblast-like cell and inhibited osteoclast precursor development and decreased committed precursor fusion to form multinucleated cells (Villa et al., 2000; Cornish, 2001). While SP, being opposite to CGRP, it also plays a role in bone resorptive cells. SP causes an acute rise of intracellular calcium in osteoclasts and stimulates the pit forming activity of rabbit osteoclasts (Goto et al., 1998; Mori et al., 1999).

On the contary, the expressions of all sensory neuronal markers, PGP 9.5, CGRP and SP-ir were decreased in OVX group and reversed by estrogen supplementation. Loss of sensory neurons containing PGP 9.5 and SP were associated with low BMD suggesting that the up-regulation of CGRP-containing neurons may be necessary for protecting bone loss in estrogen withdrawal condition. Both CGRP and SP are synthesized at the dorsal root ganglia (DRG). During pregnancy, estrogen level was increased and via estrogen receptor it could synthesize CGRP and SP on L6-S1 DRG in rats (Mowa et al., 2003A; Mowa et al., 2003B). Moreover, CGRP level was increased during pregnancy and after that it was dramatically decreased (Gangula et al., 2000) and estrogen increased the innervation of sensory nerve containing-CGRP at mammary gland (Blacklock and Smith, 2003). Additionally, DRG expresses estrogen receptor (Papka, 1997). This evidence suggested that estrogen plays role in the regulation and function of sensory nerve containing CGRP and SP. Therefore, in OVX group, immunoreactivity of three marker antibodies was decease associated with bone loss.

At the shaft part of femur where BMD is increased instead of decrease in OVX group, the pattern of PGP 9.5, CGRP and SP-ir were similar to that in the proximal part. Even though, the shaft part of femur of sham and E_2 1 µg group showed more abundant and greater intensity of SP-ir than OVX group. This pattern of immunoreactivity which showed only SP-ir and not CGRP-ir being decreased in relation to the increase of BMD in the cortical bone. Interestingly, the obvious finding in the cortical bone of OVX and estrogen supplementation group was that the PGP-ir and CGRP-ir in the periosteum layer including Volkmann's canal region were more expressed in OVX than sham group. Perhaps, up-regulation of the sensory nerves containing only CGRP at the outer surface of cortical bone was associated with

increase shaft femur BMD. It is possible that increase of CGRP containing nerves may increase bone formation rate and density in OVX group and presence of SP-ir while CRGP-ir was decreased may cause decrease in density loss in estrogen treated group. However, the clear mechanism of this evidence deserved to be further investigated.

Lastly, at the epiphyseal plate region, PGP 9.5, CGRP and rarely SP-ir were found in all groups but these three immunoractive nerves expressed in OVX and estrogen treated groups were more abundant at area below the plate. Epiphyseal plate is regions where endochondral ossification begins and we found that layer of plate were still wide especially in OVX group. It has been known that estrogen stimulates fusion of growth plate by promoting senescence of chondrocytes leading to fusion of growth plate in puberty (Savendahl, 2005). The evidence suggested that at the time I performed OVX operation animals have not reached the mature yet. To our knowledge, CGRP-containing nerve innervation is abundant at the site of new bone growth. According to the function of CGRP, CGRP plays a role in the vasodilation. It is possible that blood vessel carrying cells and growth substances to the area of new bone growth required the release of CGRP from sensory nerve. The evidence was in agreement with the observation in the bone conducting chamber where the sensory nerve grew into bone conduction chamber correlating with the growth of bone tissue in a time dependent manner (Medsen et al., 2000).

In conclusion, my study firstly demonstrated the decrease of CGRP and SP innervation at area of distal part of femur which was related low bone mass and bone density after OVX. It suggests that neural regulation may play a role in bone loss during osteoporosis. However, if the decrease of nerve innervation was compensated by up-regulation of CGRP production such as in the proximal part of the femur, it could protect bone loss induced by estrogen deficit. The lower number of SP nerves was also required to protect loss of bone mass density as shown here. These data support the evidence for a major role of nerve innervation in the local control of skeletal metabolism. In addition, the different pattern of CGRP and SP containing nerves in femur are specific to each region of bone. Since the sensory neural pathways are connected to the higher brain, the next major task in the area of bone biology at the mechanism and molecular level by which the CNS integration with peripheral nervous system in bone regulation needs to be explored. Besides the CGRP and substance P, the broad range of neurotransmitters including norepinephrine, glutamate

and NMDA the profound effects on the skeleton and may be involved in the neural control of bone metabolism. Together, these findings may shine new light on the study of the complexity of neurohormones regulating bone remodeling and uncover new potential therapeutic strategies for the treatment of osteoporosis in postmenopausal women.

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