การบำบัดด้วยเอสโตรเจนทดแทนป้องกันการสูญเสียหน้าที่ของหลอดเลือดขนาดเล็กในกระดูกและการสูญเสียมวลกระดูก

ESTRADIOL REPLACEMENT THERAPY PREVENTS BONE MICROVASCULARDYSFUNCTION AND BONE LOSS

อัจฉรียา กสิยะพัท* ATCHAREEYA KASIYAPHAT*
สุทธิลักษณ์ ประทุมราช** SUTHILUK PATUMRAJ**
ประสงค์ ศิริวิริยะกุล*** PRASONG SIRIVIRIYAKUL***

Abstract

Estrogen deficiency leads to various problems in different systems especially vascular system and bone remodeling, producing vascular disease and osteoporosis. The objective of this study was to examine roles of estradiol in postmenopausal induced-endothelial dysfunction and bone loss, the ovariectomized rat model was used. Eighteen female Wistar rats were divided into three groups of sham, Ovariectomized rats (OVX), and 17β-estradiol treated-ovariectomized rat (OVX_{E2}, 5μg/kg/day; sc). At 3 weeks after the ovariectomized surgery, the number of leukocyte adhesion in bone postcapillary venules (diameter = 15-30 um) of each group was determined by Intravital fluorescence video microscopy. Bone mass density (BMD) and bone formation marker were determined and represented by percentage of ash/dry matter and osteocalcin activity (using radioimmunoassay (RIA)), respectively. The results showed that in 3-wk OVX, leukocytes adhesion was significantly increased compared to their age-matched sham groups. Interestingly, 3-wk-OVX_{E2} leukocytes adhesion was significantly decreased. Besides, in 3-wk-OVX_{E2}, the values of BMD was significantly increased and osteocalcin activity was significantly decreased as compared with 3-wk OVX. Therefore, our findings suggested that hormone replacement therapy could effectively prevent bone microvascular dysfunction and bone loss.

* Dr., Program of Physical Education and Recreation, Faculty of Education, Chiang Mai Rajabhat University

** Assoc. Prof. Dr., Department of Physiology, Faculty of Medicine, Chulalongkorn Universit

*** Assoc. Prof. M.D., Department of Physiology, Faculty of Medicine, Chulalongkorn University

Keywords : Estradiol replacement therapy, leukocyte adhesion, bone, ovariectomized rat

บทคัดย่อ

การขาดฮอร์โมนเอสโตรเจนส่งผลเสียต่อระบบต่างๆของร่างกาย โดยเฉพาะอย่างยิ่งระบบหลอดเลือดและการสร้างและสลายของกระดูก ทำให้เกิดโรคเกี่ยวกับหลอดเลือดและโรคกระดูกพรุน การศึกษาวิจัยนี้จึงมีวัตถุประสงค์ เพื่อศึกษาบทบาทของเอสโตรเจนในหนูที่ถูกตัดรังไข่ทั้งสองข้างและทำให้เกิดภาวะการขาดเอสโตรเจน และส่งผลให้เกิดการเสียหน้าที่ของ Endothelial cell และการสูญเสียมวลกระดูก การทดลองใช้หนูขาวเพศเมียสายพันธุ์ Wistar จำนวน 18 ตัว แบ่งออกเป็น 3 กลุ่ม คือ หนูที่ผ่าตัดแต่ไม่ถูกตัดรังไข่ (Sham), หนูที่ถูกตัดรังไข่ออกทั้งสองข้าง (OVX) และหนูที่ถูกตัดรังไข่แล้วได้รับเอสโตรเจน 5 ไมโครกรัมต่อน้ำหนักตัวหนึ่งกิโลกรัม โดยวิธีฉีดเข้าใต้ผิวหนัง (OVX_{E2}) เป็นเวลา 3 สัปดาห์ภายหลังจากการตัดรังไข่ จากนั้นใช้เทคนิค Intravital fluorescence video microscopy ศึกษาผลของเอสโตรเจน ต่อจำนวนการเกาะติดของ Leukocyte ที่บริเวณส่วนปลายของหลอดเลือดฝอย หรือ Postcapillary venules (เส้นผ่าศูนย์กลาง = 15-30 ไมโครเมตร) ในกระดูก ใช้วิธีการคำนวณเปอร์เซ็นต์ของเถ้ากระดูกต่อน้ำหนักแห้งของกระดูก เพื่อตรวจสอบมวลแร่ธาตุของกระดูก และใช้เทคนิค Radioimmunoassay (RIA) เพื่อวิเคราะห์ระดับ Osteocalcin ซึ่งเป็นตัวบ่งชี้การสร้างกระดูก ผลการศึกษาพบว่า จำนวนการเกาะติดของ Leukocyte ที่บริเวณส่วนปลายของหลอดเลือดฝอย ในกลุ่ม OVX มีค่าเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ และกลุ่ม OVX_{E2} มีค่าลดลงอย่างมีนัยสำคัญทางสถิติ ตามลำดับเมื่อเทียบกับกลุ่ม Sham นอกจากนั้นในกลุ่มที่ได้รับเอสโตรเจนยังมีมวลแร่ธาตุของกระดูกสูงกว่า และระดับของ Osteocalcin ต่ำกว่ากลุ่ม OVX อย่างมีนัยสำคัญทางสถิติ ดังนั้นจากผลการทดลองสรุปได้ว่า การให้ฮอร์โมนเอสโตรเจนทดแทนสามารถป้องกันการสูญเสียหน้าที่ของหลอดเลือดขนาดเล็กในกระดูกและป้องกันการสูญเสียมวลกระดูกได้

Introduction

It is know that estrogen deficiency lead to various problems in different system including cardiovascular system and bone loss. It is believed that cardiovascular disease and bone loss were caused by endothelial dysfunction, and imbalance of bone formation and bone resorption, respectively. Estrogen clearly inhibits bone resorption, but enhances bone formation. The process of bone remodeling is determined by complex sequential interaction involving bone cells and its microcirculation (Laroche, 2002). Furthermore, the relationship among blood flow within bones, growth factors, and pro-inflammatory cytokine levels have been revealed for their roles on bone remodeling, bone formation and resorption (Passeri et al., 1993; Bismar et al., 1995 Roxane et al., 2001 and Salvatore et al., 2003). We believe that there is a close link between the abnormalities of microvascular function and subsequently bone remodeling after estrogen depletion. Therefore, in the present study we will monitor both alteration of bone microcirculation and bone loss in bilateral ovariectomized rats.

Especially, the effects of estradiol replacement therapy will be evaluated whether its molecular mechanisms on endothelial function will be accompanied by preventing bone microvascular dysfunction in associated with preserving bone remodeling or not.

Materials and methods

Animal preparation

Female Wistar rats, 10-weeks old (BW; 220-280g; obtained from National Laboratory Animal Center of Salaya Campus, Mahidol University, Bangkok), were used in this study. The animals were feed on normal food and tap water *ad libitum* under controlled environmental conditions of 12-hr light/dark period. After the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg), a bilateral ovariectomized was then performed (OVX rats) (Khemapech et al., 2003). Sham operated animals were used as controls.

Animals were divided into three groups of sham, ovariectomized rats (OVX), and OVX-treated with estradiol (17β-estradiol 5 ug/day; sc; OVX_{E2}). 17β-estradiol was given by subcutaneously injections immediately after the ovariectomized surgery for prevent estrogen depletion (Case and Davison, 1999 and Khemapech et al., 2003). All animals were fed by normal food and tap water *ad libitum* under controlled environmental conditions of 12-hr light/dark period until the experimental periods of 3 weeks.

Femur chamber preparation

On the day of experiment, the rats were anesthetized with intraperitoneal injection of 45 mg/kg of sodium pentobarbital. After the tracheostomy, polyethylene catheters (PE 20) were inserted into the left external jugular vein for intravenous drug administration. A 150 mm longitudinal skin incision was made approaching the femur from lateral. The femur was carefully exposed by blunt dissection between the flexors and extensors muscles to visualized bone vasculature. Prior to monitors of leukocyte adhesion the femur chamber was position exactly on the study area for enhanced the focusing of bone microcirculation (Hansen-Algenstaedt et al., 2005).

Intravital fluorescence video microscopy

The rat was anesthetized with intraperitoneal injection of sodium pentobarbital (45 mg/kg). A constant level of anesthesia was maintained throughout the experiment by supplement dose (20% of original dose) every 30–45 minutes. The tracheotomy was performed. The arterial blood pressure was recorded in the common carotid artery via a pressure transducer (Nihon-Kohden, Japan). The jugular vein was cannulated for injection of acridine orange (Sigma Chemical Co., USA; 5 mg/100 ml in normal saline) to label leukocytes. The bone microcirculation was observed using an epi-illumination fluorescence videomicroscope (Optiphot-2, Nikon, Japan) with a 50 W mercury lamp.

After femur chamber was put on the study area, the video images were achieved through a silicon intensified target television camera (Hamamutsu Photonics, Japan, USA), which was projected on a video monitor (GM-1411 QM, Sony, Japan) using 20 (or 40) objective lens (CF Plan Fluor, Nikon, Japan). The selected area was then recorded in real-time by a videotape recorder (SLV-X311, Sony, Japan) connected to a video timer (VTG-55, FOR-A, Japan) throughout the experimental period.

Leukocyte adhesion

Based on the recorded video images, we counted the number of leukocytes which adhered to the endothelial wall or remained stationary during 30 seconds or more in postcapillary venules (15–30 um in diameter). Using the mean total number (*N*) of adherent leukocytes measured from three venules in one rat with the length (*L,* um), we expressed the degree of leukocyte adhesion *(Cn)* in terms of cells/100 um vessel length as follows (Jariyapongskul et al., 2006):

$$Cn = \frac{N \times 100}{L}$$

Bone ash

At the end of each experiment, the right femur bone was removed and placed in 70% alcohol and dried at 100^{o}C for forty-eight hours, then weighed. After that the dried bone was burned in covered crucibles at 600^{o}C for 16 hours. Finally, the cooled bone ash was weighed and expressed by the percentage of Ash/Dry matter (Farzad et al., 2003).

Serum osteocalcin

At the end of each experiment, blood sample was collected by means of cardiac puncture, and the serum was stored at –70^{o}C until the day of analysis. Serum level of osteocalcin (OC), which is normally represented as an index of bone formation was determined by radioimmunoassay (RIA) with a commercial available kit. (Biomedical Technology Inc.) (Zeni et al., 2000).

Statistical analysis

Results were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to determine the difference of means. Tukey Post-Hoc test was used for multiple comparisons among groups. The statistical differences were considered at the probability level (*p*-value) of lower than 0.05.

Results

Leukocyte adhesion

The degrees of leukocyte adhesion (*Cn*) calculated for sham, OVX, and OVX_{E2} groups are shown in Figure 1. On 3 weeks the leukocyte adhesion were increased significantly ($p < 0.001$), compared to the corresponding. However, in estradiol treated groups, the leukocyte adhesion were reduced significantly ($p < 0.001$), compared to OVX group.

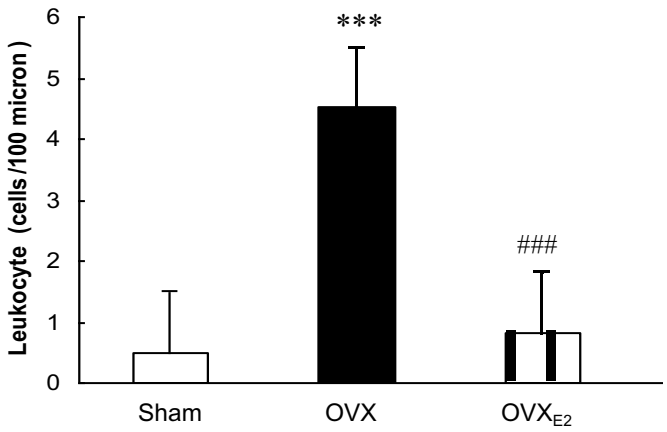
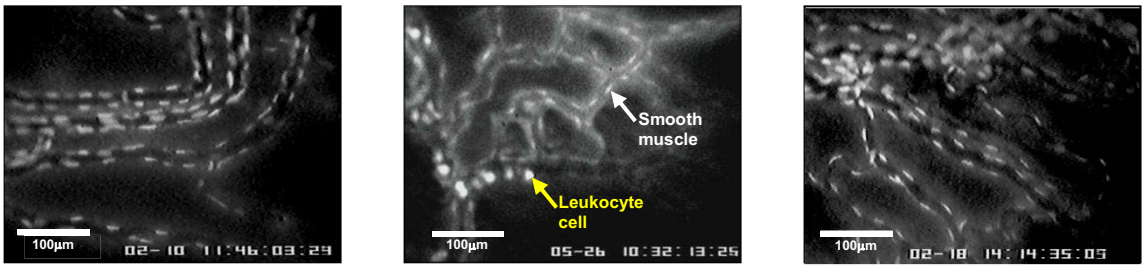


Figure 1 Effect of 17β-estradiol on leukocyte adhesion in postcapillary venule of femur bone at 3 weeks after ovariectomized rats. (n=6). ***p<0.001 vs. sham, and ### p<0.001vs. OVX.

Figure 1 showed an example of fluorescence video image to demonstrate leukocytes adhesion to the venular wall in three groups.



Sham (a) OVX (b) OVX_{E2} (c) (x20).

Figure 2 Fluorescence videomicroscopic images to demonstrate leukocyte adhesion on 3 weeks after treatment in sham (a), OVX (b), and OVX_{E2} groups (c) (x20

Bone formation marker

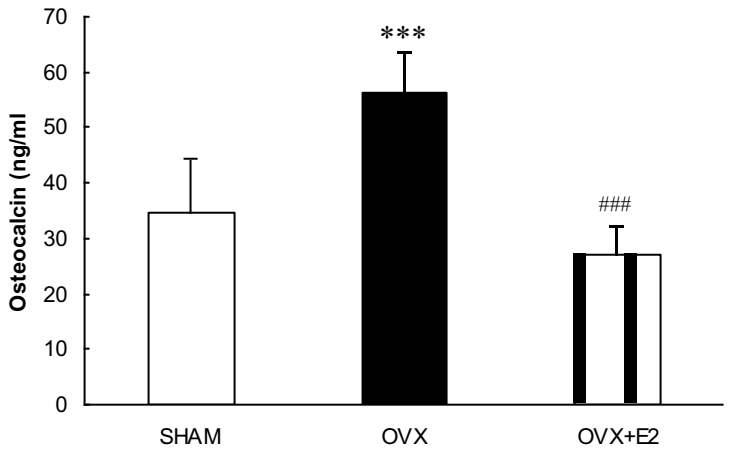


Figure 3 Effect of 17β-estradiol on serum osteocalcin at 3 weeks after ovariectomized rats. (*n*=6). ****p*<0.001 vs. sham, and ### *p*<0.001 vs. OVX.

Bone ash content

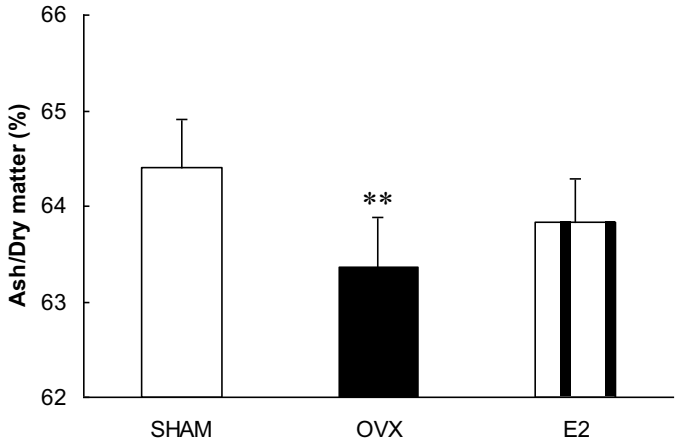


Figure 4 Effect of 17β-estradiol on bone mass at 3 weeks after ovariectomized rats. (*n*=6). ***p*<0.01 vs. sham.

Figure 4 showed the results of bone ash content of the right femur .The results demonstrated that 17β-estradiol could attenuate the decreases of bone ash content significantly as compared to 3 week- OVX group (*p*<0.05)

Discussion

This study demonstrates that estradiol replacement therapy can prevent bone microvascular dysfunction and bone loss in OVX rats. After treatment with 17β-estradiol for 3 weeks, the leukocyte-endothelial cell interaction had significantly prevented compared to OVX rats (p<0.001), suggesting that estrogen play a role in anti-inflammation properties to prevent endothelial dysfunction. Several studies have suggested that the decline in ovarian function with menopause is associated with spontaneous increase in pro-inflammatory cytokines, IL-1, IL-6 and TNFα by monocytes, bone marrow macrophages, and osteoblasts (Pacifici,1991; Kitasawa et al., 1994; Tostes, 2003). Therefore, the recruitment of leukocyte adhesion may be enhanced via the consequence of estrogen deficiency-induced pro-inflammatory cytokines production. Besides, estrogen has been demonstrated for its role in up-regulating endothelial nitric oxide synthase (eNOS) expression and so increase NO generation. Constitutive NO production by endothelium is well known for its anti-adhesive properties (Hudetz, 1999). Therefore, we believe that the recruitment of leukocyte adhesion may be enhanced by this reduction of NO bioavailability.

Roberto (2000) has showed that during ischemia in the cerebral circulation of female rats, the significant increase in basal leukocyte adhesion was obtained in estrogen depletion as compared to normal. And after treatment with estrogen, the abnormality has been attenuated. Their finding showed that ovariectomy-induced chronic estrogen depletion was associated with an enhanced leukocyte adhesion similar to our results of bone microcirculation.

Osteocalcin is a protein secreted by mature osteoblasts, and it has been shown to correlate with the bone turnover rate, including both active bone formation and resorption. Multiple studies have demonstrated a rise in osteocalcin levels in postmenopausal women with osteoporosis (Aleksandra et al., 1999). Our results showed the correlation of osteocalcin activity and bone mass density in each group, therefore, it supported the evidence that estrogen has its role on bone formation (Binbin and Shifeng, 2003).

In conclusion, the results of our experiment demonstrated that both bone mass density and endothelial dysfunction as characterized by leukocyte adhesion were occurred 3 weeks after OVX. Therefore, it may be indicated for a correlation between blood supply and bone remodeling. Interestingly, our findings suggested that estrogen replacement therapy could effectively prevent both vascular endothelial dysfunction and bone loss.

References

Aleksandra KD, Wladyslaw G, Ewa W. (1999). Osteocalcin, estradiol and prolactin concentrations in women receiving long-term estradiol replacement therapy. **Clin Invest.** 5(2): 780–786.

Binbin L and Shifeng Y. (2003). Genistein prevents bone resorption diseases by inhibiting bone resorption and stimulating bone formation. **Biol Pharm Bull**. 26: 780–786.

Bismar H, Diel I, Ziegler R and Pfilschifter J. (1995). Increased cytokine secretion by human bone marrow cells after menopause or discontinuation of estrogen replacement. **J Clin Endocrinol Metab**. 80: 3351-3355.

Case J and Davison CA. (1999). Estradiol alters relative contributions of nitric oxide and cyclooxygenase products to endothelium-dependent vasodilation. **JPET**. 291: 524–530.

Farzad Deyhim, Barbara J. Stoecker, Gerald H. Brusewitz and Bahram H. Arjmandi. (2003). The effects of estrogen depletion and isoflavones on bone metabolism in rats. **Nutr Res**. 23: 123-130.

Hansen-Algenstaedt N, Schaefer C, Wolfram L, Joscheck C, Schroeder M, Algenstaedt P and Ruther W. (2005). Femur window a new approach to microcirculation of living bone in situ. **J orthopaedic Res**. 23: 1073-1082.

Laroche M. (2002). Circulation intraosseuse: de la physiologie ‘a la pathologi. **Rev Rhum**. 69: 484-91.

Hudetz AG, Wood JD, Kampine JP. (1999). Nitric oxide syntase inhibitor augments post-ischemic leukocyte adhesion in the cerebral microcirculation in vivo. **Neural Res**. 21: 378-384.

Jariyapongskul A, Rungiaroen T, Kasetsuwan N, Patumraj S and Niimi H. (2006). Chronic changes of the iris microvasculature of streptozotocin-induced diabetic rats using fluorescence videomicroscopy. **Clin Hemorheol Microcir**. 34(1): 273-282.

Khemapech S., Patumraj S. and Siriviriyakul P. (2003). Genistein replacement therapy for vasodilation disorder in bilateral ovariectomized rats. **Clin Hemorheol Microcirc**. 29: 271–277.

Kitasawa R, Kimble RB, Vannice JL, Kung VT and Pacifici R. (1994). Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. **J Clin Invest.** 94: 2397-2406.

Pacifici R, Brown C, Puscheek E, Fredrich E, Slatopolsky E, Maggio D, McCracken R and Avioli L. (1991). Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. **Proc Natl Acad Sci USA.** 88: 5134-5138.

Passeri G, Girasole G, Jilka RL and Manolagus SC. (1993). Increased interleukin-6 production by murine bone marrow and bone cells after estrogen withdrawal. **Endocrinology**. 133: 822-828.

Roberto A. Santizo, S. Andeson, Y. Shuhua, HM. Koenig and Pelligrino DA. (2000). Effects of estrogen on leukocyte adhesion after transient forebrain ischemia. **Stroke**. 31: 2231-2235.

Roxane CS, Ronald FZ, Kevin RF and Robert CB. (2001). Evaluation of laser-Doppler perfusion imaging for measurement of blood flow in cortical bone. **J Appl Physiol.** 90: 1314-1318.

Salvatore C, Emanuela M, Laura D, Tiziana G, Rosanna DP and Zaira R. (2003). Inducible nitric oxide synthase mediates bone loss in ovariectomized mice. **Endocrinology**. 144(3): 1098-1107.

Tostes RC. (2003). Effects of estrogen on the vascular system. **Braz J Med Biol Res.** 36: 1143-1158.

Zeni S, Gomez-Acotto C, Di Greorio S and Mautalen C. (2000). Differences in bone turnover and skeletal response to thyroid hormone treatment between estrogen-depleted and repleted rats. **Calcif Tissue Int.** 67: 173-177.