

# Osteoprotective effect of hormone therapy on bone microarchitecture before impaired bone mineral density in ovariectomized rats

*Oofektomili ratlarda kemik mineral dansitesi etkilenmeden önce, hormon tedavisinin kemik mikromimari üzerine koruyucu etkisi*

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## Abstract

**Objective:** We aimed to determine the effect of hormone replacement therapy on bone microarchitecture in ovariectomized rats.

**Material and Methods:** In the Animal Ethics Committee approved-study, the effect of treatment with 17  $\beta$ -estradiol 50  $\mu$ g/kg and medroxyprogesterone 2.5 mg/kg on bone architecture and bone mineral density in rats versus ovariectomized control rats over the course of 20 days were evaluated. Femoral and lumbar bone mineral density levels and morphometric measurements were performed.

**Results:** There were no significant differences in the femoral and lumbar bone mineral density levels between the groups. In the intact control group, the trabecular structures were significantly superior to those in the other groups. Additionally, the osteoblast count was significantly higher while the osteoclast count was significantly lower than in all other groups. Two parameters reflecting trabecular bone microarchitecture, which include the trabecular count and the trabecular area, demonstrated significant improvement in the hormone replacement group when compared to the ovariectomized control group. In the hormone replacement groups, the osteoblast count was significantly higher while the osteoclast count was significantly lower than in the ovariectomized control group.

**Conclusion:** We suggest that offering estrogen alone or in combination with progestogen can be a beneficial approach in preventing early postmenopausal bone loss regardless of bone mineral density.

(J Turkish-German Gynecol Assoc 2012; 13: 261-6)

**Key words:** Hormone replacement therapy, bone density, ovariectomy, menopause, bone microarchitecture

**Received:** 24 June, 2012

**Accepted:** 27 October, 2012

## Özet

**Amaç:** Çalışmamızda oofektomili sıçanlarda hormon replasman tedavisinin kemik mikromimari üzerine etkisini değerlendirmeyi amaçladık.

**Gereç ve Yöntemler:** Çalışmanın hayvan etik komite onayı alındı. 17  $\beta$ -östradiol 50  $\mu$ g/kg ve medroksiprogesteron 2.5 mg/kg tedavisi ile; oofektomili ve oofektomi uygulanmayan sıçanlarda 20 günlük tedaviyi takiben kemik mikromimari ve kemik mineral yoğunluğa etkileri araştırıldı. Femoral ve lumbal kemik mineral yoğunluk ölçümleri yapıldı.

**Bulgular:** Gruplar arasında kemik mineral yoğunluk ölçümleri değerlendirildiğinde anlamlı fark bulunmadı. Oofektomi uygulanmayan kontrol grubunda trabeküler yapılar diğer gruplara göre anlamlı yüksek bulundu. Ayrıca oofektomi uygulanmayan kontrol grubunda, diğer gruplara göre; osteoblast sayısı anlamlı yüksek ve osteoklast sayısı anlamlı düşük bulundu.

**Sonuç:** Tek başına östrojen yada progesteron ile kombine hormon tedavisinin; erken postmenapozal dönemde kemik mineral dansitesine yansımayan kemik kayıplarının önlenmesinde etkili olduğunu düşünmekteyiz. (J Turkish-German Gynecol Assoc 2012; 13: 261-6)

**Anahtar kelimeler:** Hormon replasman tedavisi, kemik yoğunluğu, oofektomi, menopoz, kemik mikromimari

**Geliş Tarihi:** 24 Haziran 2012

**Kabul Tarihi:** 27 Ekim 2012

## Introduction

Osteoporosis is defined as a reduction in bone mass associated with impaired bone microarchitecture (1). Postmenopausal osteoporosis is the most common type of osteoporosis and causes an imbalance between osteoclastic activity and

osteoblastic function; therefore, trabecular continuity and connectivity of the trabecular bone structure are decreased resulting in increased bone fragility and increased fracture risk (2). Impaired bone microarchitecture occurs with the conversion of normal plate-like trabeculae into thinner rod-like structures (3).

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doi:10.5152/jtggga.2012.42

Bone mineral density (BMD) measurement is commonly used in practice for the diagnosis and management of postmenopausal osteoporosis (4). However, it has limitations, such as not allowing for the assessment of microarchitecture, bone geometry, mineralization and intracortical porosity (5). Whether increases in BMD contribute to bone fragility, fracture risk, and the therapeutic efficacy of osteoporosis agents is controversial (6, 7). Thus, factors other than BMD, such as bone microarchitecture, should be evaluated for management of postmenopausal osteoporosis and assessment of effects of therapeutic agents. Although new techniques have been developed for more comprehensive evaluation of bone turnover and quality, such as imaging techniques with high-resolution peripheral computerized tomography (CT); whether these novel techniques will be useful in daily practice remains to be seen (8). Clinical trials evaluating the changes of bone microarchitecture during postmenopausal osteoporosis and the effects of therapeutic agents on these changes are needed.

The hormone replacement therapy (HRT) is known to prevent accelerated bone loss (9, 10) and improve bone mass in postmenopausal osteoporosis (11). In addition to improvement in BMD, fractures were decreased with hormone therapy (12). In the conjugated ethinyl estradiol (CEE) medroxyprogesterone (MPA) arm of the Women's Health Initiative (WHI) study, active therapy significantly reduced fractures; however, the WHI study population consisted of women who were older than 70 years of age and who had undergone menopause more than 20 years previously (13). On the other hand, patients in the early stages of postmenopause with no complications are usually asymptomatic, leading to underdiagnosing and undertreatment of potential osteoporosis, and patient noncompliance to treatment. Tiihonen et al. (14) reported that women using HRT need more information about the advantages and risks of HRT to increase compliance with the treatment. This information is especially important for women who are hesitant to use HRT. Therefore it is necessary to determine the effect of widely used HRT on markers other than and with respect to BMD such as bone microarchitecture in early postmenopausal osteoporosis. We hypothesized that bone microarchitecture is impaired before impairment of BMD in postmenopausal osteoporosis and HRT has favorable effect on bone microarchitecture before its well-known effect on BMD. Demonstration of the positive effect of HRT on bone microarchitecture in animal models would provide a basis and preliminary data for further clinical studies to implement the use of HRT starting with the early stages of postmenopausal period. Therefore, in this study, we aimed to investigate the effect of HRT on bone microarchitecture with respect to its effect on BMD in a rat model with surgically induced early menopause.

## Material and Methods

In the present study, 20 adult female Sprague-Dawley rats weighing between 190 and 250 grams were used. Approval was obtained from the Animal Ethics Committee. Fifteen rats underwent bilateral dorsal ovariectomy (OVX) under combined intramuscular 10 mg/kg xylazine (Bayer Health Care, Monheim, Germany) and 60 mg/kg ketamine hydrochloride (Parke Davis, Istanbul, Turkey) anesthesia. Five rats did not undergo oopho-

rectomy. Rats were kept at the postmenopausal period for three weeks and were divided into four groups:

Group 1, control group with no OVX and no hormone therapy (n=5)

Group 2, control group with OVX and no hormone therapy (nut oil as placebo) (n=5)

Group 3, with OVX and receiving 17 $\beta$  estradiol (n=5)

Group 4, with OVX and receiving 17 $\beta$  estradiol and continuous MPA (n=5)

The following medications were administered intra-peritoneally for twenty days: nut oil 1 mL/kg, 17 $\beta$  estradiol 50 micrograms/kg (Sigma, Germany), MPA 2.5 mg/kg (Sigma, Germany). The intra-peritoneal route provided optimization and certainty of the hormone therapy dose. Twenty days later, BMD of experimental animals under general anesthesia were measured by Hologic QDR-4500A and a "small animal" program. Measurements were taken with high resolution in two different regions: the left extremity distal femoral diaphysis and the lumbar vertebrae. Intracardiac perfusion was applied to the rats under general anesthesia. Following a thoracic incision, a 20G catheter was inserted into the left ventricle, and a 10% formaldehyde fixative was given at a rate of 1 cc/sec/kg into the systemic circulation. Following the perfusion procedure, the left femurs of the animals were dissected and kept at room temperature in a 10% formaldehyde fixative for 24 hours for histomorphometric analysis. Following fixation, specimens were placed in 10% formic acid. After decalcification was completed within 7 days, they were taken into routine light microscope follow-up. From the prepared paraffin blocks, transverse sections were obtained in 3-micron thicknesses with a Leica MR 2145 microtome. For morphometric analysis, hematoxylin-eosin dyed preparations were used (15). Finally, one drop of entellan was added to preparations dried at room temperature and kept at 37°C for at least ten days to dry.

## Morphometric analyses

For morphometric analyses, five sections were serially obtained from the left hind extremity distal femoral metaphysis in the paraffin blocks of all animals included in the study. Sections were dyed with hematoxylin-eosin, and digital pictures were taken by 10x objective zoom using an Olympus microscope. The semi-automatic digital system UTHSCSA Image Tool for Windows Version 1.28 was used to measure cortex thickness, trabecular count, trabecular thickness and trabecular area. Osteoblast and osteoclast counts were obtained with 40x magnification 0.5 mm from the epiphysis plaque (16). Trabecular measurements were obtained from the distal 0.46 mm of the epiphysis plaque and equal distances from both sides of the cortex in femur preparations (17). The lengths were calculated as pixels with the aid of a program (1 pixel=128x10<sup>-8</sup> mm). All measurements were taken in accordance with the article by Parfit et al. (17).

## Morphometric measurements

For trabecular thickness ( $\mu$ m), measurements were taken at a minimum of fifty different points for every trabecula, and measurements continued to be taken until the mean values became constant (17, 18). The trabecular count was obtained by counting all trabeculae and each trabecula parallel to each

other at 0.46 mm distal to the epiphysis plaque at equal distances from both sides of the cortex (16, 17). The trabecular area (mm<sup>2</sup>) was calculated by determining the borders of the trabeculae in the region where the trabecular count was determined (17-19). Cortical thickness (µm) was calculated by mean values of fifty measurements from 3-micron sections in digital pictures of each preparation (17-19). Osteoblast and osteoclast counts were calculated in hematoxyline-eosin dyed preparations with 40x objective zoomed digital pictures using an image analysis program and counting cells around trabeculae 0.5 mm under the epiphysis plaque (17-19).

**Statistical analyses**

All statistical analyses were performed using the Microsoft SPSS 11.0 Windows program. Data were expressed as mean±standard deviation and were analyzed by the Chi-square test, the Bonferroni test and the Duncan test. A p-value of <0.05 was considered significant.

**Results**

There was no significant difference among the groups according to lumbar and femoral BMD values (p>0.05). The mean BMD values are shown in Table 1.

When the trabecular count was compared between the control groups (group 1, which is the group with no OVX and no hormone therapy and group 2, which is the group with OVX and no hormone therapy), it was found to have decreased significantly in the OVX control group without hormone therapy (group 2) (p=0.008). When the trabecular count was compared among the hormone replacement groups (group 3, which is the group with OVX and receiving 17β estradiol and group 4, which is the group with OVX and receiving 17β estradiol and continuous MPA) and in the OVX control group without hormone therapy (group 2), the trabecular count was significantly higher in hormone treatment groups (p<0.001, p=0.008 for groups 3 and 4; respectively). When the trabecular area was compared between the control groups, it was significantly higher in the control group with no OVX (group 1) than the control group with OVX (group 2) (p<0.001). Additionally, the trabecular area was significantly lower in the control group with OVX (group 2) when compared to the hormone treatment groups (p<0.001, p<0.001 for groups 3 and 4; respectively). The distribution of the trabecular structures among the groups is demonstrated in Figure 1.

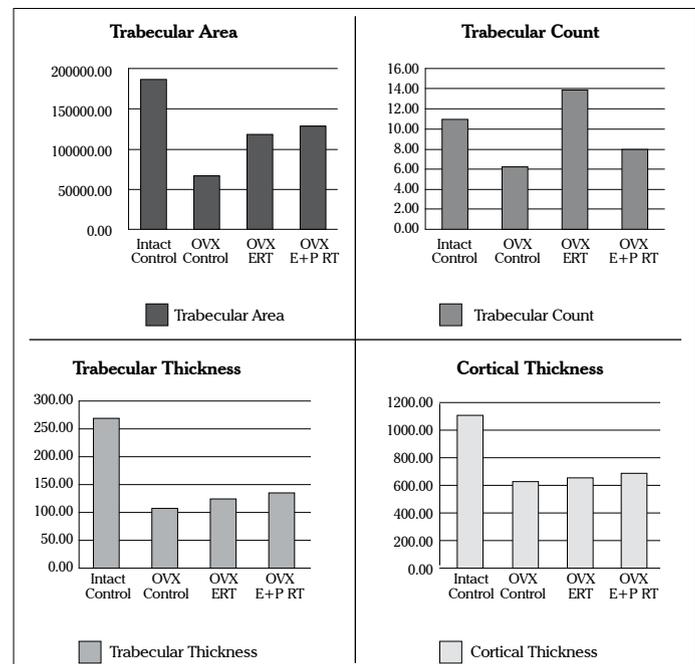
The trabecular thickness was significantly higher in the control group with no OVX (Group 1) when compared to all other groups (p<0.001). With regard to trabecular thickness, there was no significant difference among the OVX groups. The cortical thickness was significantly higher in the control group with no OVX (Group 1) than in all other groups (p<0.001), whereas there was no significant difference with respect to cortical thickness among the OVX groups (p>0.05). The results of the

morphometric analyses are shown in Table 2 and Figure 2.

According to the morphometric measurements, the osteoblast count was significantly higher in the control group with no OVX (Group 1) than in all of the OVX groups (p<0.05). When the osteoblast count was compared among the OVX groups, it was found to be significantly higher in groups 3 and 4 (hormone treatment groups) than in group 2 (control group with OVX) (p<0.01). The osteoclast count was also significantly higher in the control group with no OVX (Group 1) than in the OVX HRT groups, whereas there was no significant difference with respect to the osteoclast count between the control groups with or without OVX (p>0.05). However, when the osteoblast count was evaluated, the osteoclast count ratio was found to be significantly lower in the OVX control groups than in all other groups. In addition, there was no significant difference with respect to this ratio between the intact control group and the OVX HRT groups. The morphometric measurements are provided in Figure 3.

**Discussion**

In the present animal experiment, we found that in ovariectomized rats, bone microarchitecture, which was assessed with



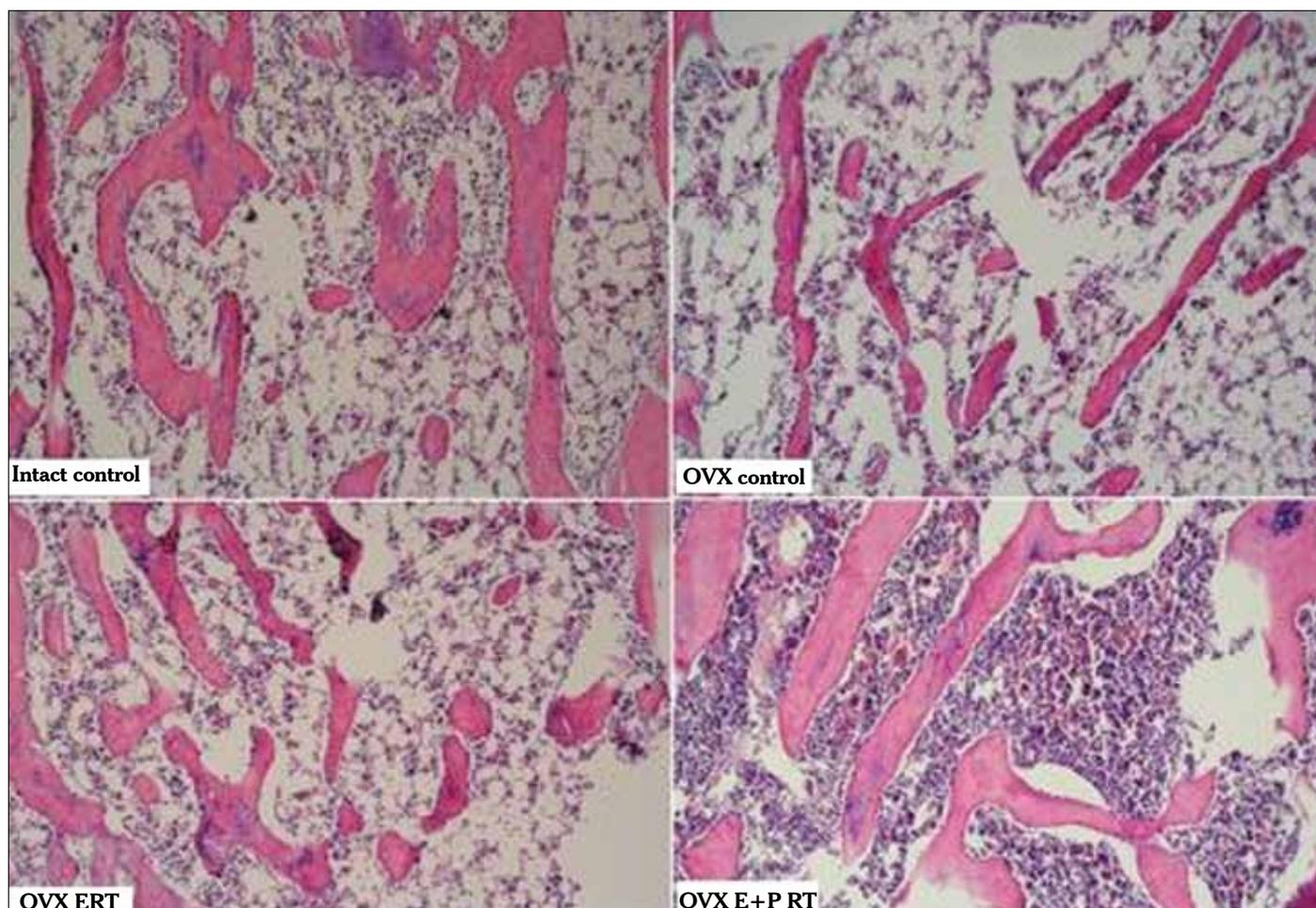
**Figure 1. Histological appearance of trabecular structures under Olympus microscope (hematoxyline-eosin, x10). Intact control: control group with no OVX and no hormone therapy, OVX control: control group with OVX and no hormone therapy (nut oil as placebo), OVX ERT: with OVX and receiving 17β estradiol, OVX E+PRT: with OVX and receiving 17β estradiol and continuous MPA**

**Table 1. Lumbar and femoral BMD values of study groups (mean±SD)**

|             | Intact control | OVX control | OVX ERT   | OVX E+P RT |
|-------------|----------------|-------------|-----------|------------|
| Lumbar BMD  | 0.15±0.03      | 0.12±0.03   | 0.15±0.02 | 0.14±0.01  |
| Femoral BMD | 0.21±0.06      | 0.19±0.04   | 0.22±0.02 | 0.24±0.08  |

**Table 2. Results of morphometric analyses of study groups (mean ± SD)**

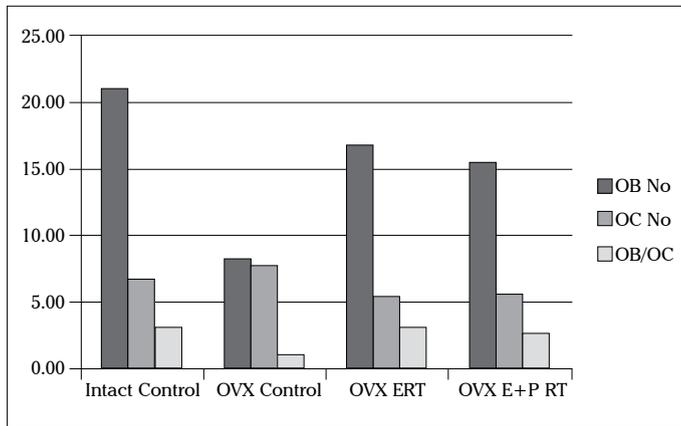
|                      | Intact control  | OVX control    | OVX ERT         | OVX E+P RT      |
|----------------------|-----------------|----------------|-----------------|-----------------|
| Trabecular count     | 11.13±1.4       | 6.28±1.34      | 14.0±2.3        | 8.04±1.26       |
| Trabecular thickness | 272.66±29.65    | 110.64±18.79   | 127.10±5.41     | 135.58±3.15     |
| Trabecular area      | 186731.2±5026.1 | 67367.8±2106.3 | 121156.8±5627.8 | 129912.8±6062.9 |
| Cortex thickness     | 1104.9±202.7    | 622.6±85.44    | 667.2±69.87     | 693.5±51.39     |



**Figure 2. Results of morphometric analyses as mean values. Intact control: control group with no OVX and no hormone therapy, OVX control: control group with OVX and no hormone therapy (nut oil as placebo), OVX ERT: with OVX and receiving 17β estradiol, OVX E+PRT: with OVX and receiving 17β estradiol and continuous MPA**

morphometric studies, deteriorates before impairment of BMD and this deterioration of bone microarchitecture was corrected with hormone therapy (17β estradiol with or without MPA). Osteoporosis is a disease with significant medical and socio-economic costs. It is characterized by an increase in the tendency for fragility fractures and an enhanced risk of other complications, such as pneumonia or thromboembolic disease due to prolonged hospitalization. Prolongation of life has made osteoporosis an important health problem (2, 20). Macroscopically, there are two types of bones in the human body skeleton: cortical bone, which constitutes 80%, and trabecular bone (cancellous). The cancellous to cortical bone ratio is approximately 50:50 in the femoral head (21). When bone loss starts due to menopause,

aging, etc., cancellous bone is affected earlier than cortical bone. Osteoporosis is described as a reduction in bone mass associated with impaired bone architecture, disruption of trabecular continuity by trabecular perforation, increased bone fragility, increased fracture risk, and thinning and increased porosity of the cortices with the conversion of normal plate-like trabeculae into thinner rod-like structures (3). These changes are the result of the combination of increased osteoclastic activity and reduced osteoblast function that characterizes postmenopausal osteoporosis. The view of affected trabecular bone can be described as stair steps that have decreased in size or been lost (1). In the present study, the intact control group's mean femoral histomorphometric parameters, such as trabecular count, tra-



**Figure 3. Results of morphometric measurements as mean values. Osteoblast and osteoclast counts were calculated using an image analysis program. Intact control: control group with no OVX and no hormone therapy, OVX control: control group with OVX and no hormone therapy (nut oil as placebo), OVX ERT: with OVX and receiving 17 $\beta$  estradiol, OVX E+PRT: with OVX and receiving 17 $\beta$  estradiol and continuous MPA. OB no: osteoblast count, OC no: osteoclast count, OB/OC: osteoblast:osteoclast count ratio**

trabecular thickness, trabecular area, and cortex thickness, were found to be significantly superior to the OVX control group. In addition, morphometric measurements, such as the osteoblast and osteoclast count, in groups 1 and 2 support an impaired microarchitecture in the OVX rat models. In their study, Bagi et al. (22) investigated the effect of oophorectomy on bone mass and endurance. The bone masses of the femoral neck of rats were evaluated by Dual Energy X-Ray Absorptiometry (DEXA) and histomorphometric parameters using various dynamic and static methods. They determined that the muscular and capsular structures of pelvic articular cartilages in rats resembled those of humans. While endocortical and cancellous bone restructuring following oophorectomy was particularly affected, the ovariectomized group was observed to have a significant decrease in trabecular count, cortical thickness and bone endurance compared with the control group. We found no correlation between impaired bone microarchitecture and femoral BMD. Similarly, in recent years, bone microarchitecture has been increasingly used to determine bone loss or fracture risk when compared to BMD. Ladinsky et al. (23) reported that structural measures obtained at the distal radius in vivo by magnetic resonance imaging (MRI) explained a significant portion of the variation in the total spinal deformity burden in postmenopausal women independent of area BMD. The National Osteoporosis Risk Assessment (NORA) study found that more than one half of women who sustained osteoporotic fractures had BMD T-scores  $\geq -2.0$ , and a significant portion had BMD levels in a range considered to be normal (24). New specialized techniques have been developed, such as high-resolution CT (hrCT), micro CT, high-resolution MR (hrMR) and microMR, that are able to provide structural information about local and systemic skeletal health, the propensity to fracture and the pathophysiology of bone fragility. While quantitative assessment of bone macrostructure can be obtained by DEXA and CT, assessment of trabecular bone microstructure may be obtained by hrCT, microCT, hrMR, and microMR (1). The rela-

tionship between osteoporosis and postmenopausal hormone replacement therapy has been widely studied. Several studies from the 1970s reported that treatment with estrogen alone or estrogen plus progestogen at the time of menopause prevented accelerated bone loss (9, 10). According to a meta-analysis published in 2002, preparations of estrogen with or without progestogen were significantly more effective than placebo in preserving and increasing BMD (11). Several follow-up studies demonstrated that discontinuation of estrogen therapy caused bone loss similar to that seen in early menopause (24). Early menopause and ovariectomy before age 45 are associated with a lower BMD and a higher osteoporotic fracture rate (25).

Data on the effect of estrogen on bone are inconsistent and range from preserving the existing bone structure (26, 27) to having a strong anabolic effect (19, 28). Lindsay et al. (29) reported that the effect of progesterone on bone is unclear. In the present study, two parameters reflecting trabecular bone microarchitecture, which include the trabecular count and trabecular area, demonstrated significant improvement in the hormone replacement group when compared to the ovariectomized control group. There was no significant difference between the two groups with respect to other parameters including trabecular and cortical thickness. In addition, there was no significant difference between treatment with estrogen with or without progesterone. Although the action of osteoprotective estrogen remains unclear, it has been suggested that, during estrogen deficiency, bone remodeling is impaired because of an increase in some cytokines, such as TNF- $\alpha$ , IL-1, IL-6, and IL-8. This indirect effect leads to bone resorption through stimulation of osteoclastogenesis (30). In the present study, we found that impaired bone microarchitecture and an imbalance between osteoblasts and osteoclasts in the OVX rats were improved by HRT independent of BMD. This finding suggests that the effect of estrogen deficiency on bone starts in the early period of menopause and that HRT reverts these changes. In a novel study, Komm et al. (31) examined the effect of daily treatment with bazedoxifene, conjugated estrogens, or both treatments combined on bone mass, bone architecture, bone strength, and the biochemical markers of bone turnover in ovariectomized rats over the course of 12 months. The investigators reported that treatment with conjugated estrogens alone or in combination with bazedoxifene completely prevented the ovariectomized-induced loss of BMD at the lumbar spine and proximal femur (31). Batukan et al. (32) found that estrogen in combination with simvastatin increased the BMD of proximal femur and lumbar vertebra effectively in rats. In addition, the WHI studies have demonstrated that estrogen with or without progestogen can prevent hip and vertebral fractures in an unselected population of women (level of evidence: A) (25).

Taking into account the duration of treatment in the present study, the main finding of this study is that the bone microarchitecture was improved in the HRT group without loss of BMD.

This study had some limitations. First, the number of rats in each study group was considerably small. We kept the total number of rats low for ethical reasons. Second, biochemical markers of bone metabolism could not be measured due to technical inadequacy in our hospital. In spite of these limitations, this animal study showed that hormone therapy produces

improvement on bone microarchitecture before its known effect on impaired bone mineral density.

## Conclusion

We found that HRT corrects impaired bone microarchitecture which develops before impairment of BMD in a rat model with surgically induced early menopause. Therefore, estrogen alone or in combination with progestogen can be a beneficial approach to preventing early postmenopausal bone loss.

## Conflict of interest

No conflict of interest was declared by the authors.

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