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Full Length Research Paper

Bone histomorphometric study of young rats following oestrogen deficiency

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Osteoporosis is a global problem which results in increased fractures risk. The reports from earlier studies were inconsistent with the aging factor as well as the time which is needed to induce bone loss post-ovariectomy. This study aimed to determine the short-term effects of estrogen deficiency on bone structural histomorphometric parameters in young rats. 30 Sprague-Dawley female rats weighing 250 to 300 g were assigned to baseline, sham-operated and ovariectomy groups. The baseline group (n = 10) was sacrificed immediately. Sham-operated rats (SO, n = 10) underwent sham operation while ovariectomised group (OVX, n =10) underwent bilateral ovariectomy. All the rats were sacrificed 6 weeks post-ovariectomy. Following sacrifice, the right femora were dissected and subjected to the histomorphometric analysis using modified Von Kossa method. Bone volume/tissue volume (BV/TV) and trabecular number (Tb.N) reduced significantly, while trabecular separation (Tb.Sp) increased significantly in the ovariectomized rats, compared to the baseline and sham groups 6 weeks post-ovariectomy (P<0.001). In the other hand, trabecular thickness (Tb.Th) was consistent among the groups (P=0.41). Estrogen deficiency resulted in marked decline in BV/TV which most probably attributed to a reduction in Tb.N. In contrast, Tb.Th was found to be preserved following estrogen loss. Hence, the period of 6 weeks post-ovariectomy was sufficient to induce osteoporosis in ovariectomized rats without affecting Tb.Th.

Key words: Bone histomorphometry, menopause, estrogen deficiency, osteoporosis, ovariectomy, trabecular bone.

INTRODUCTION

Osteoporosis is a socio-economic health problem which leads to an increased risk of fractures (Giannoudis et al., 2007). It is a metabolic disorder, characterized by decreased bone mineral density (BMD) and bone mass with microarchitectural deterioration of bone structure, leading to enhanced bone fragility (Raisz, 2005). Osteoporotic fractures carry a high rate of morbidity and mortality with the health costs rapidly increasing over the past years. WHO defines osteoporosis as BMD of subjects with a T-score at or below -2.5 standard deviation, below the peak adult bone mass (Kanis, 1994).

Osteoporosis can be classified according to the aetiology into primary postmenopausal osteoporosis (type I), primary senile osteoporosis (type II) and secondary osteoporosis (Gali, 2001). Postmenopausal osteoporosis is the commonest cause of osteoporosis. The primary role of estrogen deficiency in the pathogenesis of postmenopausal osteoporosis was discovered earlier by Albright (1947). The pathogenesis of postmenopausal osteoporosis is manifested by an increase in bone remodeling and an uncoupling between resorption by osteoclasts and formation by osteoblasts. The excessive bone resorption by osteoclasts occurs without adequate new bone formation by osteoblasts which lead to bone loss (Wronski et al., 1989). The

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remodeling process occurs in a small area called the basic multicellular unit (BMU) (Frost, 1998). BMUs are temporary anatomic structures that can conserve or reduce bone strength and mass (Frost, 1998). Earlier studies showed that estrogen deficiency led to activation of BMUs, induction of osteoblast apoptosis and suppression of osteoclast apoptosis (Riggs et al., 2002). Earlier studies have linked aging with bone loss, where it was found that aging reduces bone formation while estrogen deficiency increases bone resorption (Baldock et al., 1999).

Estrogen inhibits osteoclast differentiation and activity through inhibition of expression of pro-inflammatory cytokines expression such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) (Chen et al., 2009). Postmenopausal estrogen loss induced production of macrophage colony-stimulating factor (M-CSF) and various cytokines from marrow stromal cells (Chen et al., 2009). These pro-inflammatory cytokines are involved in osteoclastogenesis (Weitzmann and Pacifici, 2006). Estrogen inhibits osteoclastic bone resorption by inducing osteoclasts apoptosis (Kameda et al., 1997).

The ovariectomised rat is still considered as a reliable animal model of postmenopausal osteoporosis (Frost and Jee, 1992). Various approaches are commonly used in many studies to evaluate changes in micro-architecture and bone mineral density (BMD) namely, micro-computed tomography (Micro-CT), histomorphometry and dual energy X-ray absorptiometry (DEXA). Histomorphometric study of the bone is a quantitative method used to study the histology and bone remodeling process which cannot be obtained from other tests such as bone turnover markers (Vedi and Compston, 2003). It is mainly used to evaluate trabecular bone more than cortical bone. Trabecular bone volume (BV/TV, %), trabecular thickness (Tb.Th, µm), trabecular number (Tb.N, mm^{-1}) and trabecular separation (Tb.Sp, µm) are the parameters used to evaluate trabecular bone structure. The main aim of the present study was to elucidate whether a period of 6 weeks following ovariectomy was sufficient to significantly affect the bone histomor-phometric parameters in young rat model.

MATERIALS AND METHODS

Osteo-Bed Solution A Resin and benzoyl peroxide plasticized were purchased from Polysciences, Germany and were used for the preparation of the resin for the embedding process. Silver nitrate was purchased from Sigma, USA and was used for the Von Kossa staining procedure. Anesthetic agents used for surgical procedures were Xylazil (Troy Laboratories, Australia) and Ketamil (Troy Laboratories, Australia).

This study was approved by the Animal Ethics Committee of Universiti Kebangsaan Malaysia (UKM). 30 female Sprague Dawley rats weighing 200 to 250 g were used in this study and they were housed individually in cages at room temperature and normal 12-h light-dark cycle. All the animals had free access to water and rat chow *ad libitium*. They were acclimatized for 2 weeks before the intervention.

Experimental protocol

The rats were divided randomly into baseline (control), sham-operated (SO) and ovariectomy (OVX) groups. They were anaesthetized with a combination of xylazil and ketamine (1:1) at a dose of 0.1 ml/100 g rat weight which was given intramuscularly. Bilateral ovariectomy was performed on rats from the OVX group using the ventral approach. The fur of the abdomen was shaved and the skin was sterilized using alcohol 70% and Povidone iodine solution. Midline abdominal incision was made to gain access to the peritoneal cavity. The uterine tubes were ligated and the ovaries were removed. The SO group of rats underwent sham operation, where the ovaries were exposed as mentioned earlier, gently manipulated but not excised and left *in situ*. All the surgical procedures were performed according to a previous surgical protocol (Devareddy et al., 2008). The incision was then closed with sutures under aseptic technique. Daily dressing with Povidone iodine was done and enrofloxacin 5% (Baytril, Bayer) at a dose of 10 mg/kg was given intramuscularly daily for 7 days. Fusidic cream was applied topically on the wound to prevent wound infection. Success of the surgery was confirmed by marked atrophy of the rat's uterus.

Measurement of structural histomorphometry

After the rats were sacrificed, the right femora were dissected, cleaned and fixed in Neutral buffered formalin 10% for at least 24 h. The distal third of each femur was divided in the sagittal plane using bone cutting tools with a low-speed saw (Black & Decker Rotary Tools, USA). One half of the bone was used for the study. Bone samples were infiltrated with Osteo-Bed Solution A Resin according to Polysciences osteo-Bed Embedding Kit protocol (Catalog #17734). Following the polymerization process, the excess resin was trimmed and the resin blocks were sectioned at the thickness of 8 µm by using microtome Leica RM 2235 (Leica, Germany). Staining process was started by using the modified Von Kossa staining method by which slides were immersed in 5% silver nitrate as per previous protocol (Hermizi et al., 2009).

The modified Von Kossa's staining method was adopted to measure the structural histomorphometric parameters of bone. All histomorphometric measurements were described according to the standardized system proposed by the American Society for Bone and Mineral Research (ASBMR) nomenclature committee (Parfitt et al., 1987), that is, trabecular bone volume (BV/TV, %), trabecular thickness (Tb.Th, µm), trabecular number (Tb.N, mm^{-1}) and trabecular separation (Tb.Sp, µm) (Table 1).

Following the staining, all the histomorphometric measurements were performed at the metaphyseal region, which was located 3 to 7 mm from the lowest point of the epiphyseal plate and 1 mm from the lateral cortex (Baldock et al., 1999). This area is known as the secondary spongiosa area, which is rich in newly formed trabecular bone. The earlier mentioned histomorphometric parameters were measured at 100x magnification and optical microscopic images were taken using Pixelink colour camera and analyzed with an image analysis system linked to a light microscope using a software package, video T-Morphology 5.1. Three sections per specimen were measured and all histomorphometric measurements for each specimen were assessed blindly.

Statistical analysis

Statistical analysis was performed using SPSS software package version 17. Normal distribution of all variables was examined by

Table 1. Table showing structural histomorphometry parameters of bone.

Structural histomorphometry parameter	Unit	Formula
Bone volume/tissue volume	%	BV/ TV x 100
Trabecular Thickness (Tb.Th)	µm	BV/ (½ BS)
Trabecular Number (Tb.N)	mm^{-1}	(BV/ TV) / Tb.Th
Trabecular Separation (Tb.Sp)	µm	(1/ Tb.N) - Tb.Th

BV, Bone volume; TV, tissue volume; BS, bone surface. Source: Adopted from Vedi and Compston (2003).

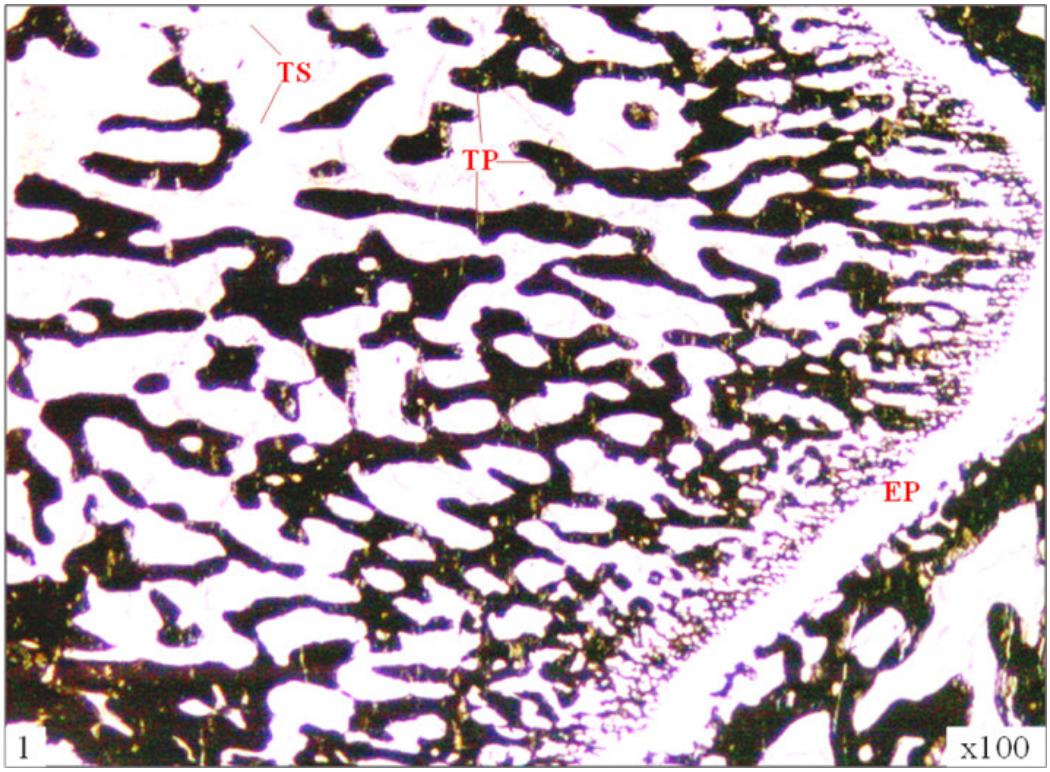

Figure 1. Von Kossa stained section at the metaphyseal region of distal rat femur at lower magnification (100x) for baseline group, showed preservation of normal trabecular architecture. Trabecular plate (TP); trabecular separation (TS); epiphyseal plate (EP).

using the Shapiro-Wilk test. Normally, distributed (parametric) variables were analyzed by using analysis of variance (ANOVA) followed by Tukey's post-hoc test. Level of significance was taken at $P<0.05$. All data were presented as mean ± SEM. Pearson's correlation study was used for parametric data and differences were considered significant at $P<0.01$.

RESULTS

Von Kossa stained sections of the distal part of rat femur at the lower magnification (100x), revealed normal trabecular architecture with preservation of trabecular plate and connectivity between trabeculae in the baseline and the SO groups (Figures 1 and 2). However, in the OVX group, there was deterioration of trabecular bone structure accompanied by extensive removal of trabeculae with perforation of the trabecular plates at the metaphyseal region. In addition, there was widening of the spaces between the trabeculae (trabecular separation) as a result of trabecular loss (Figure 3). All these findings were indicative of osteoporotic changes following oestrogen deficiency.

Bone histomorphometry measurements

Following six weeks of ovariectomy, BV/TV and Tb.N were significantly decreased in the OVX group as compared to the baseline and SO groups ($P<0.001$). A very high positive correlation was observed between BV/TV

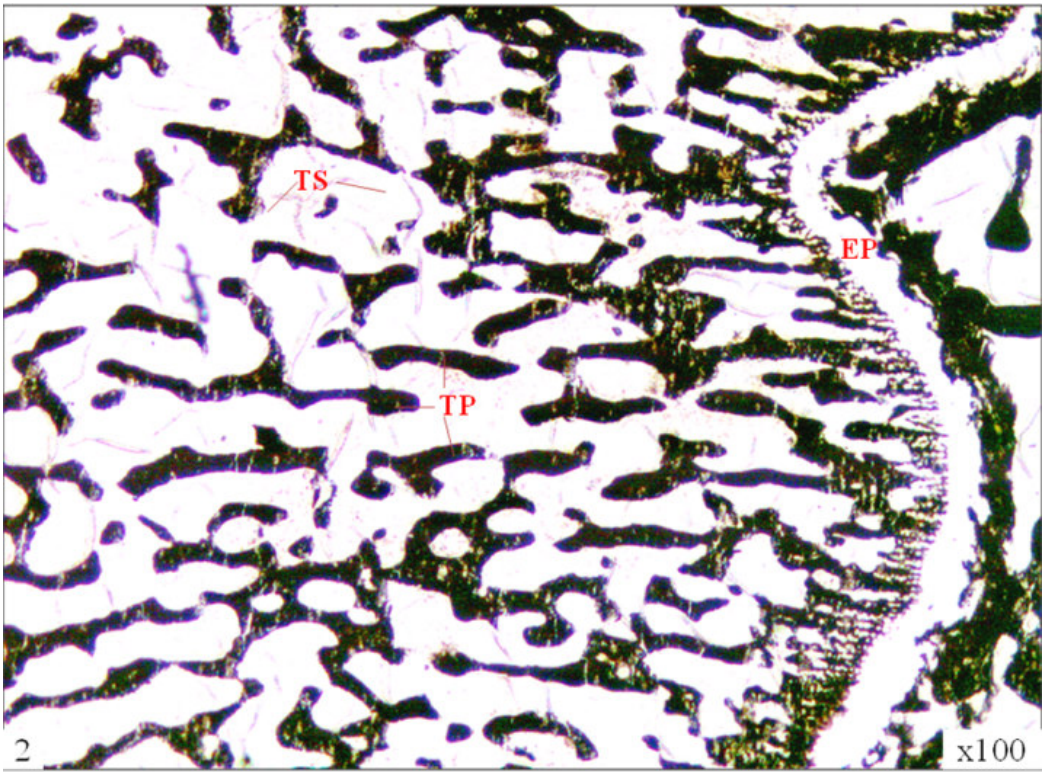

Figure 2. Von Kossa stained sections at the metaphyseal region of distal rat femur at lower magnification (100x) SO group, revealed maintenance of trabecular network. Trabecular plate (TP); trabecular separation (TS); epiphyseal plate (EP).

and trabecular number which was statistically significant at $P<0.01$ ($r = 0.924$). In addition, Tb.Sp was significantly increased in the OVX group as compared to the baseline and SO groups ($P<0.001$). However, no significant differences were observed in mean Tb.Th among all the groups ($P = 0.41$). The mean values of all structural parameters were summarized in Table 2.

DISCUSSION

The ovariectomised rat is a similar model for post-menopausal osteoporosis in women (Frost and Jee, 1992). Estrogen deficiency leads to significant bone loss and reduction in BMD in ovariectomised rats (Frost and Jee, 1992). Furthermore, the ovariectomised mature rats showed similar bone changes with menopausal women which was mainly due to disturbance of bone remodeling (Turner et al., 2001). Trabecular bone is concentrated mainly in the vertebrae and the epiphysis of long bones. Approximately, 20 to 30% of trabecular bone and 3% of compact bone are renewed yearly (Safadi and Khurana, 2010). Trabecular bone is 8 times more active metabolically than cortical bone; hence, trabecular bone contributes to 80% of bone turnover, while the compact bone contributes to 20% of bone turnover (Steiner et al., 1996). This explains why osteoporosis affects mainly the trabecular bone (Parfitt, 2002). Fractures of the vertebra, wrist and hip are most common osteoporotic fractures in the elderly as these bones are mainly made up of trabecular bone (Schuit et al., 2004).

The OVX group revealed a significant decrease in BV/TV and Tb.N, and significantly higher Tb.Sp as compared to the baseline and age-matched SO groups. The estrogen deficient state was associated with an increased loss of trabecular bone as shown by reduction in Tb.N and BV/TV. There was a high positive correlation between BV/TV and Tb.N. The reduction in Tb.N led to a decrease in BV/TV and an increase in Tb.Sp in the estrogen deficient state. This may indicate that BV/TV was mainly determined by Tb.N. The reductions in Tb.N and BV/TV were probably attributed to the increase in bone resorption more than to the decrease in bone formation. The estrogen deficient state led to activation of BMUs, induction of osteoblast apoptosis and suppression of osteoclast apoptosis (Riggs et al., 2002). The estrogen deficiency state induced oxidative stress and subsequent bone loss by increasing the level of hydrogen peroxide (H_2O_2), whereby H_2O_2 induced osteoclastogenesis and signals bone loss (Muthusami et al., 2005; Lean et al., 2005). Oxidative stress also induced release of cytokines such as IL-6 and M-CSF which induce osteoclastic differentiation and proliferation (Parhami, 2003). Goss et al. (2009) reported that the ovariectomised-control group showed significantly lower BV/TV and Tb.N and

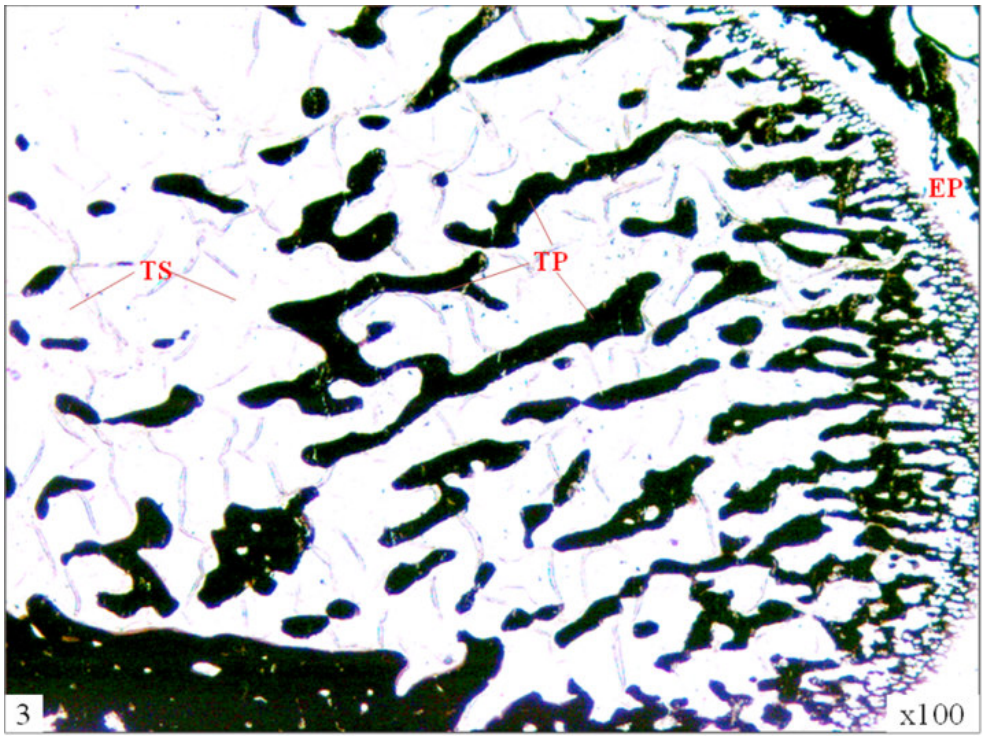

Figure 3. Von Kossa stained sections at the metaphyseal region of distal rat femur at lower magnification (100x) OVX group, showed removal of trabeculae with widening of spaces between trabecular plates. Trabecular plate (TP); trabecular separation (TS); epiphyseal plate (EP).

Table 2. Summary of the mean values of structural histomorphometry parameters after 6 weeks of ovariectomy.

Group	BV/ TV (%)	Tb.Th (µm)	Tb.N (mm-¹)	Tb.Sp (µm)
Baseline	48.3±1.24	48.9±3.29	10.5±0.34	50.0±1.60
SO	45.5±1.017	42.9±2.03	10.7±0.48	51.3±2.65
OVX	25.4±0.55* #	42.5±1.83	6.05±0.16* #	124.2±2.67* #

Baseline group, rats sacrificed at zero time; SO (sham-operated) and OVX (ovariectomised) groups, rats were sacrificed 6 weeks post-ovariectomy.
* P<0.001 as compared to the SO group; # P<0.001 as compared to the baseline group.
All analysis was done by using one way ANOVA test followed by Tukey's post-hoc test. Values were expressed as mean ± SEM.

significantly higher Tb.Sp as compared to the normal control group, 16 weeks post-ovariectomy.

The same pattern was observed in other studies which found that the ovariectomised-control group had a significant decrease in BV/TV and Tb.N and an increase in Tb.Sp as compared to the treated ovariectomised and sham groups, 8 weeks post-ovariectomy (Lane et al., 2002; Rubinacci et al., 2008). This was similar to other findings at 20 weeks post-ovariectomy (Zhang et al., 2009; Wang et al., 2001). Bone loss in the estrogen deficient state occurred mainly due to increased bone resorption locally and perforation of trabecular plates (Abe et al., 1999). This was in accordance with the observation of Baldock et al. (1999) which found that trabecular bone loss in ovariectomised rats was mainly due to a decrease in Tb.N. Bone loss in the early stage of ovariectomy was mainly attributed to the reduction of Tb.N rather than reduction of Tb.Th.

There was no significant difference in the Tb.Th between OVX, age-matched SO and baseline groups. The estrogen deficient state did not have significant effects on Tb.Th in the OVX group. Eriksen et al. (1994) concluded that estrogen deficiency did not affect Tb.Th in OVX rats, indicating that estrogen loss did not interfere with bone formation during the early stage of ovariectomy. Preservation of Tb.Th had been previously reported in postmenopausal women (Kleerekoper et al., 1985). This was in accordance with previous studies

which showed that Tb.Th in the normal control and ovariectomised-control groups were consistent at the early stages of ovariectomy (Goss et al., 2009; Rubinacci et al., 2008). Baldock et al. (1999) concluded that Tb.Th was preserved in the age-matched sham group and increased slightly in the ovariectomised group at the late stage of ovariectomy due to contraction of the remodeling space. The maintenance of Tb.Th is most likely attributed to the thickness of cortical bone which may conceal any osteoporotic changes in the trabecular bone. However, the results of some earlier studies were different from ours, whereby it was reported that estrogen loss had significantly decreased Tb.Th in the late stage of ovariectomy (Zhang et al., 2009; Nian et al., 2009). This may indicate that trabecular thickness is affected by aging in ovariectomised rats. Hence, we concluded that the estrogen deficient state may not interfere with bone formation during the early period of ovariectomy.

There was no significant difference between the baseline and SO groups in all the structural histomorphometric parameters. Aging is considered as a factor which may contribute to osteoporosis. Mosekilde et al. (1993) concluded that estrogen loss together with aging resulted in significant bone loss as compared to the effect of normal aging. Earlier studies have shown that trabecular bone volume remained constant at 25 to 30% in the sham group by 12 months of age and the trabecular bone volume was decreased significantly to about 10% at the age of 21 months (Wronski et al., 1989). This same pattern was reported by Hermizi et al. (2009) who found no significant difference in all the structural histo-morphometric parameters between the baseline and control groups. At the termination of the pilot study, the rats were about 6 months of age and were still considered to be young adults. In order to have significant bone loss due to the aging process, the rat's age probably should have exceeded 12 months. Based on these results, it would be better to use young adult rats during assessment of bone changes in the estrogen deficient state in order to eliminate the aging factor.

In this study, the estrogen deficient state induced trabecular bone loss by increasing bone resorption locally and inducing perforation of trabecular plates. This was probably attributed to the estrogen loss and over-production of ROS. The estrogen deficient state was associated with overproduction of cytokines such as IL-6 and M-CSF which induced osteoclastic differentiation (Chen et al., 2009). Trabecular bone loss occurred mainly due to a decrease in Tb.N and BV/TV as well as an increment in Tb.Sp in the early post-ovariectomy period. There was a high positive correlation between BV/TV and Tb.N. This may indicate that BV/TV was determined mainly by Tb.N rather than by Tb.Th. In the estrogen deficient state, Tb.Th was preserved in the early stage after ovariectomy. Age related bone loss was slower and time dependent as compared to ovariectomy related bone loss (Mosekilde et al., 1993). Previous research reports have proved that aging correlated to bone loss and aging resulted in extensive bone loss in ovariectomised rats (Sibonga et al., 2000). Estrogen loss involved mainly bone resorption by osteoclasts rather than bone formation by osteoblasts. Trabecular bone loss in the estrogen deficient state was mainly due to reduction of Tb.N and BV/TV rather than by trabecular thinning at 6 weeks post-ovariectomy.

In conclusion, the estrogen deficient state resulted in significant bone loss and reduction of BV/TV which was most probably due to the decrease in Tb.N. Trabecular bone loss in ovariectomised rats occurred as a result of reduction in Tb.N rather than reduction in Tb.Th. It is important to take into account the aging factor when we use older rats as aging can result in marked bone loss. This study had confirmed that 6 weeks of estrogen deficiency post-ovariectomy was sufficient to cause significant bone loss in the rat model. Hence, the period of 6 weeks post-ovariectomy was sufficient to induce osteoporosis in the young adult rat model. Further studies may be needed to confirm this.

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