The Effect of Estrogen on the Restoration of Bone Mass and Bone Quality in Ovariectomized Rats*

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Summary: To evaluate the effect of estrogen on its ability to restore the bone mass and bone quality in ovariectomized rats by examining the changes of bone morphology and histomorphometry, 3-month-old rats were divided randomly into 4 groups: normal control, ovariectomized (OVX), sham-operated (Sham-O) and OVX plus estrogen (OVX+E_2). Treatment initiated from the day 8 weeks after operation and continued for 12 weeks. Bone morphology and histomorphometry were examined afterwards. Results showed that comparing to control group, the trabecular bone in OVX appeared thinner and reduced in the amount. The connectivity between trabecula was decreased and the structure disordered. The free-end of trabecula was increased. The cavity of bone marrow enlarged. After treatment with estrogen, above changes improved remarkably by different degree, although did not reach the normal face. The bone histomorphometry results damonstrated that estrogen treatment increased bone mass and the amount of trabecula by 129 % and 132 % respectively ($P<0.05$). The activity of bone resorption decreased significantly and the rate of bone formation increased to 203 %. These results suggest that treatment of ovariectomized rats with estrogen can not only increase bone mass, also improve the bone structure and enhance the property of bone mechanics.

Key words: estrogen; ovariectomized rats; morphology; histomorphometry; osteoporosis

There are abundantly evidences bone experiences a rapid loss after menopause. Although this bone loss was considered due to several factors, estrogen deficiency was regarded with main responsibility[1]. Studies showed that estrogen deficiency affected the skeletal sites with mainly cancellous bone, such as vertebra, hip, the ends of long bones etc[2], resulting of the high risk fracture in of these parts. With the development of the study on the pathogenesis of osteoporosis, it was observed that bone material quality, usually considered invariant, probably contributes more to fragility than is recognized. Clinical practices have revealed that low bone mass probably accounted only for less than half of all of osteoporotic fractures[3]. Based on above recognition this study was designed to investigate the bone microanatomical change and mechanism of bone loss after ovariectomy in rat and assess the effect of estrogen on these changes.

1 MATERIALS AND METHODS

1.1 Animals

Thirty-two Sprague-dawley rats, aged 3 months and weighing between 210-230 g (purchased from Experimental Animal Center of Tongji Medical University) were involved in this experiment. They were randomized in 4 groups: normal control group(N), ovariectomized control group (OVX), sham-operated control (Sham-O) group and estrogen treated group (OVX + E_2). The rats of the latter three groups were anesthetized by 1 % sodium pentobarbital 0.2 ml/100 g body weight. Bilateral ovaviectomies were performed on OVX and OVX+E_2 rats through dorsal approach. The rats in the Sham-O group were subjected to sham surgery. The rats were breed in cages and allowed free access to water and pelleted diets. The treatment started at 8 weeks after the surgery. The rats in normal control group, OVX group and Sham-O group received an intramuscular injection of 0.9 % sodium chloride 0.1 ml per rat once a week. The rats in the OVX + E_2 group received subcutaneous injection of estradiol benzoate 0.5 mg/kg per rat once a week.

1.2 Sample Preparation

The rats were killed 12 weeks after treatment. All rats received a subcutaneous injection of 25 mg/kg of tetracycline on the day 14 and 13 and 5 mg/kg of calcein on the day 4 and 3 before sacrifice.

1.3 Histology

The second lumber vertebrae (L_2) were defreshed and fixed in polyformalin for 24 h. Then, the bones were decalcified in 10 % EDTA for 15 days. The decalcified samples were embedded in paraffin and cut into thin sections. The sections were stained with hematoxyin and eosin.

1.4 Histomorphometry

The left tibiae were cut into three parts using a low-speed metallurgical saw. The proximal third of each tibia was cut in the frontal longitudinal plane, then they were dehydrated in 70 % alcohol for 24 h and thereafter embedded in methylmethacrylate. After polymerization, the samples were cut into 5 μm thin sections and 10 μm thick sections. The thick

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sections were used to observe the trabecular number and structure. After dissolving in the methylmethacrylate, the thin sections were stained using goldener staining for bone cell morphology. Bone histomorphometry parameters included: (1) Static parameters. Trabecular area (Tb. Ar, %); Trabecular thickness (Tb. Th, μm); Trabecular number (Tb. N, mm^{-1}); Trabecular separation (Tb. Sp, μm). (2) Dynamic parameter of bone formation. Laber perimeter (L. Pm, %); Bone formation rate (BFR/TV, %). (3) Dynamic parameters of bone resorption. Osteoclast number (N. OC, mm^{-2}); osteoclast number/bone length (N. OC/Tb. Pm, mm^{-1}).

1.5 Statistical analysis

Data were expressed as $\bar{x}\pm s$. The formula "$(x_2 \div x_1)\times 100-100$" was used to calculate percentage. The significance of the difference between the two groups was determined by t test.

2 RESULTS

2.1 Wet Weight of the Rat Uterus

The data were showed in table 1.

2.2 Histology

The character of bone in normal and Sham-O groups was rich of trabecula that was dense and connected each other to form the woven pattern, among which the marrow cavity existed and it is scarcely to see the free-end of trabecula (fig. 1, 2). However, the amount of trabecula in OVX group decreased apparently and trabecula became thinner. The arrangement of trabecula losed the oriented and the joints between trabeculae reduced, the free-end was easy to see, associated with enlarged marrow cavity (fig. 3). Treatment with estrogen could effectively restore the changes happened in OVX group. It showed that in OVX + E_2 group the trabecula was increased and looked thicker. The organization of trabecula became relative order and the area of marrow cavity decreased with the more connection of trabecula. Although the free-end of trabecula was markedly reduced, it still did not reach the level of the normal group (fig. 4).

Table 1 The wet weight of the rat uterus in each group

Groups	n	Wet weight of uterus (g)
N	5	0.42±0.05
Sham	5	0.41±0.035
OVX	5	0.16±0.02△
OVX+E_2	5	0.35±0.04*

* $P<0.01$ as compared with OVX group
△ $P<0.01$ as compared with normal control group

2.3 Histomorphometry

The results of histomorphometry in each were shown in table 2.

Table 2 Histomorphometric analysis-static parameters

Parameters	N	Sham-O	OVX	%-N	OVX+E_2	%-N	%-O
%Tb. Ar	30.31±3.90	29.42±5.21	6.20±1.84	−80***	14.21±10.08	−53*	129*
Tb. Th	66.93±5.52	65.34±6.14	65.70±6.31	−2	64.11±4.91	−4	2
Tb. N	4.52±0.25	4.39±0.38	0.96±0.32	−79***	2.23±1.64	−51*	132*
Tb. Sp	154.99±16.26	163.54±21.31	1079.06±411.53	596**	538.10±288.19	274*	−50*

%-N: the percentage change as compared with normal control group; %-O: the percentage change as compared with OVX group
* $P<0.05$ ** $P<0.01$ *** $P<0.001$

As shown in table 2, the trabecular area and number in OVX group were decreased significantly when compared with the normal control group, and the trabecular separation increased, indicating that trabecula became sparser ($P<0.01$—0.001). However, thickness of trabecula did not exhibit significant changes. In comparison with OVX group, there were significant increases in the traecular area and number after treated with estrogen in (OVX + E_2) group, and the trabecula separation decreased, indicating that trabecular became dense ($P<0.05$), but did not reach the level of the normal group ($P<0.05$).

Table 3 Histomorphometric analysis-dynamic parameters

Parameters	N	Sham-O	OVX	%-N	OVX+E_2	%-N	%-O
%L. Pm	10.72±3.84	10.32±3.62	14.79±3.26	38*	13.87±1.27	29*	−6
BFR/TV	16.53±13.05	15.96±11.96	5.19±3.89	−76*	11.77±11.16	−29	203*
OC. No	8.42±2.15	8.35±2.85	10.50±3.17	25*	4.60±2.94	−45*	−56**
N. OC/Tb. Pm	0.34±0.08	0.36±0.07	0.41±0.13	23*	0.05±0.03	−86**	−89***

%-N: The percentage change as compared with normal control group; %-O: The percentage change as compared with OVX control group
* $P<0.05$ ** $P<0.01$ *** $P<0.001$

Table 3 showed that both bone formation activity and bone resorption activity in OVX group were increased whereas the bone formation rate in bone tissue decreased ($P < 0.05$). As compared with OVX group, the bone formation activity in OVX+E_2 group had no more change, but the bone resorption activity restrained significantly and the bone formation rate of bone tissue increased significantly ($P < 0.05$).

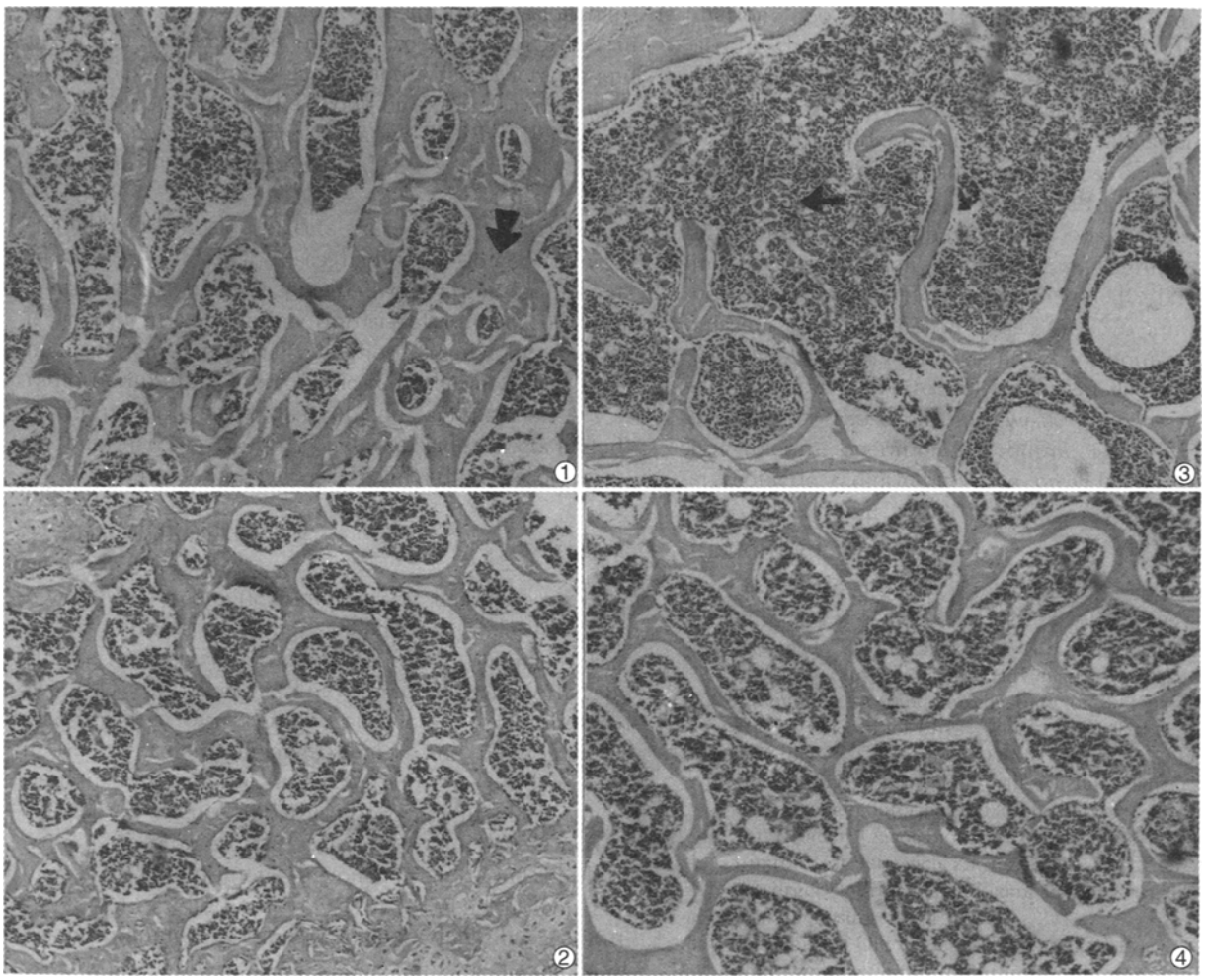

Fig. 1,2 In normal and Sham-O groups, trabecula was rich, dense and connected each other to form the woven pattern. The free-end of trabecula was seldom seen (Arrow: trabecula).

Fig. 3 In OVX group, trabecula decreased apparently and became thinner. The arrangement of trabecula losed and the joints between trabeculae reduced; the free-end was conspicuous, being associated with enlarged marrow cavity (Arrow: marrow cavity).

Fig. 4 In OVX + E_2 group the trabecula was increased and looked thicker. The organization of trabecula became relative orderly and the area of marrow cavity decreased with the more connection of trabecula.

3 DISCUSSION

Bone morphology and histomorphometry are the powerful research approaches in the investigation of changes in cortical as well as cancellous bone caused by metabolic bone diseases or by pharmaceutical interventions. They can give the observer the objective and visible results of tested bone. In this study, the indices from both morphology and histomorphometry showed clearly that in OVX rats both the trabecular number and volume were decreased and the distance between trabeculae increased, space of marrow cavity enlarged, which demonstrated the ovariectomy caused an obvious bone lose and the architecture of trabecula change. Based on the dynamic parameter of histomorphometry, the L. Pm%, which represent the number of osteoblast, N. OC, the number of osteoclast and N. OC/Tb. Pm, the number of osteoclast per mm trabecula appeared to be increased markedly in the OVX rats, but BFR/TV which reflects bone formation rate was decreased significantly as compared with N group, suggesting that after ovariectomy, bone turnover sustained increased. It displayed

one hand, bone formation and bone remodeling increased, in the other hand, bone resorption also increased, but the increase of bone formation did not compensated the bone loss. So, the final result was net bone loss. Our observation was in accordance with precious studies[1] which told that after menopausal, although both bone formation and resorption are increased, resorption is predominant over formation, bone loss is due to this excessive resorption. In addition, the high bone turnover led to the excessive remodeling lacuna, resulting in thinning trabecula associated with a loss of connectivity of trabeculae and easy to break. All of these would impair bone quality and increase the risk of fracture[5].

It is well accepted that hormone replacement therapy (HRT) to postmenopausal women can effectively prevent osteoporosis and reduce the risk of osteoporotic fracture. In this study, we observed morphologically that the loss of trabecular mass was inhibited effectively by estrogen treatment. Comparing to the OVX group, the samples from OVX+E_2 animals demonstrated a marketable increase in trabecular thickness and trabecular connection, decrease in marrow cavity. Well organized trabecula were also observed in this group. Calculated by bone histomorphometry, it showed that bone volume and number of trabecula were increased, the separation rate of trabecula was decreased, i. e., trabecula became dense. The above changes demonstrated that treatment with estrogen not only increased bone mass, but improved bone quality and enhanced mechanic strengthen of bone. In addition, dynamic parameter of histomorphometry revealed that the treatment with estrogen although did not made the critical effect on bone formation, bone resorption was significantly inhibited, so, bone gain finally, which addressed that protected effect of estrogen on bone was contributed to the inhibition of bone resorption and the improvement of bone structure.

The exact pathogenesis of postmenopausal osteoporosis is still uncertain. Cytokins that are produced in bone or by tissues adjacent to bone, such as IL-1, TNF-a, IL-6 etc., may play a very important role[6,7]. In which, IL-1 and TNF-α were the effective components to stimulate bone resorption by working on bone marrow stromal cell to increase M-CSF secretion. M-CSF together with IL-1 and TNF-α could alter early preosteoclast to differentiate to late preosteoclast which would become mature active osteoclast mediated by osteoblast. Meanwhile in the presence of IL-1 and TNF-α, cellular action of osteoblast on osteoclast would be enhanced significantly. IL-6, attracting many studies on its role on osteoporosis recent years, has been wildly considered to be a strong stimulating factor on bone resorption by acting positively on osteoclast formation and activity. The production of various cytokins in either monocytes or marrow stromal cells as well as osteoblastic cell lines is stimulated by systemic hormones such as gonadal hormones and local factors. By in vitro studies, it has been confirmed that estrogen possesses the ability to inhibit monocyte and marrow stromal cell to produce above cytokins, decreases the sensitivity of stromal cells to IL-1 and TNF, blocks the interaction between cytokins. In addition, estrogen was also found to inhibit the ability of osteoblast on promoting maturation of late preosteoclast and worked directly on osteoclast to reduce its activity[8].

REFERENCES

1 Dempster D W, Lindsay R. Pathogenesis of osteoporosis. Lancet, 1993,341:79

2 Dempster D W, Shane E, Horbert W *et al.* A simple method for correlative light and scanning electron microscopy of human iliac crest bone biopsies: qualitative observations in normal and osteoporotic subjects. J Bone Miner Res, 1986,1:15

3 Parfitt A M. Trabecular bone architecture in the pathogenesis and prevention of fracture. Am J Med,1987,82: 68

4 Rocker R R. Techniques and Interpretation. In: Rocker R R (ed). Bone histomorphometry. Florida: CRC Press, 1983. 14-24

5 Frost H M. On rho, a marrow mediator and estrogen: their roles in bone strength and "mass" in humen females, osteopenias and osteoporosis. J Bone Miner Metab, 1998, 16:113

6 Miyaura C, Kusano K, Masuzawa T *et al.* Endogenous bone-resorbing factors in estrogen deficiency: cooperative effects of IL-1 and IL-6. J Bone Miner Res, 1995, 10: 1365

7 Kitazawa R, Kimble R B, Vannice J L *et al.* Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. J Clin Invest, 1994,94:2397

8 Stavros C M, Robert L J. Bone marrow, cytokines, and bone remodeling. The New Eng J of Med, 1995, 332 (5):305

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