Effect of Epimedium-derived Phytoestrogen on Bone Turnover and Bone Microarchitecture in OVX-induced Osteoporotic Rats

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Summary To investigate the preventive effect of epimedium-derived phytoestrogen (PE) on osteoporosis induced by ovariectomy (OVX) in rats, 11-month-old female Wistar rats were randomly divided into Sham, OVX and PE groups. One week after OVX, daily oral administration of PE (0.4 g·kg⁻¹·day⁻¹) started in PE group, and rats in Sham and OVX groups were given vehicle accordingly. The administrations lasted for 12 weeks. The biological markers including serum osteocalcin (OC) and urinary deoxypyridinoline (DPD) for bone turnover were evaluated at the end of the 12th week. On the 13th week, all the rats were sacrificed. The right proximal tibiae were removed, subjected to micro CT for determination of trabecular bone structure and then bone histomorphometry was performed to assess bone remodeling. The OVX rats were in a high bone turnover status as evidenced by increased bone formation markers and bone resorption markers. Treatment with PE could suppress the high bone turnover rate in OVX rats. Micro CT data revealed that PE treatment could ameliorate the deterioration of the micro-architecture of proximal tibiae induced by OVX, as demonstrated by greater bone volume, increased trabecular thickness and less trabecular separation in PE group in comparison with OVX group. The static and dynamic parameters of bone histomorphometry indicated that there were significant increases in bone formation variables and significant decreases in bone resorption variables between PE and OVX groups. The findings suggest that PE has a beneficial effect on trabecular bone in OVX rat model and this effect is possibly associated with stimulation of bone formation as well as inhibition of bone resorption.

Key words osteoporosis; phytoestrogen; rat

Osteoporosis is a global health problem, characterized by a reduction in bone mass and deterioration of bone microstructure, consequently leading to a reduction of bone strength and increased risk of bone fracture[1]. For the postmenopausal women, current strategies to prevent and treat osteoporosis focus on the estrogen replacement therapy (ERT) and non-estrogenic drug therapy. ERT used to be one of the most effective anti-osteoporotic drugs, however, recent evidence suggested that prolonged ERT may be associated with increased risk of development of breast and ovarian cancers[2]. The rationale of non-estrogenic drug therapy is directed along two basic approaches, namely agents preventing bone resorption and that stimulating bone formation. However, some of these drugs have serious side effects and the cost is too high for patients in the developing countries. Thus, the alternative approach for prevention and treatment of osteoporosis is urgently needed. Phytoestrogens, plant-derived non-steroidal compounds with estrogen-like biological activity, have attracted much attention because of their potential beneficial role on osteoporotic bone and less estrogen-related side effects[3]. In this study, effect of the phytoestrogen compounds from epimedium on bone turnover and bone micro-architecture was evaluated.

1 MATERIALS AND METHODS

1.1 Animals and Experimental Design Twenty-seven 11-month-old Wistar female rats (The Center of Experimental Animals, Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China) were housed with three animals in each cage and acclimated under standard conditions at 22°C and in 50%–60% humidity. Rats were allowed free access to tap water and commercial standard chow. After acclimation for 1 month, rats were divided into sham-operated group (Sham, n=9), bilateral ovariectomized group (OVX, n=9) and OVX plus herbal PE group (PE, n=9). One week after surgery, PE, which was commercially available (Guizhou Xianling Pharmaceutical Co., China), was administrated orally at 0.4 mg/kg bw/day in PE group. The treatment lasted for 12 weeks. At the end of the experiment, the right tibiae were removed, dissected free of soft tissues and kept in 70% ethanol, ready for pQCT, micro CT scan and bone histomorphometry. All rats received subabdominal injection...
of tetracycline (30 mg/kg) for 14 d and calcein (5 mg/kg) for 4 d before sacrifice.

1.2 Biochemical Markers for Bone Turnover

Serum osteocalcin (OC) was measured by radioimmunoassay (RIA) and the protocol was recommended by the supplier of the reagents (Biomedical Technologies, Stoughton, MA, USA). Urinary deoxypyridinoline (DPD) was assayed by high performance liquid chromatography. The values of DPD were expressed as a ratio to urinary creatine (Cr). The coefficients of variation of OC and DPD were 3.1% and 4.2%, respectively.

1.3 Micro CT

The right proximal tibiae were measured with a desktop micro CT (µCT-40, Scanco Medical, Bassersdorf, Switzerland) with an isotropic resolution of 20 µm in all three spatial dimensions. The starting scanning line was 1 mm below the growth plate and the region of interest for evaluation covered 1.5 mm length distal from the starting scanning line. Such a region of interest was chosen because it could avoid the bias from the primary spongiosa. In order to obtain the original 3-D image of trabecular bone, a threshold value of 224 was set to separate the trabecular bone from cortical bone in the analysis system. The trabecular bone was separated from the cortical bone in 2-D images. On the original 3-D images, the bone volume (BV, mm³) and the tissue volume (TV, mm³) were measured directly. The normalization of trabecular bone volume (BV/TV, %) was calculated so as to compare samples with different sizes. The trabecular structural variables including trabecular thickness (Tb.Th, µm), trabecular number (Tb.N, 1/mm) and trabecular separation (Tb.Sp, µm), structure model index (SMI) were directly measured on 3-D images. SMI was a parameter to quantify the characteristic form of a 3-dimensional structure of plates and rods.

1.4 Bone Histomorphometry

After micro CT scan, the bone samples were fixed in 70% ethanol, dehydrated in increasing concentrations of ethanol and embedded in methyl methacrylate. After polymerization, the blocks were trimmed with saw microtome (Leitz 6000, Germany) to a thickness of 100 µm. Undecalcified sections, 10 µm thick, were obtained with a polycut E microtome (Ultratmiller Polycut E, Jung, Germany). Golder’s Trichrome staining was performed on the polycut sections. Unstained slices were examined under fluorescent microscope. Static and dynamic variables were analyzed by using a semi-automatic image analysis osteomasure system (Leica Q500MC, Germany). The static variables included total tissue area (T.Ar), trabecular bone area (B.Ar) and perimeter (B.Pm). The dynamic parameter included single (sL.Pm) and double-labeled perimeter (dL,Pm), osteoid perimeter (O.Pm), interlabel width (Ir.L.W) and erosive perimeter (E.Pm). These indices were used to calculate the bone formation parameters: osteoid surface/bone surface (OS/BS), mineralizing surface/bone surface (MS/BS), mineral apposition rate (MAR) and the bone absorption parameter eroded surface/bone surface (ES/BS,%).

1.5 Statistical Analysis

All the data were expressed as X ± s. One way analysis of variance (ANOVA) with post hoc test was used to evaluate the differences of variables among groups. A P<0.05 was considered to be significant. All statistical evaluations were performed by using SPSS version 12.0 (SPSS, Chicago, IL, USA).

2 RESULTS

2.1 Biochemical Markers for Bone Turnover

OVX significantly increased serum osteocalcin and urinary DPD levels (P<0.05, vs sham-operated group), indicating the induction of high bone turnover rate in rats by OVX. Herbal formula PE could result in a significant reduction in serum DPD level (P<0.05 vs sham-operated group) and maintain OVX-induced bone formation in serum OC level (table 1).

Table 1 The biochemical markers for bone turnover in different groups (n=9)

<table>
<thead>
<tr>
<th>Groups</th>
<th>OC (nmol/L)</th>
<th>DPD (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX</td>
<td>6.27±0.23</td>
<td>84.96±8.97</td>
</tr>
<tr>
<td>Sham</td>
<td>5.48±0.22</td>
<td>64.30±24.31*</td>
</tr>
<tr>
<td>PE</td>
<td>6.20±0.23*</td>
<td>68.37±10.07*</td>
</tr>
</tbody>
</table>

P<0.05 as compared with OVX group; *P<0.05 as compared with sham group

2.2 Trabecular Bone Microstructure Evaluated by Micro CT

The micro CT image showed that the microstructure of the proximal tibia was disconnected in OVX group, as compared with that of the sham group. In the PE group, the interconnection was much better than that in the OVX group. The BV/TV, Tb.Th, Tb.N and SMI in the OVX group were significantly lower than those in the sham-operated group. The treatment with PE increased BV/TV, Tb.Th significantly when compared with those in the OVX group, while it did not improve Tb.N. The increase in Tb.Sp after OVX was suppressed by administration of herbal formula PE and the Tb.Sp in the XLGB group decreased significantly in comparison with the OVX group. No significant differences were found in microCT variables between the PE and the sham group (table 2).

Table 2 A comparison of trabecular bone micro-architectural indices among different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>BV/TV (%)</th>
<th>Tb.N (µm⁻¹)</th>
<th>Tb.Th (µm)</th>
<th>Tb.Sp (µm)</th>
<th>SMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX</td>
<td>0.06±0.02</td>
<td>1.98±0.59</td>
<td>0.07±0.01</td>
<td>0.6±0.12</td>
<td>3.15±0.49</td>
</tr>
<tr>
<td>Sham</td>
<td>0.16±0.03*</td>
<td>3.11±0.87*</td>
<td>0.1±0.02</td>
<td>0.3±0.04*</td>
<td>1.15±0.14*</td>
</tr>
<tr>
<td>PE</td>
<td>0.09±0.03*</td>
<td>2.02±0.19*</td>
<td>0.11±0.0*</td>
<td>0.42±0.08*</td>
<td>2.13±0.25*</td>
</tr>
</tbody>
</table>

*P<0.05 as compared with OVX group; #P<0.05 as compared with sham group
2.3 The Static and Dynamic Parameters of Bone Histomorphometry

In OVX and PE group, newly formed unmineralized bone (osteoid) as well as eroded surface due to bone resorption was observed by means of Goldner’s staining. The static and dynamic histomorphometric parameters of bone formation osteoid surface (OS/BS), mineralizing surface (MS/BS) and mineral apposition rate (MAR) were significantly higher in the OVX group than those in sham-operated group. After treatment with PE for 12 weeks, OS/BS, MS/BS and MAR increased substantially as compared with those in OVX group and there were no significant differences in these parameters between PE and sham-operated group (table 3). ES/BS, a bone resorption variable in the OVX group decreased significantly compared with that in sham-operated group. The decrease could be suppressed by the treatment of PE, as there was a significant increase in this parameter in PE group (table 3).

Table 3 A comparison of trabecular bone histomorphometric parameters among different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>OS/BS (%)</th>
<th>MAR (µm/d)</th>
<th>ES/BS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX</td>
<td>21.4±3.12</td>
<td>1.65±0.33</td>
<td>5.43±0.28</td>
</tr>
<tr>
<td>Sham</td>
<td>18.5±3.18</td>
<td>1.23±0.12</td>
<td>4.72±0.32</td>
</tr>
<tr>
<td>PE</td>
<td>20.7±2.12</td>
<td>1.60±0.21</td>
<td>4.95±0.27</td>
</tr>
</tbody>
</table>

*P<0.05 as compared with OVX group; **P<0.05 as compared with sham group

3 DISCUSSION

More and more attentions have been paid to the traditional Chinese medicine in terms of the effect to prevent and treat osteoporosis. Epimedium-derived phytoestrogen (PE), mainly composed of genistein and daidzein, is widely used in clinical practice and claimed to be effective in strengthening bone and muscle in postmenopausal woman[6]. Up to now, there is no scientific report about the preventive effect of PE on osteoporosis. In this study, we tried to assess the effect of PE on the bone turnover and bone micro-architecture, by using a standard OVX-induced osteoporotic model. The OVX rats were in a high bone turnover status, as evidenced by the significant increase of serum osteocalcin, the marker of bone formation and a significant decrease of urinary DPD, a marker of bone resorption.

Bone micro-architecture is one of the most important determining factors on bone strength. In this study, trabecular bone micro-architecture was assessed by high resolution micro CT and it showed that the structural indices, including BV/TV, Tb.Th decreased significantly because of the estrogen deficiency in OVX rats. This result was consistent with our previous study[3-5]. Treatment with PE was able to improve the relative bone volume, Tb.Th and decrease the Tb.Sp but the Tb.N remained unchanged during this period of study. This was probably because the increase of bone volume was a result of thickening existing trabeculae instead of restoration of the damaged trabeculae. However, in a recent study by Yao et al[7], their findings indicated that the increase of trabecular bone volume after the treatment of basic fibroblast growth factor (bFGF) in OVX rats resulted from restoring trabecular number and connectivity. The discrepancy in the change of trabecular number in OVX rats between the treatment of bFGF and PE is probably due to the different effects of the two agents on trabecular bone. Though not beneficial to the trabecular number, administration of herbal formula PE could suppress the trend of deterioration of trabecular bone micro-architecture.

In OVX rats, estrogen deficiency accelerates bone remodeling with a predominance of bone resorption over bone formation, leading to the deterioration of trabecular bone structure and a reduction of bone mass. Biochemical markers can only give an indication of the overall balance of bone resorption and formation. Histomorphometry, however, allows assessment of bone remodeling at the cellular and tissue levels[8], therefore remains an important tool to evaluate the long-term effects of therapeutic agents on bone quality and remodeling. To determine the possible mechanism of the beneficial effect of herbal preparation PE on trabecular bone in OVX-induced osteoporotic rats, the static and dynamic parameters of the proximal tibiae were assessed. Our histomorphometric analysis suggested that OVX rats were in a high bone turnover status, which was consistent with the result of biological markers. OS/BS, MS/BS and MAR, the parameters for bone formation were significantly improved in the PE group. The ex vivo histomorphometric data together with the in vivo biochemical markers for bone formation indicate that PE could stimulate bone formation. In the meanwhile, in this study, we observed that PE could decrease urinary DPD and ES/BS, the bone resorption markers. The micro CT analysis showed that trabecular separation in OVX group was significantly lower than that in the PE group, this phenomenon suggests that herbal formula PE improves the quality of trabecular bone at the proximal tibiae, probably through the mechanism of stimulation of bone formation and inhibition of bone resorption.

In conclusion, PE could block the bone loss and ameliorate the deterioration of trabecular micro-architecture induced by OVX in old rats. This beneficial effect of PE on trabecular bone is associated with the stimulation of bone formation as well as inhibition of bone resorption.

REFERENCES

formula "XLGB" prevents OVX-induced deterioration of musculoskeletal tissues at the hip in old rats. J Bone Miner Metab, 2005, 23 (Suppl): 55

5 Zhang G, Qin L, Hung W Y et al. Flavonoids derived from herbal Epimedium Brevicornum Maxim prevent OVX-induced osteoporosis in rats independent of its enhancement in intestinal calcium absorption. 2006, Bone, 38:818

6 Qin L. Research and development of herbal formulae for prevention and treatment of osteoporosis. International Bone and Research Instructional Course and Hands-on Workshop, Hong Kong. 2002, pp112


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