Summary. Puerarin and zinc play a key role in preventing osteoporotic-related bone loss. Previous research on puerarin or zinc mainly focused on the anti-osteoporotic effects of long bone. However, it is obscure for puerarin or zinc to prevent mandibular osteoporosis. Here, we explore the effects on additive coadministration of puerarin and zinc on preventing mandibular bone loss in ovariectomized rats, and evaluate the underlying mechanisms \textit{ex vivo}. Rats were ovariectomized and administrated puerarin, zinc or both. After 12 weeks, bone mineral density (BMD) and histomorphometrical parameters of mandibles were measured by micro-CT. The mechanical properties were determined using a three-point bending test. Then, osteogenic differentiation of primary bone marrow stromal cells (BMSCs) and osteoclastogenesis of bone marrow mononuclear were performed \textit{ex vivo}. The culture supernatant and serum level of bone biochemical markers including osteoprotegerin (OPG), osteopontin (OPN), receptor activator of nuclear factor (NF)-κB ligand (RANKL), and tartrate-resistant acid phosphatase (TRAP) were detected by ELISA. Culture supernatant and serum levels of calcium were measured using a Plasma Emission Spectrometer. One-way ANOVA was used for statistical analyses. The results showed that administration of puerarin plus zinc prevented the decrease in mandibular BMD and bone morphometrical parameters more effectively than single use of puerarin or zinc ($p<0.05$), which was similar to the biomechanical tests ($p<0.05$). Furthermore, puerarin and zinc additively up-regulated OPG, OPN protein levels, Ca ion level and down-regulated RANKL, TRAP protein levels. In conclusion, puerarin and zinc additively prevent mandibular bone loss through inhibiting osteoclastogenesis in ovariectomized rats, which will shed more light on the potential use of puerarin and zinc in the prevention/treatment of oral bone loss clinically.

Key words: Puerarin, Zinc, Mandible, Osteoporosis, Osteoclastogenesis

Introduction

Osteoporosis is a systemic disease of excessive skeletal fragility characterized by loss of bone mass and bone micro-architectural deterioration. Recently, much research has concluded that osteoporosis could be a risk factor for the progression of oral disease, such as periodontitis and oral bone loss (Brennan-Calanan et al., 2008; Bartold et al., 2010; Bashutski et al., 2010). Two major pharmacologic treatments of osteoporosis are anabolic agents like teriparatide, or anti-resorptive agents like bisphosphonates, denosumab, and raloxifene (Gupta and March, 2016; Lindsay et al., 2016; Sanderson et al., 2016). Although these therapies have increased bone mineral density (BMD) and reduced the risk of fractures, we must be aware of long-term safety and efficacy. For instance, bisphosphonates have potential side effects on bisphosphonate-related
osteonecrosis of the jaw (BRONJ) (Gonen and Yilmaz \citeyear{Asan2016}; Graves et al., 2016; Kaibuchi et al., 2016). Thus, new safe and effective modalities are required for osteoporotic treatment.

Puerarin (PubChem CID: 5281807) is a phytoestrogen extracted from Pueraria plants such as P. lobata (Willd.) Ohwi used in traditional Chinese medicines (TCM), which has fewer side effects and is more suitable for long-term use compared with the chemically synthesized medicines. Many reports have validated that this compound has an isoflavone structure and exerts antiosteoporotic effects in vitro and in vivo (Sheu et al., 2012; Wang et al., 2012; Yang et al., 2012; Li et al., 2014).

Zinc, as a kind of nutritional trace element, plays an important role in bone metabolism, and zinc deficiency is thought to accelerate the development of osteoporosis (Otsuka et al., 2003; Gurban and Mederle, 2011; Zheng et al., 2014). Uchiyama and Yamaguchi have shown that co-administrated genistein (an isoflavone phytoestrogen) and zinc suppress receptor activator of nuclear factor kappa-B ligand (RANKL) signaling-related gene expression in vitro (Uchiyama and Yamaguchi, 2007a, b). This implies that coadministration of genistein and zinc may indirectly inhibit osteoclast differentiation. Consistent with genistein isoflavone, we hypothesize that coadministration of puerarin and zinc may also inhibit osteoclastogenesis induced by osteoporosis.

In the study, we explore the effects on additive coadministration of puerarin and zinc on preventing mandibular bone loss in the OVX rats, and evaluate the underlying mechanisms ex vivo.

Materials and methods

Ethical approval of the study protocol

All animal research was approved by the Animal Care and Use Committee of Peking University Health Science Center (approval number: LA2015121; Beijing, China).

Animals and administration procedure

Eight-week-old female SD rats (n=72) were assigned randomly into six groups (12 per group): (1) sham: sham surgery, orally administrated with normal saline vehicle; (2) OVX: bilaterally ovariectomized, orally administrated with normal saline vehicle; (3) OVX-E: the OVX rats were intraperitoneally injected with 10 μg/kg body weight (BW) 17β-estradiol (dissolved in plant oil) (Purity: 98.4%, Sigma-Aldrich, Saint Louis, MO, USA) once every other day; (4) OVX-P: puerarin (dissolved in normal saline) (Analytically pure, Sigma-Aldrich, Saint Louis, MO, USA) was given by gavage once every other day at 0.25 mg/kg BW after surgery; (5) OVX-Z: ZnSO₄ (dissolved in normal saline) (Purity: 99.9%, Sigma-Aldrich, Saint Louis, MO, USA) was given by gavage once every other day at 0.25 mg/kg BW after surgery; (6) OVX-ZP: dosage and administration of puerarin + ZnSO₄ as for groups 4 and 5. All above-mentioned treatments were administrated to the OVX rats 3 days after surgery. 12 weeks after treatment, the rats were exsanguinated before euthanasia. Serum was separated and stored at -80°C for detection of osteoprotegerin (OPG), osteopontin (OPN), Ca, RANKL, and tartrate-resistant acid phosphatase (TRAP) level. The bilateral mandible, femur, and tibia of rats were thoroughly dissected free from soft tissue and kept for further analysis.

Microtomographic histomorphometry and BMD detected by micro-CT

The mandibles were scanned by micro-CT of Inveon MM system (Siemens, Munich, Germany) as previously described (Liu et al., 2016). In brief, images were acquired at a voxel of 8.82 μm × 8.82 μm × 8.82 μm, 80 kV voltage, 500 μA current, and 1500 ms exposure time in each of the 360 rotational steps. Parameters were calculated by an Inveon Research Workplace (Siemens, Munich, Germany) as follows: bone volume/total volume (BV/TV), bone surface area/bone volume (BS/BV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and BMD adjusted through phantom. The ROI (region of interest) of mandible was in the trabecula of the mandible below the first molar in all three directions, as previously described (Fig. 1) (Liu et al., 2015b). The bone morphometric parameters on mandibles of randomly selected 6 out of the 12 rats in one group were performed in triplicate by three independent assessors.

Biomechanical testing

The mandibles scanned by micro-CT were subjected to three-point bend tests. The tests proceeded until failure at a plunger speed of 1.0 mm/min using a servohydraulic testing device (Instron 4302, Instron, Norwood, Mass), as previously described (Liu et al., 2015b). The tested area of the mandible was the basal bone in the mandible below the molars. The parameters were recorded as follows: the maximum load, stiffness, energy to ultimate load, and elastic modulus.

Isolation, culture, and osteogenic differentiation of bone marrow stromal cells (BMSCs)

All reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA) unless stated otherwise. Bone marrow cells from the tibia of randomly selected 6 out of the 12 rats in each group were cultured in the α-MEM medium containing 10% FBS, 100 U/mL penicillin G and 100 mg/mL streptomycin (GIBCO Laboratories, Grand Island, NY, USA) at 5×10⁵ cells/mL. When the BMSCs were cultured up to passage 3-5, the culture medium was replaced with new α-MEM supplemented separately with 10 mM β-sodium glycerophosphate +
0.05 M ascorbic acid + 10 nM dexamethasone for osteogenic differentiation. 7 days after culture, culture supernatants were collected and stored at -80°C. OPG, OPN, Ca, and RANKL were detected through culture supernatants of osteogenic media. There were 6 samples detected in each replicate. All cell-based experiments were repeated at least three times (Liu et al., 2014).

Osteoclastogenesis ex vivo

Bone marrow cells from the femur of randomly selected 6 out of the 12 rats in each group were obtained as above. The suspending cells in media containing 10 ng/ml M-CSF were seeded in the culture dishes overnight. Non-adherent cells were cultured at 10^6 cells/mL in osteoclastogenic media \([\alpha\text{-MEM} + 10\% FBS, 10\ ng/ml \text{ M-CSF}, 50\ ng/ml \text{ RANKL}]\). 7 days after culture, culture supernatants were stored at -80°C until analysis. TRAP were tested by the culture supernatants of osteoclastogenic media. There were 6 samples detected in each replicate. All cell-based experiments were repeated at least three times (Kamel Mohamed et al., 2005).

Assay for biochemical markers

After osteogenic differentiation or osteoclastogenesis ex vivo, the related conditioned medium was discarded and the normal medium was used to detect bone biochemical markers. Then, culture supernatant and serum levels of OPG, OPN, RANKL, and TRAP were detected using the relevant enzyme-linked immunoassay (ELISA) kits (Abcam, Cambridge, MA, USA) (IDS, Frankfurt, Germany). The intra-assay coefficients of variations for OPG, OPN, RANKL, and TRAP assays were 3.8%, 5.2%, 6.9%, and 4.9%, respectively. The inter-assay coefficients of variations for OPG, OPN, RANKL, and TRAP tests were 6.7%, 7.4%, 9.2%, and 8.6%, respectively (Rubin et al., 2002; Liu et al., 2015a). Culture supernatant from osteogenic media and serum levels of calcium were measured using a Plasma Emission Spectrometer (iCAP 6000; Thermo Fisher Scientific, Waltham, MA, USA) (Li et al., 2014). Samples were measured in duplicate (at least).

Statistical analyses

Data are expressed as the mean ± standard deviation (SD). SPSS v19.0 (IBM, Armonk, NY, USA) was used for statistical analyses. One-way ANOVA by a Tukey’s post hoc test was performed, and p-values<0.05 were considered statistically significant.

Results

The effect of puerarin and zinc on BMD in mandible

To assess bone mass in the six groups, BMD was determined by micro-CT (Fig. 2). Micro-CT analyses demonstrated that the OVX group had a dramatically decreased BMD in bone trabeculae of mandibles compared with the sham group (p<0.001) and the OVX-E group (p<0.001). The OVX-P and OVX-Z groups had a lower BMD than the OVX-E group (p<0.01, respectively). However, the BMD of the OVX-ZP group significantly increased compared with the OVX-P and OVX-Z groups (p<0.05, respectively) (Fig. 2a).

Considering another analysis site, the OVX group showed a significantly decreased BMD compared with the sham and OVX-E group in the inferior margin of the mandible (p<0.001, respectively). Interestingly, the other four OVX groups (OVX-E, OVX-P, OVX-Z, and OVX-ZP groups) had a significant increase in BMD compared with the sham group (p<0.05, respectively). However, there were no significant differences among the OVX-P, OVX-Z, and OVX-ZP groups (Fig. 2b).

The effect of puerarin and zinc on microtomographic histomorphometry in mandible

The bone morphometry of the trabeculae of mandibles below the first molar was evaluated by micro-CT (Fig. 3) (Table 1). The OVX group showed a significantly decreased BV/TV and increased BS/BV compared with the sham and OVX-E groups (p<0.001).
Puerarin and zinc prevent mandibular bone loss

Table 1. Bone histomorphometry of mandibles from the six groups after 12 weeks of treatment.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BV/TV (%)</th>
<th>BS/BV (1/mm)</th>
<th>Tb.Th (mm)</th>
<th>Tb.N (1/mm)</th>
<th>Tb.Sp (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sham</td>
<td>32.74±4.74</td>
<td>40.66±0.45</td>
<td>0.042±0.004</td>
<td>6.05±0.49</td>
<td>0.123±0.013</td>
</tr>
<tr>
<td>OVX</td>
<td>22.29±2.94</td>
<td>56.13±2.88</td>
<td>0.048±0.002</td>
<td>6.34±1.25</td>
<td>0.110±0.010</td>
</tr>
<tr>
<td>OVX-E</td>
<td>34.09±3.55</td>
<td>38.12±2.00</td>
<td>0.053±0.003</td>
<td>6.49±0.68</td>
<td>0.103±0.015</td>
</tr>
<tr>
<td>OVX-P</td>
<td>27.46±2.85</td>
<td>45.58±3.26</td>
<td>0.045±0.002</td>
<td>6.19±0.35</td>
<td>0.117±0.004</td>
</tr>
<tr>
<td>OVX-Z</td>
<td>28.07±2.76</td>
<td>40.21±3.11</td>
<td>0.050±0.004</td>
<td>5.64±0.63</td>
<td>0.129±0.019</td>
</tr>
<tr>
<td>OVX-ZP</td>
<td>36.33±3.17</td>
<td>37.67±2.16</td>
<td>0.052±0.002</td>
<td>7.50±0.32</td>
<td>0.089±0.008</td>
</tr>
</tbody>
</table>

Data are means ± SD, n=6 per group. * p<0.05 vs. sham, # p<0.05 vs. OVX, $ p<0.05 vs. OVX-E, & p<0.05 vs. OVX-P, % p<0.05 vs. OVX-Z.

Fig. 2. BMD of bone trabeculae of mandibles (a), and the inferior margin of mandibles (b) in the six treatment groups. Data are means ± SD, n=6 per group. * p<0.05 vs. sham, # p<0.05 vs. OVX, $ p<0.05 vs. OVX-E, & p<0.05 vs. OVX-P, % p<0.05 vs. OVX-Z.

Fig. 3. Representative images of changes in bone microarchitecture of mandibles in the transverse plane in rats.
and 0.001, respectively), which means that the OVX-induced osteoporotic rat model was performed. Surprisingly, the Tb.Th of the OVX and OVX-E groups were higher than that of the sham group (p<0.05 and 0.05, respectively).

Notably, the OVX-ZP group had significantly increased BV/TV and Tb.N and decreased Tb.Sp compared with the OVX-P and OVX-Z groups (OVX-P group: BV/TV p<0.01, Tb.N p<0.05, Tb.Sp p<0.05; OVX-Z group: BV/TV p<0.05, Tb.N p<0.01, Tb.Sp p<0.01). Moreover, the OVX-ZP and OVX-Z groups decreased significantly in BS/BV and increased in Tb.Th compared with the OVX-P group (OVX-ZP group: BV/TV p<0.01 and 0.01, respectively; OVX-Z group p<0.05 and 0.05, respectively). The same trends in bone morphometry of the mandibles in the six groups were also observed in analysis of the trabeculae of the mandibles below all three molars (Fig. 4).

**Mechanical tests in mandible**

To evaluate mechanical effects in the six groups after 12 weeks of treatment, three-point bend tests were performed in the mandibles (Fig. 5). The OVX group showed a dramatic decrease in all four mechanical parameters compared with the sham and OVX-E groups (maximal load: p<0.01 and 0.01, respectively; stiffness: p<0.01 and 0.05, respectively; energy to ultimate load: p<0.001 and 0.001, respectively; elastic modulus: p<0.01 and 0.01, respectively). Besides, the OVX-Z and OVX-ZP groups exhibited significantly more energy to ultimate load compared with the OVX-P group (OVX-Z group p<0.05, OVX-ZP group p<0.01). With respect to maximal load, stiffness, and elastic modulus, there were no significant differences among the OVX-P, OVX-Z, and OVX-ZP groups. Generally, the four parameters of mechanical tests in the OVX-ZP group were similar to those of the sham group.

**The serum bone biochemical markers in rats of six groups after 12 weeks of treatment in vivo**

To investigate underlying mechanisms on mandibular bone regeneration in the OVX rats, five serum bone biochemical markers were detected (Table 2). In brief, the OVX group had a significant decreased serum OPN, OPG, and Ca levels, and significantly increased serum TRAP and RANKL levels compared with the sham and OVX-E groups (p<0.01, respectively). Besides, the OVX-ZP group had a significantly increased serum OPG level compared with the OVX-P group (p<0.05). Moreover, the OVX-ZP group had a significant increase in serum Ca level compared with the OVX-P and OVX-Z groups (p<0.05 and 0.05, respectively).

Inversely, the serum TRAP levels in the OVX-ZP group was lower than that of the OVX-P and OVX-Z groups (OVX-P group p<0.01; OVX-Z group p<0.001). Moreover, the OVX-Z group significantly increased in serum TRAP level and the level was higher than the OVX-P group (p<0.05). Consistent with serum TRAP level, the serum RANKL level in the OVX-ZP group.

**Table 2. Bone biochemical markers in rats’ serum after 12 weeks of treatment.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OPN (μg/L)</th>
<th>OPG (ng/L)</th>
<th>Ca (mg/L)</th>
<th>TRAP (pg/L)</th>
<th>RANKL (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sham</td>
<td>62.56±1.25</td>
<td>1464.93±150.24</td>
<td>54.82±4.34</td>
<td>3353.41±44.16</td>
<td>36.04±0.64</td>
</tr>
<tr>
<td>OVX</td>
<td>60.04±2.24a</td>
<td>1239.53±37.48a</td>
<td>45.27±3.30a</td>
<td>3623.18±55.28a</td>
<td>42.41±0.98a</td>
</tr>
<tr>
<td>OVX-E</td>
<td>63.92±1.70a,b</td>
<td>1700.58±174.43b</td>
<td>61.05±2.88b</td>
<td>2816.46±63.95b</td>
<td>33.98±0.80b</td>
</tr>
<tr>
<td>OVX-P</td>
<td>67.95±1.84b,c</td>
<td>1447.92±139.74c</td>
<td>54.65±7.15b</td>
<td>3042.25±103.48a,b,c</td>
<td>35.13±0.98b,c</td>
</tr>
<tr>
<td>OVX-Z</td>
<td>67.33±1.18a,b,c</td>
<td>1500.86±113.41b,c</td>
<td>55.07±5.17b</td>
<td>3134.37±54.38a,b,c,d</td>
<td>36.52±1.38b,c,d</td>
</tr>
<tr>
<td>OVX-ZP</td>
<td>69.32±1.29a,b,c</td>
<td>1632.21±146.23a,b,d</td>
<td>66.17±8.42a,b,d,e</td>
<td>2945.35±72.30a,b,c,d,e</td>
<td>30.39±0.60a,b,c,d,e</td>
</tr>
</tbody>
</table>

Data are means ± SD, n=6 per group. a: p<0.05 vs. sham, b: p<0.05 vs. OVX, c: p<0.05 vs. OVX-E, d: p<0.05 vs. OVX-P, e: p<0.05 vs. OVX-Z.

**Table 3. Bone biochemical markers in culture supernatant from the six groups.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OPN (μg/L)</th>
<th>OPG (ng/L)</th>
<th>Ca (mg/L)</th>
<th>TRAP (pg/L)</th>
<th>RANKL (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sham</td>
<td>23.91±0.53</td>
<td>486.41±75.32</td>
<td>49.75±8.16</td>
<td>1218.55±23.94</td>
<td>12.66±0.36</td>
</tr>
<tr>
<td>OVX</td>
<td>21.65±0.69a</td>
<td>384.97±33.83a</td>
<td>36.55±3.58</td>
<td>1445.67±24.99a</td>
<td>16.25±0.42a</td>
</tr>
<tr>
<td>OVX-E</td>
<td>25.75±0.39ab</td>
<td>515.93±56.68ab</td>
<td>52.70±5.11b</td>
<td>1092.57±29.01ab,b</td>
<td>11.57±0.29ab,b</td>
</tr>
<tr>
<td>OVX-P</td>
<td>24.62±0.45ab,c</td>
<td>490.67±73.89b</td>
<td>42.25±8.37</td>
<td>953.19±35.29ab,b,c</td>
<td>11.45±0.30ab,b,d</td>
</tr>
<tr>
<td>OVX-Z</td>
<td>23.97±0.48ab,c,d</td>
<td>467.19±60.87b,c</td>
<td>46.47±9.76</td>
<td>1145.24±31.77ab,c,d</td>
<td>11.45±0.30ab,b,d</td>
</tr>
<tr>
<td>OVX-ZP</td>
<td>26.52±0.33ab,b,c,d,e</td>
<td>581.30±60.47ab,b,c,d,e</td>
<td>61.40±13.13b,d,e</td>
<td>744.13±34.18ab,b,c,d,e</td>
<td>7.53±0.28ab,b,c,d,e</td>
</tr>
</tbody>
</table>

Data are means ± SD, n=6 per group. a: p<0.05 vs. sham, b: p<0.05 vs. OVX, c: p<0.05 vs. OVX-E, d: p<0.05 vs. OVX-P, e: p<0.05 vs. OVX-Z.
Fig. 4. Bone histomorphometry on the trabeculae of mandibles below all three molars after 12 weeks of treatment. Parameters of bone microarchitecture (BV/TV, BS/BV, Tb.Th, Tb.N, Tb.Sp) were measured in the trabeculae of mandibles below all three molars using micro-CT. Data are means ± SD, n=6 per group. * p<0.05 vs. sham, # p<0.05 vs. OVX, $ p<0.05 vs. OVX-E, & p<0.05 vs. OVX-P, % p<0.05 vs. OVX-Z.
was lower than that of the OVX-P and OVX-Z groups (p<0.001 and 0.001, respectively). Besides, the serum RANKL level in the OVX-P group had a significant decrease compared with the OVX-Z group (p<0.05).

**Bone biochemical markers in culture supernatant from the six groups ex vivo**

To further confirm the reason for attenuating mandibular bone loss in the OVX rats using puerarin and (or) zinc, *ex vivo* experiments were performed (Table 3). The OVX group had significantly decreased supernatant OPN, OPG levels and increased supernatant TRAP, RANKL levels compared with the sham and OVX-E groups (p<0.01, respectively). However, the supernatant Ca level of the OVX-E group was higher than that of the OVX group (p<0.001).

With respect to supernatant OPN, OPG, and Ca

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**Fig. 5.** Biomechanical measurements of mandibles in a three-point bending test. Parameters of maximum load (a), stiffness (b), energy to ultimate load (c), and elastic modulus (d) were measured in the basal bone of the mandible below the molars. Data are means ± SD, n=6 per group. * p<0.05 vs. sham, # p<0.05 vs. OVX, $ p<0.05 vs. OVX-E, & p<0.05 vs. OVX-P.
levels, the OVX-ZP group had significantly higher supernatant OPN and OPG levels than the OVX-P and OVX-Z groups (p<0.001, respectively). Moreover, the supernatant OPN level in the OVX-Z group had a significant increase compared with the OVX-P group (p<0.01). Similar to the serum level, the supernatant TRAP and RANKL levels in the OVX-ZP group were lower than the OVX-P and OVX-Z groups (p<0.001, respectively). Besides, the OVX-P group had a significant decrease in supernatant TRAP and RANKL levels compared with the OVX-P group (p<0.001, respectively).

Discussion

Alveolar bone loss is associated with systematic bone loss induced by osteoporosis (Liu et al., 2015b; Hsu et al., 2016). Some research has validated that alveolar bone loss makes it difficult for tooth retention and therefore causes malnutrition (Payne et al., 1999; Sheiham et al., 2001; Ervin and Dye, 2009). Furthermore, poor diet elevated a risk of chronic diseases such as type 2 diabetes, obesity, and some cancers and exacerbates osteoporosis. Thus, the maintenance of alveolar bone plays a key role in health. As is well known, the jaw is both morphologically and functionally different from the other bones of the axial or peripheral skeleton. It also arises from a different embryonic germ layer (neuroectoderm) compared with bone of the axial and appendicular skeleton, which arise from mesoderm (Mavropoulos et al., 2007). Moreover, the growth of jaw is mainly through endochondral ossification, but it is intramembranous ossification for bone of the axial and appendicular skeleton. So, the therapeutic strategy for osteoporosis of jaw is not exactly the same as osteoporosis on bone of the axial and appendicular skeleton clinically. For instance, the drugs of osteoporotic treatment such as bisphosphonates had a potential side effect on osteonecrosis of the jaw rather than bone of the axial and appendicular skeleton. Hence, safety and efficacy of treatment for osteoporosis of the jaw should be explored in depth.

Puerarin, which attenuated / prevented lower limb bone loss induced by osteoporosis has been widely reported in many studies (Li et al., 2016; Yuan et al., 2016). Our previous research has shown that puerarin enhanced bone mass by promoting osteoblastogenesis and slightly lowering bone marrow adiposity in the OVX rats (Li et al., 2016). However, the protective effect of puerarin on jaw is still obscure. To the best of our knowledge, there are no reports that puerarin prevented oral bone loss induced by osteoporosis. However, Yang et al. have reported that puerarin decreased bone loss and collagen destruction in rats with ligature-induced periodontitis (Yang et al., 2015). Our results showed that puerarin prevented mandibular bone loss in the OVX rats, not only BMD but also bone 3D morphometry. Moreover, inspired by coadministration genistein (a phytoestrogen) and zinc performed by Uchiyama and Yamaguchi (Uchiyama and Yamaguchi, 2007a,b), our research also showed that coadministration of puerarin and zinc had a more beneficial effect on improving bone formation compared with use of each treatment alone in the OVX rats, especially in bone histomorphometry. Furthermore, mechanical tests, such as a ‘gold standard’ of bone protective effect, also proved that coadministration of puerarin and zinc prevented the decrease in mandibular mechanical property more effectively than the use of each treatment alone.

OPG, as a member of the TNF receptor superfamily, plays a key role in inhibiting osteoclast differentiation, osteoclast resorptive function and promoting osteoclast apoptosis (Joanna Tyrovola, 2008). Moreover, previous study concluded that the antiosteoporotic effects of puerarin were due to inhibit the osteoclastogenesis through up-regulating the ratio of OPG/RANKL (Tiyasatkulkovit et al., 2012, 2014; Wang et al., 2014; Li et al., 2016; Yuan et al., 2016). Hence, we concluded that serum OPG levels in the OVX-Z, OVX-P and OVX-ZP groups were higher than that of the OVX group. Our results also confirmed that puerarin upregulated the OPG protein level and down-regulated the RANKL protein level ex vivo and in vivo, which was similar to the aforementioned study. Notably, co-gavage of puerarin and zinc raised the ratio of OPG/RANKL more effectively comparing with a gavage of each treatment alone. However, Brzóska et al. have reported that zinc protected from increase in sRANKL concentration and the sRANKL/OPG ratio, and decrease in OPG concentration of bone and serum in the rats model of cadmium-induced bone metabolism disorders (Brzoska and Rogalska, 2013). We concluded that the better antiosteoporotic effects may be mediated by both through upregulating the ratio of OPG/RANKL synergistically.

Besides, co-administrated puerarin and (or) zinc decreased the TRAP protein level in this study. The conclusion was similar to previous research (Kishi and Yamaguchi, 1994; Michihara et al., 2012). There is a controversy on serum calcium and osteoporosis (Arjmandi et al., 1996; North American Menopause, 2001). Our research demonstrated that co-gavage puerarin and zinc increased the serum calcium level. That means that puerarin and zinc may prevent OVX-induced bone loss through improving the calcium absorption from the GI tract.

Interestingly, ex vivo research showed that the OVX-ZP group had a dramatic increase in OPN level compared with the OVX-Z and OVX-P group. However, there was no significant difference in OPN level among three groups in vivo. Furthermore, to the best of our knowledge, there are no relevant reports. Further investigation will focus on contradictory conclusions.

In this study, we investigated the mandibular antiosteoporotic effects of coadministered puerarin and zinc using the OVX rats as a model for the first time. The results showed that puerarin and zinc additively prevented the decrease in mandibular BMD and
parameters of bone morphometry, and therefore improved the mechanical ability of mandible. Further experiments showed that the underlying mechanisms on mandibular antosteoporotic effects were due to the inhibition of the osteoclastogenesis through OPG up-regulation and RANKL down-regulation. The results in this study will shed more light on the potential use of puerarin and zinc in the prevention/treatment of osteoporosis.

Acknowledgements. This work was supported by the National Natural Science Foundation of China (grant numbers: 30901671).

Conflict of Interest. All authors declare no conflicts of interest regarding the publication of the paper.

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Accepted December 14, 2016