

# Puerarin and zinc additively prevent mandibular bone loss through inhibiting osteoclastogenesis in ovariectomized rats

Hao Liu<sup>1,3,4</sup>, Wei Li<sup>2,3,4</sup>, Shengnan Jia<sup>2,3,4</sup> and Binbin Li<sup>2,3,4</sup>

<sup>1</sup>The Core Laboratory, <sup>2</sup>Department of Oral Pathology, Peking University School and Hospital of Stomatology, <sup>3</sup>National Engineering Laboratory for Digital and Material Technology of Stomatology and <sup>4</sup>Beijing Key Laboratory of Digital Stomatology, Beijing, China

**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 *ex vivo*. Rats were ovariectomized and administered puerarin, zinc or both. After 12 weeks, bone mineral density (BMD) and histomorphometry 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 *ex vivo*. The culture supernatant and serum level of bone biochemical markers including osteoprotegerin (OPG), osteopontin (OPN), receptor activator of nuclear factor (NF)- $\kappa$ B 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

Offprint requests to: Dr. Binbin Li, Department of Oral Pathology, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Avenue, Haidian District, Beijing 100081, China. e-mail: [kqlibinbin@aliyun.com](mailto:kqlibinbin@aliyun.com)

DOI: 10.14670/HH-11-855

osteonecrosis of the jaw (BRONJ) (Gonen and Yilmaz Asan, 2016; 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 should 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 50 mg/kg BW after surgery; (5) OVX-Z: ZnSO<sub>4</sub> (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 + ZnSO<sub>4</sub> 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 5×10<sup>5</sup> 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  $-80^{\circ}\text{C}$ . 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 [ $\alpha$ -MEM + 10% FBS, 10 ng/ml M-CSF, 50 ng/ml RANKL]. 7 days after culture, culture supernatants were stored at  $-80^{\circ}\text{C}$  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  $\pm$  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

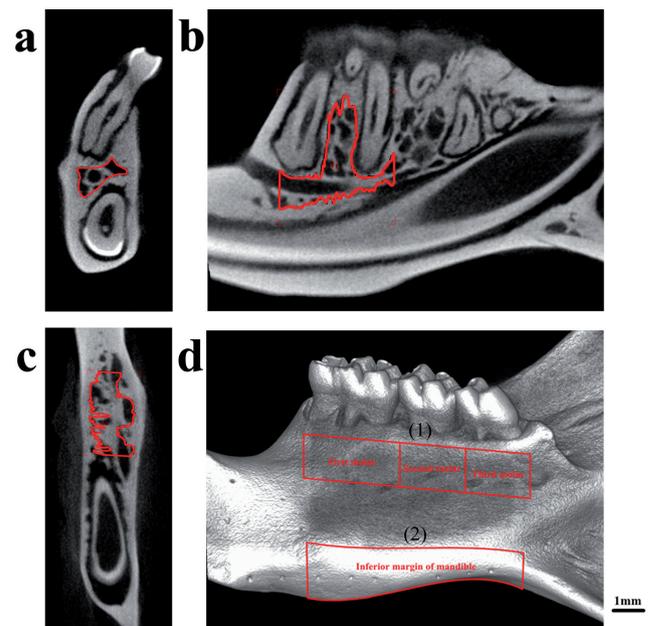
To assess bone mass in the six groups, BMD was determined by micro-CT (Fig. 2). Micro-CT analyses

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 ( $p < 0.001$



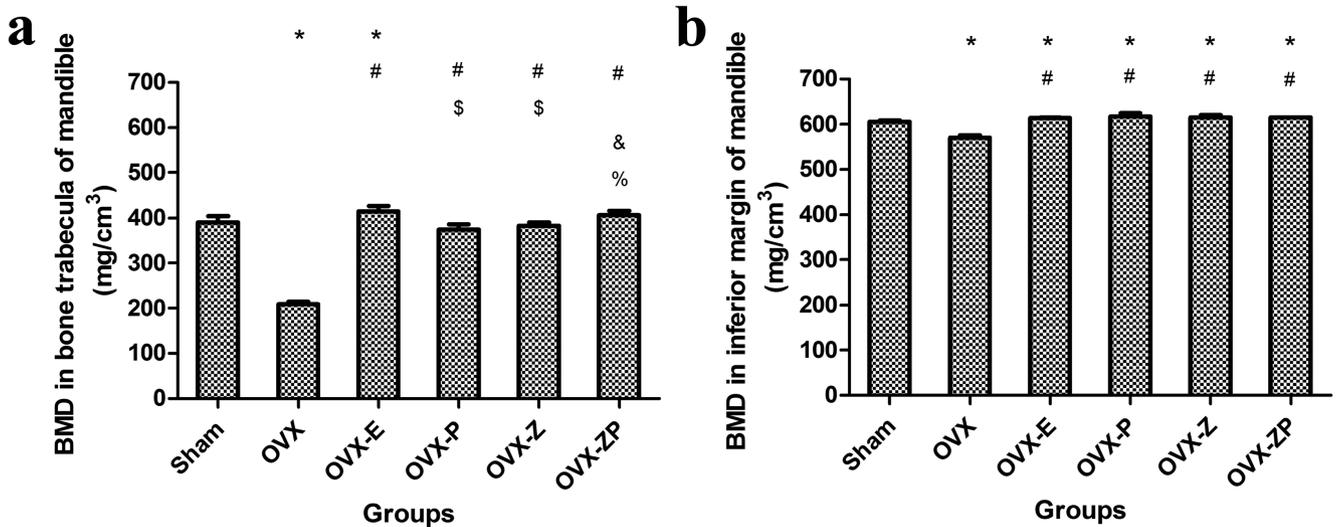
**Fig. 1.** The regions of interest (ROI) on mandible in micro-CT analysis and biomechanical testing. The black outline represents the ROI in the coronal view (a), sagittal view (b), and transverse view (c) of mandible. (d) Represents the ROI in analyses of biomechanics and micro-CT in the third-dimension. (1) The basal bone below the molars in the mandible on the tongue side, the site for biomechanical analysis and the ROI for the trabecula of the mandible in the sagittal plane; (2) the inferior margin of the mandible, another ROI for BMD in the mandible. Scale bar: 1 mm.

*Puerarin and zinc prevent mandibular bone loss*

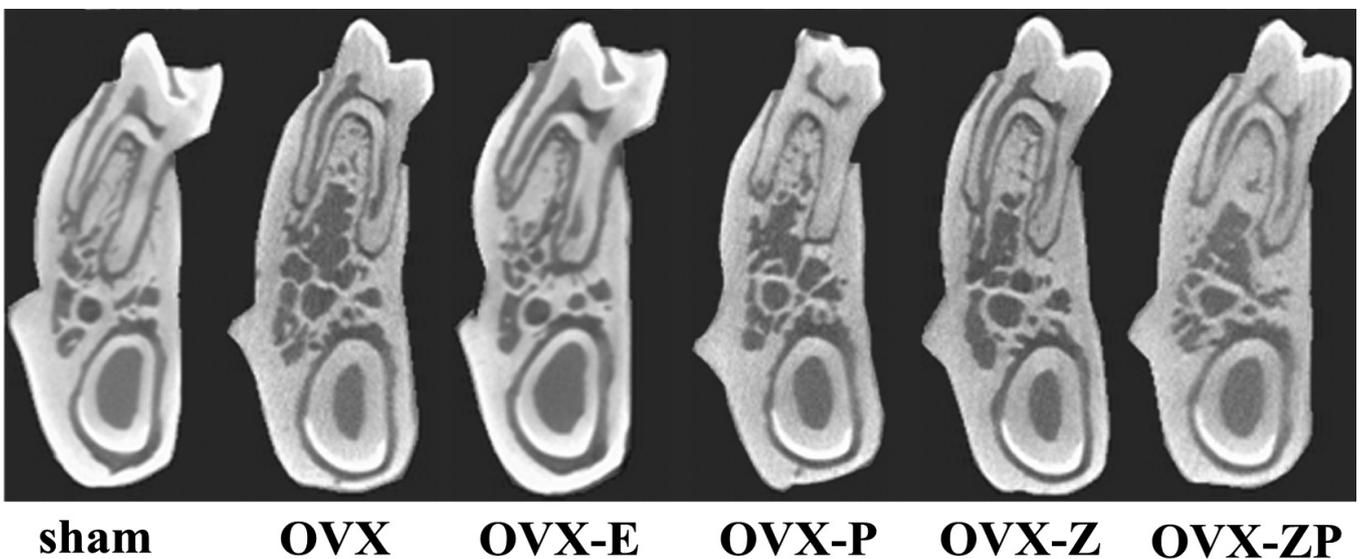
**Table 1.** Bone histomorphometry of mandibles from the six groups after 12 weeks of treatment.

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

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

## Puerarin and zinc prevent mandibular bone loss

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  $p < 0.01$  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  $p < 0.05$ ; 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.

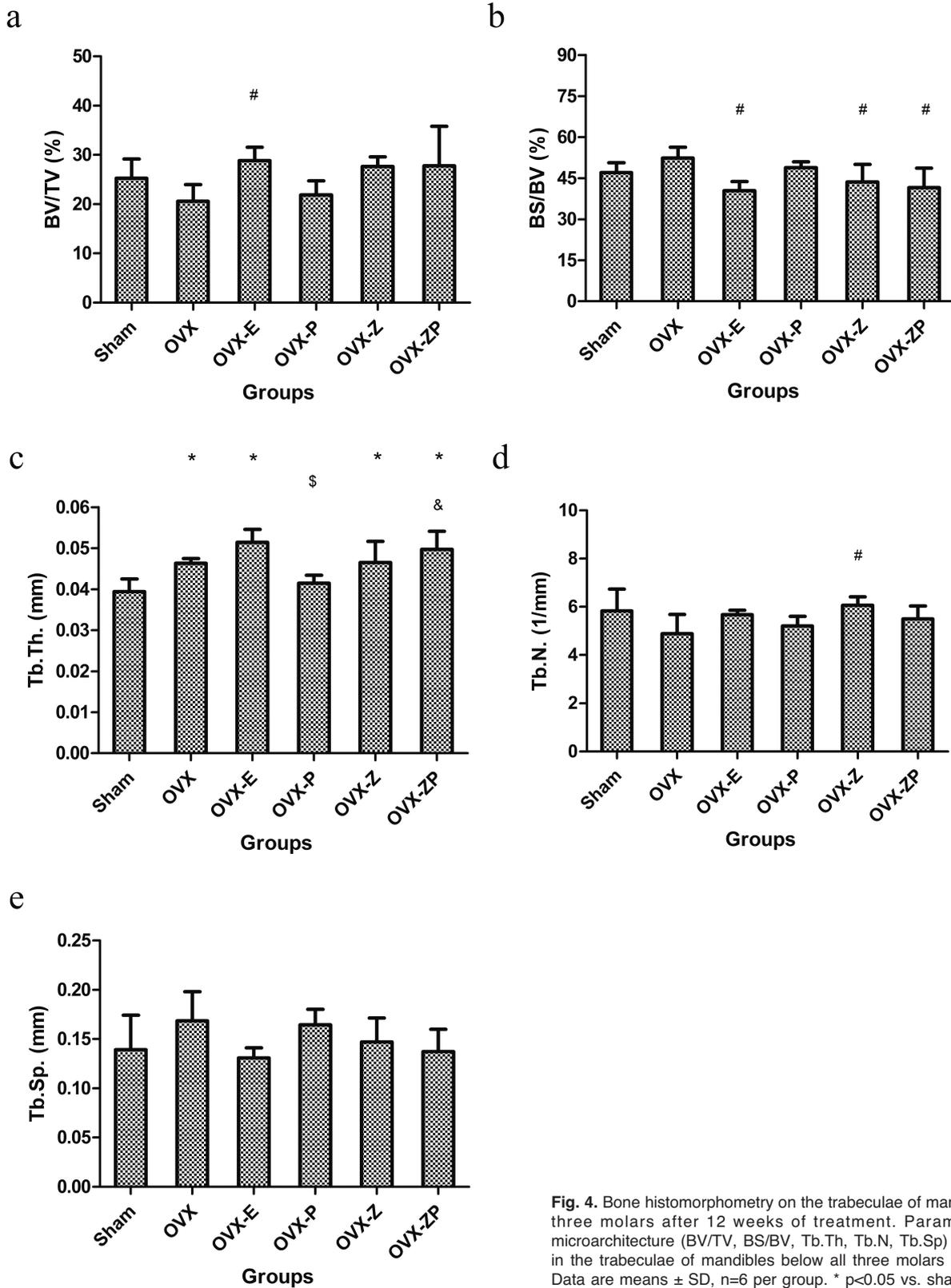
Parameter	OPN ( $\mu\text{g/L}$ )	OPG (ng/L)	Ca (mg/L)	TRAP (pg/L)	RANKL ( $\mu\text{g/L}$ )
sham	62.56 $\pm$ 1.25	1464.93 $\pm$ 150.24	54.82 $\pm$ 4.34	3353.41 $\pm$ 44.16	36.04 $\pm$ 0.64
OVX	60.04 $\pm$ 2.24 <sup>a</sup>	1239.53 $\pm$ 37.48 <sup>a</sup>	45.27 $\pm$ 6.30 <sup>a</sup>	3623.18 $\pm$ 85.28 <sup>a</sup>	42.41 $\pm$ 0.49 <sup>a</sup>
OVX-E	63.92 $\pm$ 1.70 <sup>a,b</sup>	1700.58 $\pm$ 174.43 <sup>a,b</sup>	61.05 $\pm$ 2.88 <sup>b</sup>	2816.46 $\pm$ 63.95 <sup>a,b</sup>	33.98 $\pm$ 0.80 <sup>a,b</sup>
OVX-P	67.95 $\pm$ 1.84 <sup>b,c</sup>	1447.92 $\pm$ 139.74 <sup>b,c</sup>	54.65 $\pm$ 7.15 <sup>b</sup>	3042.25 $\pm$ 103.48 <sup>a,b,c</sup>	35.13 $\pm$ 0.95 <sup>b,c</sup>
OVX-Z	67.33 $\pm$ 1.18 <sup>a,b,c</sup>	1500.86 $\pm$ 113.41 <sup>b,c</sup>	55.07 $\pm$ 5.17 <sup>b</sup>	3134.37 $\pm$ 54.38 <sup>a,b,c,d</sup>	36.52 $\pm$ 1.35 <sup>b,c,d</sup>
OVX-ZP	69.32 $\pm$ 1.29 <sup>a,b,c</sup>	1632.21 $\pm$ 146.23 <sup>a,b,d</sup>	66.17 $\pm$ 9.42 <sup>a,b,d,e</sup>	2945.35 $\pm$ 72.30 <sup>a,b,c,d,e</sup>	30.39 $\pm$ 0.60 <sup>a,b,c,d,e</sup>

Data are means  $\pm$  SD, n=6 per group. <sup>a</sup>:  $p < 0.05$  vs. sham, <sup>b</sup>:  $p < 0.05$  vs. OVX, <sup>c</sup>:  $p < 0.05$  vs. OVX-E, <sup>d</sup>:  $p < 0.05$  vs. OVX-P, <sup>e</sup>:  $p < 0.05$  vs. OVX-Z.

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

Parameter	OPN ( $\mu\text{g/L}$ )	OPG (ng/L)	Ca (mg/L)	TRAP (pg/L)	RANKL ( $\mu\text{g/L}$ )
sham	23.91 $\pm$ 0.53	486.41 $\pm$ 75.32	49.75 $\pm$ 8.16	1218.55 $\pm$ 23.94	12.66 $\pm$ 0.36
OVX	21.65 $\pm$ 0.69 <sup>a</sup>	384.97 $\pm$ 33.83 <sup>a</sup>	36.55 $\pm$ 3.58	1445.67 $\pm$ 24.99 <sup>a</sup>	16.25 $\pm$ 0.42 <sup>a</sup>
OVX-E	25.75 $\pm$ 0.39 <sup>a,b</sup>	515.93 $\pm$ 56.65 <sup>b</sup>	52.70 $\pm$ 9.51 <sup>b</sup>	1092.57 $\pm$ 29.01 <sup>a,b</sup>	11.57 $\pm$ 0.29 <sup>a,b</sup>
OVX-P	24.62 $\pm$ 0.45 <sup>a,b,c</sup>	490.67 $\pm$ 73.85 <sup>b</sup>	42.25 $\pm$ 8.37	953.19 $\pm$ 35.29 <sup>a,b,c</sup>	9.77 $\pm$ 0.27 <sup>a,b,c</sup>
OVX-Z	23.97 $\pm$ 0.48 <sup>b,c,d</sup>	467.19 $\pm$ 60.87 <sup>b,c</sup>	46.47 $\pm$ 9.76	1145.24 $\pm$ 31.77 <sup>a,b,c,d</sup>	11.45 $\pm$ 0.30 <sup>a,b,d</sup>
OVX-ZP	26.52 $\pm$ 0.33 <sup>a,b,c,d,e</sup>	581.30 $\pm$ 60.47 <sup>a,b,c,d,e</sup>	61.40 $\pm$ 13.13 <sup>b,d,e</sup>	744.13 $\pm$ 34.18 <sup>a,b,c,d,e</sup>	7.53 $\pm$ 0.28 <sup>a,b,c,d,e</sup>

Data are means  $\pm$  SD, n=6 per group. <sup>a</sup>:  $p < 0.05$  vs. sham, <sup>b</sup>:  $p < 0.05$  vs. OVX, <sup>c</sup>:  $p < 0.05$  vs. OVX-E, <sup>d</sup>:  $p < 0.05$  vs. OVX-P, <sup>e</sup>:  $p < 0.05$  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  $\pm$  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.

Puerarin and zinc prevent mandibular bone loss

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

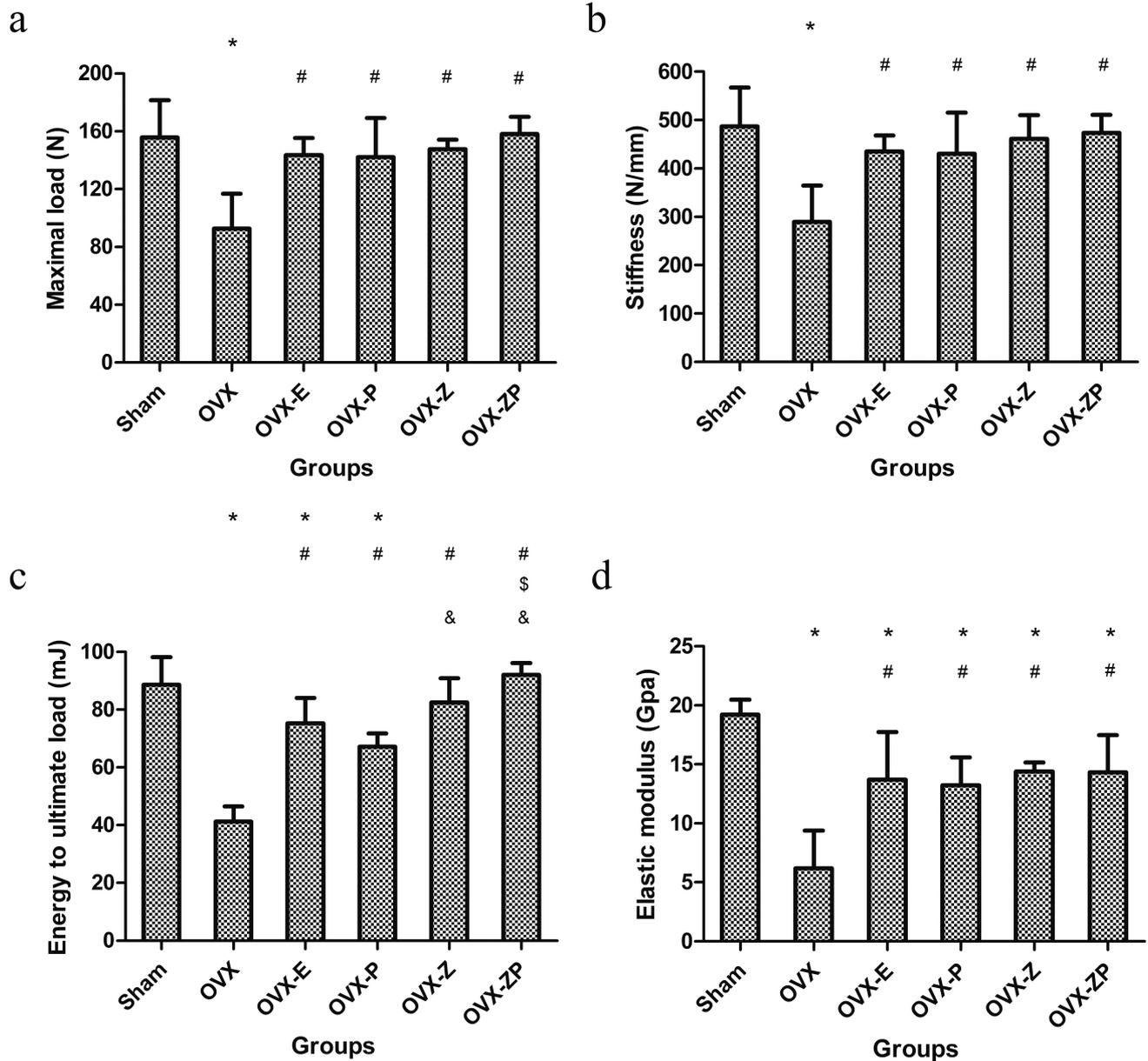


Fig. 5. Biomechanical measurements of mandibles in a three-point bending test. Parameters of maximum load (a), stiffness (b), energy to ultimate load (c), and elastic modulus (d) were measured in the basal bone of the mandible below the molars. Data are means  $\pm$  SD,  $n=6$  per group. \*  $p < 0.05$  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 well 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 (Brzóska 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 coadministrated 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

## *Puerarin and zinc prevent mandibular bone loss*

parameters of bone morphometry, and therefore improved the mechanical ability of mandible. Further experiments showed that the underlying mechanisms on mandibular antiosteoporotic 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.

### References

- Arijmandi B.H., Alekel L., Hollis B.W., Amin D., Stacewicz-Sapuntzakis M., Guo P. and Kukreja S.C. (1996). Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. *J. Nutr.* 126, 161-167.
- Bartold P.M., Cantley M.D. and Haynes D.R. (2010). Mechanisms and control of pathologic bone loss in periodontitis. *Periodontol.* 2000 53, 55-69.
- Bashutski J.D., Eber R.M., Kinney J.S., Benavides E., Maitra S., Braun T.M., Giannobile W.V. and McCauley L.K. (2010). Teriparatide and osseous regeneration in the oral cavity. *N. Engl. J. Med.* 363, 2396-2405.
- Brennan-Calanan R.M., Genco R.J., Wilding G.E., Hovey K.M., Trevisan M. and Wactawski-Wende J. (2008). Osteoporosis and oral infection: Independent risk factors for oral bone loss. *J. Dent. Res.* 87, 323-327.
- Brzoska M.M. and Rogalska J. (2013). Protective effect of zinc supplementation against cadmium-induced oxidative stress and the rank/rankl/opg system imbalance in the bone tissue of rats. *Toxicol. Appl. Pharmacol.* 272, 208-220.
- Ervin R.B. and Dye B.A. (2009). The effect of functional dentition on healthy eating index scores and nutrient intakes in a nationally representative sample of older adults. *J. Public Health Dent.* 69, 207-216.
- Gonen Z.B. and Yilmaz Asan C. (2016). Treatment of bisphosphonate-related osteonecrosis of the jaw using platelet-rich fibrin. *Cranio* 2016, 1-5.
- Graves L.L., Bukata S.V., Aghazadehsanai N., Chang T.I., Garrett N. and Friedlander A.H. (2016). Patients receiving parenteral bisphosphonates for malignant disease and having developed an atypical femoral fracture are at risk of concomitant osteonecrosis of the jaw: An evidence-based review. *J. Oral Maxillofac. Surg.* 74, 2403-2408.
- Gupta A. and March L. (2016). Treating osteoporosis. *Aust Prescr* 39, 40-46.
- Gurban C.V. and Mederle O. (2011). The opg/rankl system and zinc ions are promoters of bone remodeling by osteoblast proliferation in postmenopausal osteoporosis. *Rom. J. Morphol. Embryol.* 52, 1113-1119.
- Hsu P.Y., Tsai M.T., Wang S.P., Chen Y.J., Wu J. and Hsu J.T. (2016). Cortical bone morphological and trabecular bone microarchitectural changes in the mandible and femoral neck of ovariectomized rats. *PLoS One* 11, e0154367.
- Kaibuchi N., Iwata T., Yamato M., Okano T. and Ando T. (2016). Multipotent mesenchymal stromal cell sheet therapy for bisphosphonate-related osteonecrosis of the jaw in a rat model. *Acta Biomater.* 42, 400-410.
- Kamel Mohamed S.G., Sugiyama E., Shinoda K., Hounoki H., Taki H., Maruyama M., Miyahara T. and Kobayashi M. (2005). Interleukin-4 inhibits rankl-induced expression of nfatc1 and c-fos: A possible mechanism for downregulation of osteoclastogenesis. *Biochem. Biophys. Res. Commun.* 329, 839-845.
- Kishi S. and Yamaguchi M. (1994). Inhibitory effect of zinc compounds on osteoclast-like cell formation in mouse marrow cultures. *Biochem. Pharmacol.* 48, 1225-1230.
- Li B., Liu H. and Jia S. (2014). Puerarin enhances bone mass by promoting osteoblastogenesis and slightly lowering bone marrow adiposity in ovariectomized rats. *Biol. Pharm. Bull.* 37, 1919-1925.
- Li H., Chen B., Pang G., Chen J., Xie J. and Huang H. (2016). Anti-osteoporotic activity of puerarin 6"-o-xyloside on ovariectomized mice and its potential mechanism. *Pharm. Biol.* 54, 111-117.
- Lindsay R., Kregge J.H., Marin F., Jin L. and Stepan J.J. (2016). Teriparatide for osteoporosis: Importance of the full course. *Osteoporos. Int.* 27, 2395-2410.
- Liu Y.S., Ou M.E., Liu H., Gu M., Lv L.W., Fan C., Chen T., Zhao X.H., Jin C.Y., Zhang X., Ding Y. and Zhou Y.S. (2014). The effect of simvastatin on chemotactic capability of sdf-1alpha and the promotion of bone regeneration. *Biomaterials* 35, 4489-4498.
- Liu H., Li W., Liu Y., Zhang X. and Zhou Y. (2015a). Co-administration of aspirin and allogeneic adipose-derived stromal cells attenuates bone loss in ovariectomized rats through the anti-inflammatory and chemotactic abilities of aspirin. *Stem Cell. Res. Ther.* 6, 200.
- Liu H., Gao K., Lin H., Zhang Y. and Li B. (2015b). Relative skeletal effects in different sites of the mandible with the proximal tibia during ovariectomy and the subsequent estrogen treatment. *J. Oral Implantol.* 41 Spec No, 386-390.
- Liu H., Li W., Liu Y.S. and Zhou Y.S. (2016). Bone micro-architectural analysis of mandible and tibia in ovariectomised rats: A quantitative structural comparison between undecalcified histological sections and micro-ct. *Bone Joint Res.* 5, 253-262.
- Mavropoulos A., Rizzoli R. and Ammann P. (2007). Different responsiveness of alveolar and tibial bone to bone loss stimuli. *J. Bone Miner. Res.* 22, 403-410.
- Michihara S., Tanaka T., Uzawa Y., Moriyama T. and Kawamura Y. (2012). Puerarin exerted anti-osteoporotic action independent of estrogen receptor-mediated pathway. *J. Nutr. Sci. Vitaminol. (Tokyo)* 58, 202-209.
- North American Menopause S. (2001). The role of calcium in peri- and postmenopausal women: Consensus opinion of the north american menopause society. *Menopause* 8, 84-95.
- Otsuka M., Marunaka S., Matsuda Y., Ito A., Naito H., Ichinose N., Kokubo T., Nakamura T. and Higuchi W.I. (2003). Effect of particle size on zinc release from zinc containing tricalcium phosphate (zntcp) in zn-deficient osteoporosis rats. *Biomed. Mater. Eng.* 13, 103-113.
- Payne J.B., Reinhardt R.A., Nummikoski P.V. and Patil K.D. (1999). Longitudinal alveolar bone loss in postmenopausal osteoporotic/osteopenic women. *Osteoporos. Int.* 10, 34-40.
- Rubin J., Ackert-Bicknell C.L., Zhu L., Fan X., Murphy T.C., Nanes M.S., Marcus R., Holloway L., Beamer W.G. and Rosen C.J. (2002). IGF-I regulates osteoprotegerin (opg) and receptor activator of nuclear

- factor-kappab ligand *in vitro* and opg *in vivo*. *J. Clin. Endocrinol. Metab.* 87, 4273-4279.
- Sanderson J., Martyn-St James M., Stevens J., Goka E., Wong R., Campbell F., Selby P., Gittoes N. and Davis S. (2016). Clinical effectiveness of bisphosphonates for the prevention of fragility fractures: A systematic review and network meta-analysis. *Bone* 89, 52-58.
- Sheiham A., Steele J.G., Marceles W., Lowe C., Finch S., Bates C.J., Prentice A. and Walls A.W. (2001). The relationship among dental status, nutrient intake, and nutritional status in older people. *J. Dent. Res.* 80, 408-413.
- Sheu S.Y., Tsai C.C., Sun J.S., Chen M.H., Liu M.H. and Sun M.G. (2012). Stimulatory effect of puerarin on bone formation through co-activation of nitric oxide and bone morphogenetic protein-2/mitogen-activated protein kinases pathways in mice. *Chin. Med. J. (Engl.)* 125, 3646-3653.
- Tiyatatkulkovit W., Charoenphandhu N., Wongdee K., Thongbunchoo J., Krishnamra N. and Malaivijitnond S. (2012). Upregulation of osteoblastic differentiation marker mRNA expression in osteoblast-like umr106 cells by puerarin and phytoestrogens from *Pueraria mirifica*. *Phytomedicine* 19, 1147-1155.
- Tiyatatkulkovit W., Malaivijitnond S., Charoenphandhu N., Havill L.M., Ford A.L. and VandeBerg J.L. (2014). *Pueraria mirifica* extract and puerarin enhance proliferation and expression of alkaline phosphatase and type I collagen in primary baboon osteoblasts. *Phytomedicine* 21, 1498-1503.
- Tyrovola J.B., Spyropoulos M.N., Makou M. and Perrea D. (2008). Root resorption and the OPG/RANKL/RANK system: A mini review. *J. Oral Sci.* 50, 367-376.
- Uchiyama S. and Yamaguchi M. (2007a). Genistein and zinc synergistically enhance gene expression and mineralization in osteoblastic mc3t3-e1 cells. *Int. J. Mol. Med.* 19, 213-220.
- Uchiyama S. and Yamaguchi M. (2007b). Genistein and zinc synergistically stimulate apoptotic cell death and suppress rankl signaling-related gene expression in osteoclastic cells. *J. Cell. Biochem.* 101, 529-542.
- Wang P.P., Zhu X.F., Yang L., Liang H., Feng S.W. and Zhang R.H. (2012). Puerarin stimulates osteoblasts differentiation and bone formation through estrogen receptor, p38 mapk, and wnt/beta-catenin pathways. *J. Asian Nat. Prod. Res.* 14, 897-905.
- Wang Y., Yang C., Xie W.L., Zhao Y.W., Li Z.M., Sun W.J. and Li L.Z. (2014). Puerarin concurrently stimulates osteoprotegerin and inhibits receptor activator of nf-kappab ligand (rankl) and interleukin-6 production in human osteoblastic mg-63 cells. *Phytomedicine* 21, 1032-1036.
- Yang F., Zhang R., He F., Wang X.X., Zhao S. and Yang G. (2012). Osteoblast response to puerarin-loaded porous titanium surfaces: An *in vitro* study. *J. Biomed. Mater. Res. A.* 100, 1419-1426.
- Yang X., Zhang H., Wang J., Zhang Z. and Li C. (2015). Puerarin decreases bone loss and collagen destruction in rats with ligature-induced periodontitis. *J. Periodontol Res.* 50, 748-757.
- Yuan S.Y., Sheng T., Liu L.Q., Zhang Y.L., Liu X.M., Ma T., Zheng H., Yan Y., Ishimi Y. and Wang X.X. (2016). Puerarin prevents bone loss in ovariectomized mice and inhibits osteoclast formation *in vitro*. *Chin. J. Nat. Med.* 14, 265-269.
- Zheng J., Mao X., Ling J., He Q. and Quan J. (2014). Low serum levels of zinc, copper, and iron as risk factors for osteoporosis: A meta-analysis. *Biol. Trace Elem. Res.* 160, 15-23.

Accepted December 14, 2016