Life Sciences 166 (2016) 20–26

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Coadministration of puerarin (low dose) and zinc attenuates bone loss and suppresses bone marrow adiposity in ovariectomized rats

Hao Liu [a,b,1], Wei Li [b], Xiyuan Ge [a], Shengnan Jia [b], Binbin Li [b,*]

[a] The Core Laboratory, Peking University School and Hospital of Stomatology, Beijing 100081, China
[b] Department of Oral Pathology, Peking University School and Hospital of Stomatology, Beijing 100081, China

ARTICLE INFO

Article history:
Received 17 June 2016
Received in revised form 17 September 2016
Accepted 28 September 2016
Available online 30 September 2016

Keywords:
Puerarin
Zinc
Osteoporosis
Osteogenesis
Adipogenesis

ABSTRACT

Aims: Puerarin is a phytoestrogen that shows osteogenic effects. Meanwhile, zinc stimulates bone formation and inhibits bone resorption. The study aims to investigate the effects of coadministration of puerarin (low dose) and zinc on bone formation in ovariectomized rats.
Materials and methods: Co-administration or use alone of puerarin (low dose) and/or zinc were gavaged in OVX rats. The estrogen-like effects were detected by the uterus weight, the histologic observation and the IGF-1 protein expression. The osteogenic effects were determined by bone histomorphometric and mechanical parameters, osteogenic and adipogenic blood markers, and so on.
Key findings: The results showed that oral administration of puerarin (low dose) plus zinc didn't significantly increase uterus weight. The glandular epithelial of endometrium had no proliferation and no protein expression of IGF-1. Moreover, co-administration attenuated bone loss and biomechanical decrease more than single use of puerarin or zinc ($p < 0.05$). Next, combined administration of puerarin and zinc promoted the serological level of osteocalcin, bone marrow stromal cell (BMSC) proliferation, and the expression of alkaline phosphatase (ALP), and suppressed the serological level of adiponectin and adiposity in bone marrow (BM).
Significance: In conclusion, co-administrated puerarin (low dose) and zinc can partially reverse OVX-induced bone loss and suppress the adiposity of BM in rats, which shed light on the potential use of puerarin and zinc in the treatment of osteoporosis.

1. Introduction

Postmenopausal osteoporosis is widely recognized as a major public health problem. The prevalence of osteoporosis has increased rapidly among the elderly as life expectancy has increased in industrialized countries [1].

Current therapeutic approaches for osteoporosis are anabolic agents such as parathyroid hormone [2] (which stimulate bone formation), and antiresorptive agents such as bisphosphonates, raloxifene (which inhibit bone resorption) [3]. These therapies have been shown to increase bone mineral density (BMD) and reduce the risk of fractures, but their long-term safety and efficacy are ongoing concerns [4]. Moreover, drugs for the treatment of osteoporosis remain costly [5]. Thus, new, safe, effective, low-cost modalities are desired to ameliorate osteoporotic conditions.

Phytoestrogens have structural similarity to estrogen and can bind to estrogen receptors (ERs). Puerarin (PubChem CID: 5281807, Fig. 1a) is a phytoestrogen extracted from *Pueraria lobata* (Willd.) Ohwi, a wild creeper leguminous plant used in Chinese traditional medicine; this compound shows many important biological activities and has an isoflavone structure. *Pueraria* can be used to treat various diseases including cerebral ischemia, diabetes, and cardiovascular diseases in China [6]. Puerarin from *P. lobata* exerts antiosteoporotic effects in vitro and in vivo [7–10].

Recently, Rachon D et al. have reported that high-dose consumption of puerarin may expose women with an intact uterus to the risk of endometrial hyperplasia because of its uterotropic effect [11]. Another study also exhibited that supplementation of puerarin at doses of 150–600 mg/kg increased uterus weight in ovariectomized mature rats and immature female mice [12]. Moreover, uterotrophic and vaginal cornification assays revealed that puerarin exhibited dose-dependent estrogenic side effects [13]. Therefore, a high-dose of puerarin may raise the risk of estrogen-related cancers such as breast cancer and endometrium cancer. We speculate that low-dosage application of puerarin may down-regulate its adverse estrogenic effect, but the osteogenic activity may decrease accordingly. Therefore, we wish to assess

* Corresponding author at: Department of Oral Pathology, Peking University School and Hospital of Stomatology, 22 Zhongguancun South Avenue, Haidian District, Beijing 100081, China.
E-mail address: kqlibinbin@aliyun.com (B. Li).
[1] These authors also contributed equally to this work.

http://dx.doi.org/10.1016/j.lfs.2016.09.024
0024-3205/

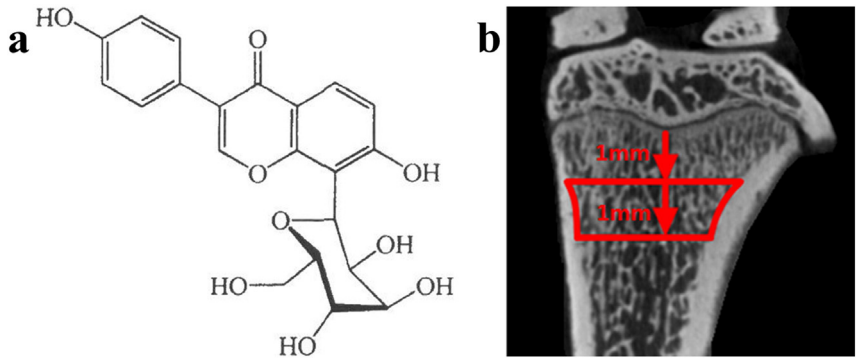

Fig. 1. The chemical structure of puerarin (a) and the regions of interest (ROI) (b) in tibias in micro-CT analysis. The outline in Fig.1b represents the ROI in the coronal direction of tibia.

how to produce greater osteogenic activity with lower adverse estrogenic effects.

Puerarin's phenol hydroxyl and carbonyl on the benzene ring (Fig. 1a) both have the ability to coordinate metal ions. Nutritional trace elements, e.g., zinc, are thought to be of particular importance in bone formation, quality and status [14]. Our previous research demonstrated that zinc enhanced bone metabolism in ovariectomized rats and exerted anabolic osteoblastic/adipocytic marrow effects ex vivo [15]. We previously showed that zinc inhibited pit formation by isolated neonatal osteoclasts in a biphasic manner [16]. Moreover, Uchiyama et al. reported that genistein (a phytoestrogen) and zinc synergistically enhance osteogenic ability and suppress receptor activator of nuclear factor kappa-B ligand (RANKL) signaling-related gene expression in vitro [17,18]. Therefore, we hypothesize that coadministration of puerarin and zinc may attenuate osteoporosis-induced bone loss in vivo.

In this study, we design experiments to evaluate the effects of coadministration of puerarin and zinc on attenuating tibial bone loss in OVX rats, and investigate the underlying mechanism of these effects ex vivo.

2. Materials and methods

2.1. Reagents

α-Modified Eagle's medium (αMEM), fetal bovine serum (FBS) and penicillin-streptomycin (5000 U/mL penicillin; 5000 μg/mL streptomycin) were purchased from GIBCO Laboratories (Grand Island, NY, USA). Puerarin, $ZnSO_4$, estradiol, 3-[4,5-dimetylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Oil-red O, β-sodium glycerophosphate, ascorbic acid, insulin, and dexamethasone were purchased from Sigma–Aldrich (St. Louis, MO, USA). Insulin-like growth factor 1 (IGF-1) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animals and administration procedure

All animal experiments were approved by the Animal Care and Use Committee of Peking University Health Science Center (approval number: LA2014213; Beijing, China).

Eight-week-old female Sprague Dawley rats (n = 90) were obtained from Vital River Inc. (Beijing, China) and assigned randomly into six groups of fifteen: (1) sham: sham surgery; (2) OVX: bilaterally ovariectomized, negative control; (3) OVX-E: OVX rats were injected i.p. with 10 μg/kg body weight (BW) estradiol (dissolved with oil) once every 2 days after ovariectomization, positive control; (4) OVX-P: puerarin (dissolved with normal saline) was orally administrated once every 2 days at 50 mg/kg BW after ovariectomization; (5) OVX-Z: $ZnSO_4$ (dissolved with normal saline) was orally administrated once every 2 days at 0.25 mg/kg BW after ovariectomization; (6) OVX-ZP: puerarin (50 mg/kg BW) and $ZnSO_4$ (0.25 mg/kg BW) in the normal saline were orally administrated once every 2 days after ovariectomization. The sham and OVX groups were treated with normal saline vehicle. Bilateral ovariectomy or sham operation was undertaken by standard methods. The above mentioned treatment/vehicle was performed at one week post-surgery. After 12 weeks of treatment, the rats were anesthetized and exsanguinated before euthanasia. Serum was separated and stored at −80 °C until analysis. The uteruses of rats were separated for weighing and histological analysis. The bilateral tibias from each animal were thoroughly dissected free from soft tissue following euthanization, frozen at −80 °C, and later used for micro computed tomography (micro-CT) and biomechanical analyses.

2.3. Immunohistochemistry for uterine IGF-1 in rats

Uterotropic effects of estrogens are part due to the activation of the uterine IGF-1 expression, thus uterine IGF-1 was detected by immunohistochemistry as previously described [19,20]. In brief, the uterus were dehydrated and subsequently embedded in paraffin. The 5 μm thickness sections were dewaxed and subsequently dehydrated. After antigen retrieval through boiling in citrate buffer (pH 6.0), sections were incubated with anti-IGF-1 monoclonal antibody at 4 °C overnight. Next, the sections were incubated with second antibody about 30 min at 37 °C. At last, immunoreactivity was visualized by diaminobenzidine and the sections were counterstained through hematoxylin. Purified IgG was used as a negative control.

2.4. Bone microtomographic histomorphometry evaluated by micro-CT

Micro-CT was undertaken by Inveon MM system (Siemens, Munich, Germany) as previously described [21]. Briefly, images were acquired at an effective pixel size of 8.82 μm, voltage 80 kV, current 500 μA, and exposure time of 1500 ms in each of the 360 rotational steps. Parameters were calculated using an Inveon Research Workplace (Siemens, Munich, Germany), as follows: bone volume/total volume (BV/TV) refers to the total amount of bone region present in relation to the analyzed bone volume; bone surface area/bone volume (BS/BV) is the relation between the overall trabecular bone surface and the bone volume analyzed of mineralized bone; trabecular thickness (Tb.Th) is figured out by means of a technique that fills with spheres the structure analyzed and by the transformation of the distance it is calculated the mean thickness of the osseous structures; trabecular number (Tb.N) implies the number of times that trabeculae are crossed by means of length in a randomly selected way across the bone volume analyzed; trabecular separation (Tb.Sp) represents the average diameter of all non-overlapping spheres that fit inside the marrow space [22]. For tibia, the ROI was 1–2 mm distal to the proximal epiphysis as previously described (Fig. 1b) [23].

2.5. Biomechanical testing

Following the micro-CT measurements, the left tibias were subjected to three-point bend tests. The tests proceeded at a plunger speed of 1.0 mm/min using a servohydraulic testing device (Instron 4302, Instron, Norwood, Mass), as previously described [23]. Load-deformation curves were recorded during the bending process. The analysis area of the tibia was 3 mm distal to the proximal epiphysis. The parameters were calculated as follows: The maximum load, stiffness, energy to ultimate load, and elastic modulus.

2.6. Bone marrow stromal cell (BMSC) isolation, and osteogenic and adipogenic differentiation

To isolate and culture BMSCs, bone marrow (BM) cells from the femur were obtained as described previously [24]. Briefly, the tips of bone were removed with a rongeur, and the marrow was harvested by inserting a syringe needle (27-gauge) into one end of the bone and flushing with 1 mL αMEM. Cells were cultured in maintenance medium (αMEM containing 10% FBS, 100 U/mL penicillin G and 100 mg/mL streptomycin) at 5×10^5 cells/mL in 24-well plates (0.5 mL/well) at 37 °C. After two days, the culture medium was replaced with new αMEM supplemented separately with 10 mM β-sodium glycerophosphate + 0.05 M ascorbic acid + 10 nM dexamethasone (for osteogenic differentiation), or 10 μg/mL insulin + 10^{-7} M dexamethasone (for adipogenic differentiation). Culture supernatants from BMSCs cultured for 7 days were collected and stored at − 80 °C until analysis.

2.7. MTT assays and Oil-red O staining

To examine cell proliferation, the MTT assay was performed. Briefly, BMSCs cells were cultured to the logarithmic phase and thereafter seeded in 96-well plates (2×10^4 cells per well) for 24 h. Then, the medium was removed and 20 μL MTT solution (500 μg/mL) was added to each well, followed by 4 h incubation. MTT solution was replaced by 150 μL dimethyl sulfoxide to dissolve blue formazan crystals, and absorbance was measured at 570 nm using a microplate reader.

Adipocyte differentiation was determined by Oil-red O staining as previously described [10]. In brief, the BMSCs subject to adipogenic differentiation were cultured in 96-well plates, fixed in formalin and washed. Next, cells were stained with 0.6% Oil-Red O solution for 30 min. After removing the staining solution, the dye retained in the cells was eluted into isopropanol just before measurement of the optical density at 490 nm.

2.8. Assay of biochemical markers in serum and culture supernatant

Serum levels of osteocalcin (OC) and adiponectin, alkaline phosphatase (ALP) in culture supernatant were measured using relevant ELISA kits (eBioscience, San Diego, CA, USA). Samples were measured at least in duplicate.

2.9. Statistical analyses

Data are shown as the means ± standard deviation. SPSS v16.0 (IBM, Armonk, NY, USA) was used for statistical analyses. One-way ANOVA with post hoc LSD was performed, and p-values < 0.05 were considered significant.

3. Results

3.1. The evaluation of uterine wet weight and IGF-1 expression among 6 groups

To evaluate whether low dose of puerarin was safe, the uterine wet weight were calculated (Fig. 2a). The uterine wet weight of OVX group decreased significantly than that of sham group ($p < 0.05$). However, the OVX-E group had a highest uterine wet weight compared with the other group ($p < 0.01$, respectively). Therefore, it was successful to perform the OVX model in rats. Notedly, there was no significant difference on uterine wet weight among OVX, OVX-P, OVX-Z, and OVX-ZP groups, which means that uterine wet weight didn't significant increase when low dose of puerarin was administrated.

To further explore the safety of puerarin, the expression of uterine IGF-1 were performed by the immunohistochemistry. There was no positive staining of IGF-1 in the glandular epithelial of endometrium. From the point of the morphological change, the epithelial appeared flat in OVX group, while the normal epithelial in Sham group showed tall-columnar in shape. The cells presented short-cylindrical features after the estrogen administration. The epithelial in the uterus of OVX-P and OVX-Z group were both cuboidal. After the co-administration of puerarin and Zn, the epithelial cells returned back to be flat (Fig. 2b).

3.2. The effect of puerarin and zinc on bone microarchitecture in tibia

To assess bone microarchitecture in the six groups, bone morphometry were performed by micro-CT (Figs. 3 and 4). Micro-CT analyses of the proximal tibia demonstrated that the OVX group exhibited significantly less BV/TV than the sham group (OVX group $p < 0.001$), which demonstrated the utility of the rat OVX model. Besides, the OVX-ZP group exhibited a significant difference in tibia microarchitecture compared with the OVX group (increase: BV/TV and Tb.Th $p < 0.01$, Tb.N $p < 0.05$; decrease: BS/BV and Tb.Sp $p < 0.001$). Of note, the OVX-P group had a dramatically decreased BV/TV and a significantly increased Tb.Sp compared with the OVX-ZP group (BV/TV $p < 0.05$; Tb.Sp $p < 0.001$). However, the BV/TV in OVX-Z group was lower than that of OVX-ZP group ($p < 0.05$).

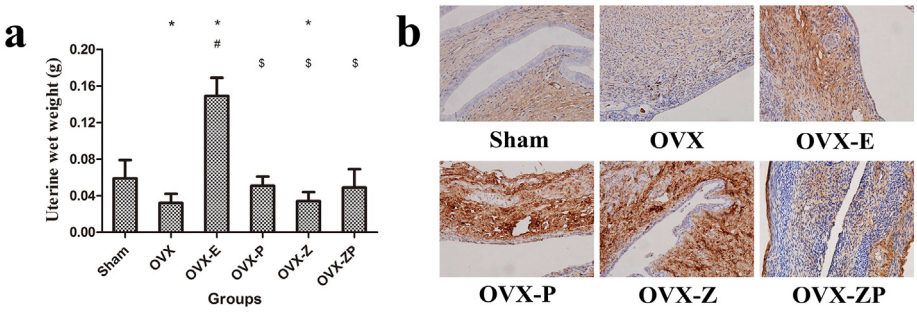

Fig. 2. The wet weight (a) and histologic characteristics (b) of the uterus among 6 groups. (b) The IGF-1 expression in the glandular epithelial of endometrium was detected by IHC. The cells were tall-columnar (Sham), flat (OVX), low-columnar (OVX-P), low-columnar (OVX-Z) and flat (OVX-ZP), comprehensively (40×). Data are means ± SD, n = 15 per group. *$p < 0.05$ vs. sham, #$p < 0.05$ vs. OVX, $$p < 0.05$ vs. OVX-E.

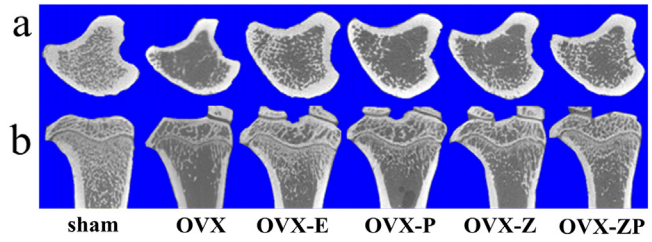

Fig. 3. Representative images of changes in bone microarchitecture of tibias in rats. (a) The bone microarchitecture of tibias of the six treatment groups in the transverse plane; (b) the bone microarchitecture of tibias in the coronal plane.

3.3. Mechanical tests of tibia

To evaluate mechanical effects, three-point bend tests were performed in tibias (Fig. 5). In detail, the OVX-ZP group had a significantly increased maximum load, stiffness, energy to ultimate load, and elastic modulus compared with the OVX group (maximum load and stiffness $p < 0.001$; energy to ultimate load $p < 0.01$; elastic modulus $p < 0.05$). Moreover, the maximum load of the OVX-ZP group increased markedly compared with the OVX-P and OVX-Z groups (OVX-P group $p < 0.01$, OVX-Z group $p < 0.05$). The OVX-ZP group showed a significant increase in stiffness compared with the OVX-Z group ($p < 0.05$).

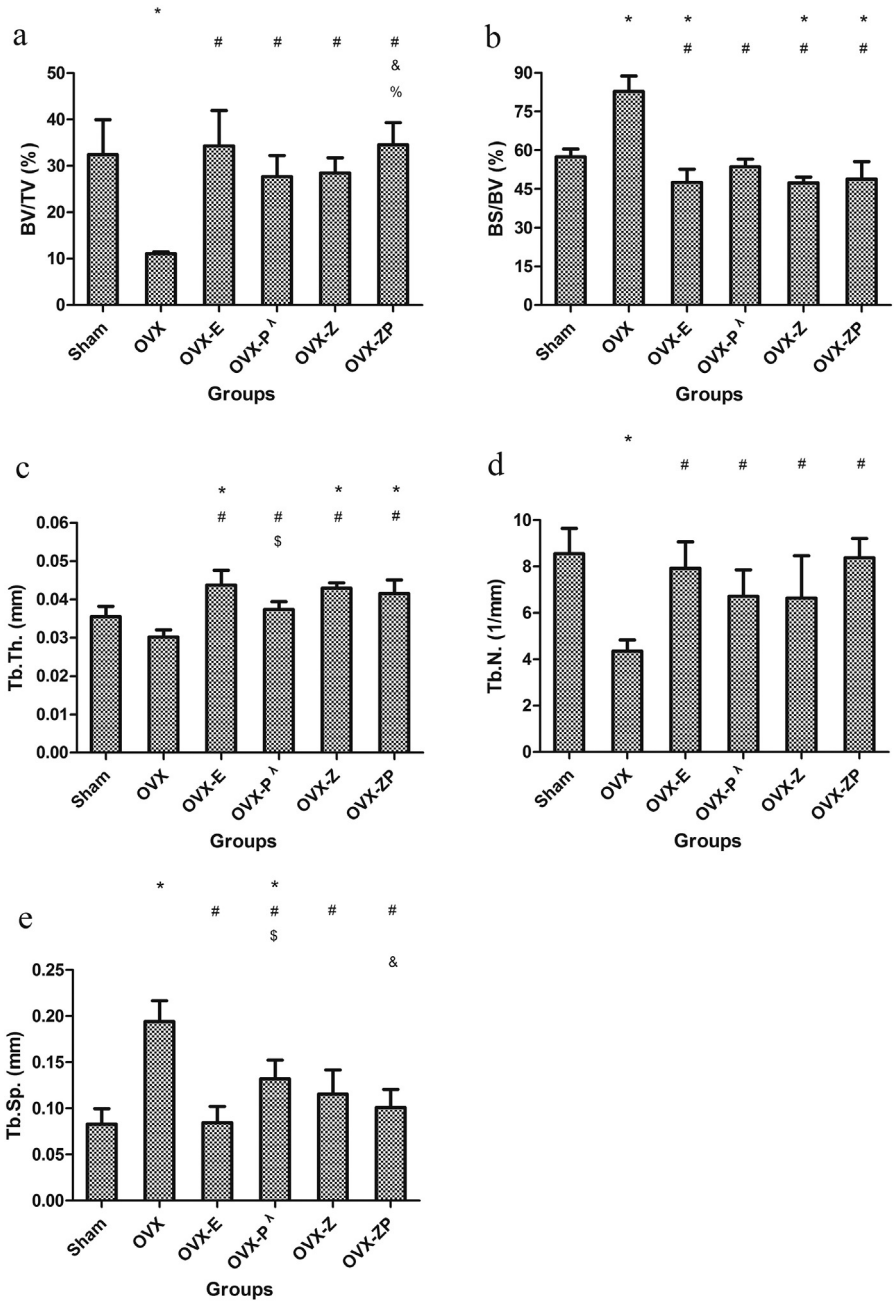

Fig. 4. Bone histomorphometry of tibias from the six groups after 12 weeks of treatment. The parameters of bone microarchitecture (BV/TV, BS/BV, Tb.Th, Tb.N, Tb.Sp) were measured in the trabecular bone of the proximal tibia (1–2 mm distal to the proximal epiphysis) using micro-CT. Data are means ± SD, n = 15 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. λ: the data were cited from our published article [10].

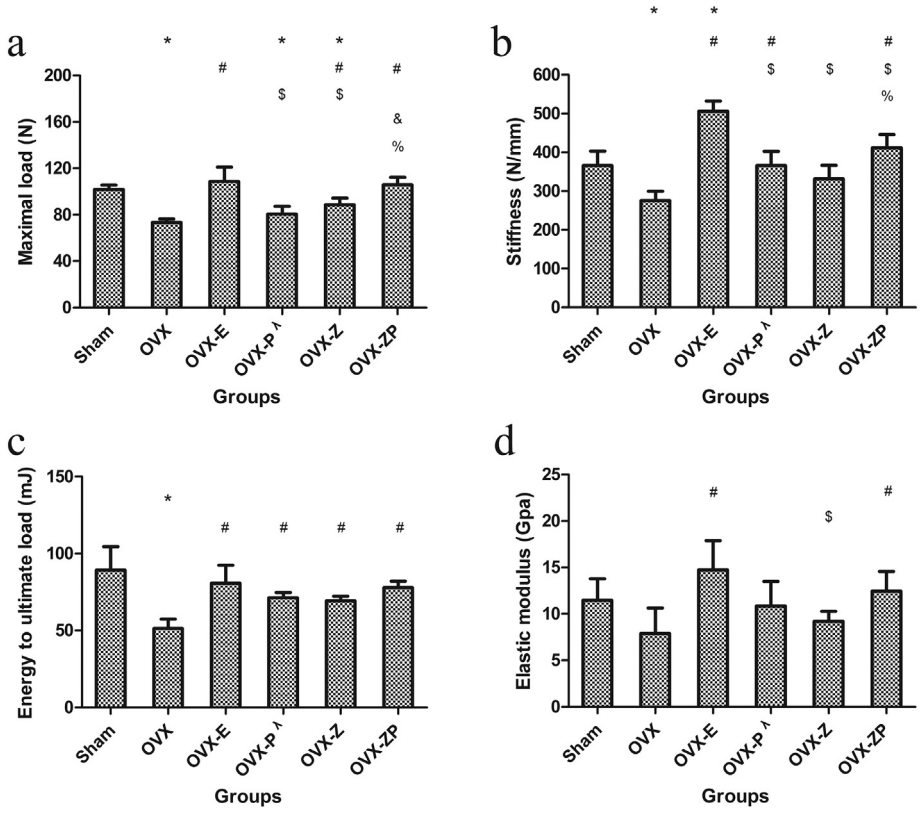

Fig. 5. Biomechanical measurements of tibias in a three-point bending test. Parameters of maximum load (a), stiffness (b), energy to ultimate load (c), and elastic modulus (d) were measured in the tibias 3 mm distal to the proximal epiphysis. Data are means ± SD, n = 15 per group. $^{*}p < 0.05$ vs. sham, $^{\#}p < 0.05$ vs. OVX, $^{\$}p < 0.05$ vs. OVX-E, $^{\&}p < 0.05$ vs. OVX-P, $^{\%}p < 0.05$ vs. OVX-Z. λ: the data were cited from our published article [10].

3.4. Serological assessment of osteocalcin and adiponectin

Levels of osteocalcin, a marker of bone formation, were significantly higher in the OVX-P, OVX-Z, and OVX-ZP groups than in the sham, OVX, and OVX-E groups ($p < 0.001$, respectively) (Fig. 6a). However, serum levels of adiponectin in the OVX, OVX-P, OVX-Z, and OVX-ZP groups were significantly lower than those in the sham group (OVX, OVX-P, and OVX-Z group, $p < 0.05$, OVX-ZP group, $p < 0.001$) (Fig. 6b). Moreover, adiponectin levels in the OVX-ZP group notably decreased compared with the OVX, OVX-E, and OVX-Z groups (OVX and OVX-Z group $p < 0.05$, OVX-E group $p < 0.01$) (Fig. 6b).

3.5. Effects of puerarin and zinc on proliferation and differentiation of BMSCs ex vivo

To evaluate the effects of BMSC proliferation in the six groups, MTT assays were performed (Fig. 6c). All the OVX groups had a lower rate on cell proliferation than the sham group except the OVX-ZP group (OVX, OVX-P, and OVX-Z groups $p < 0.001$, OVX-E group $p < 0.01$). Moreover, the OVX group showed significantly decreased cell proliferation compared with the other OVX groups (OVX-E and OVX-ZP groups $p < 0.001$, OVX-P and OVX-Z group $p < 0.01$). Of note, BMSC proliferation in the OVX-ZP group was higher than that in the OVX-E, OVX-P and OVX-Z groups (OVX-E $p < 0.01$, OVX-P and OVX-Z group $p < 0.001$).

To assess effects on osteogenesis and adipogenesis, culture supernatant levels of ALP were evaluated to measure osteogenesis and Oil-red O staining was performed to assess adipogenesis. The ALP in supernatant of the OVX group was lower than that in the other OVX groups (OVX-E, OVX-P, OVX-Z, and OVX-ZP groups) ($p < 0.001$, respectively). Notably, the OVX-ZP group showed a significant increase in ALP compared with the OVX-E and OVX-P groups (OVX-E $p < 0.01$, OVX-P $p < 0.05$) (Fig. 6d). Oil-red O staining of the OVX, OVX-E, and OVX-P groups resulted in a higher OD value compared with the sham group ($p < 0.001$, respectively). OVX-Z and OVX-ZP groups showed a significant decrease compared with the OVX and OVX-E groups ($p < 0.001$). Moreover, the OVX-Z and OVX-ZP groups had markedly decreased OD values compared with the OVX-P group ($p < 0.001$) (Fig. 6e).

4. Discussion

In the current study, we demonstrate for the first time that coadministration of puerarin at a low dose and zinc attenuates bone loss and suppresses bone marrow adiposity in ovariectomized rats (a classical animal model mimicked postmenopausal osteoporosis). The results also show that the combined administration was more advantageous in improving osteogenesis and suppressing adipogenesis than single treatment with either zinc or puerarin.

To fully explore the underlying mechanism of this coadministration, a series of in vivo and ex vivo experiments were performed. Coadministration of puerarin (low dose) and zinc promoted BMSC proliferation, the expression of ALP, and serological levels of osteocalcin. Moreover, combined administration also suppressed serological levels of adiponectin and adiposity in bone marrow.

Several recent lines of evidence support the hypothesis that puerarin enhances osteoblastic differentiation by multiple mechanisms in vitro [25–27]. Moreover, both our previous research and that of Tanaka, T. et al. showed that puerarin (*Pueraria lobata* vine ethanol extracts) enhanced bone mass by promoting osteoblastogenesis in vivo [10,28]. Our findings in this study further confirmed above conclusions through bone morphometry, biomechanics, and osteogenic mechanisms systematically. However, Cho et al. have showed that low dose puerarin (50 mg/kg) administrated to OVX rats had a weaker osteogenic effect than high-dose puerarin (500 mg/kg) [29]. But, high-dose puerarin

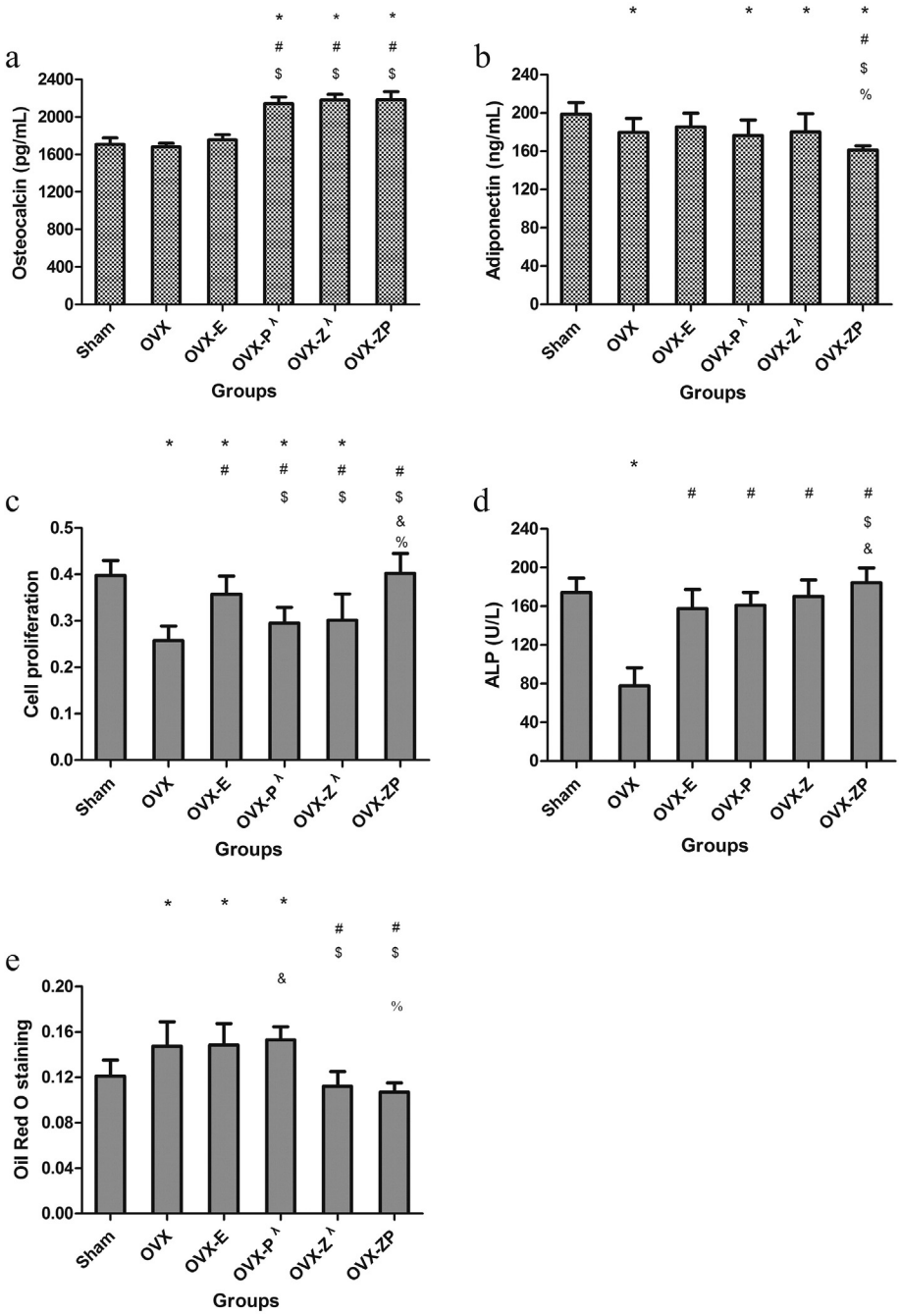

Fig. 6. The evaluation on mechanisms of osteogenesis and adipogenesis. Serum levels of osteocalcin (a marker of osteogenic differentiation) (a) and adiponectin (a marker of adipogenic differentiation) (b) were detected after 12 weeks of treatment. BMSC proliferation was evaluated by MTT assay (c). Supernatant levels of ALP (a marker of bone formation) during osteogenic differentiation of BMSCs (d). Oil-red O staining was evaluated after adipogenic differentiation of BMSCs (e). Data are means ± SD from experiments carried out in triplicate, n = 15 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. λ: the data were cited from our published article [10,15].

enhanced the risk of endometrial hyperplasia due to its uterotropic effect [11], which it was conflicted. So, to use puerarin, one must balance the beneficial osteogenic effects with the adverse effects.

Zinc is a trace element that plays essential roles in multiple metabolic pathways. A growing body of evidence showed that zinc supplementation improved bone formation and stimulated collagen synthesis and mineralization [30,31]. Our experiments suggested that zinc had an excellent effect in attenuating bone loss in OVX rats, which was consistent with most other research.

Uchiyama et al. discovered that genistein (another phytoestrogen) and zinc synergistically enhanced osteogenic ability through increasing the expression of ALP, osteocalcin and α1 collagen in vitro [17]. Like the study of Uchiyama et al., our research also exhibited synergistic osteogenic effects between puerarin and zinc were enhanced by improving the expression of ALP and serological levels of osteocalcin ex vivo. Moreover, we further showed that co-administration showed more excellent biomechanical ability compared with use alone, which was of great significance to osteoporotic prevention or treatment.

Surprisingly, the BW and uterus wet weight had no difference among OVX-P, OVX-Z, and OVX-ZP, which mean that low dose of puerarin didn't have a malignant effect on uterus. Obviously, co-administration of puerarin (low dose) and zinc balance the beneficial

osteogenic effects with the adverse effects (uterus weight increase) excellently, which had more advantage in osteoporotic treatment compare with high dose of puerarin use only [29].

However, in our work, the serological levels of osteocalcin were not significantly different among the OVX-P, OVX-Z, and OVX-ZP groups, so we speculated that the osteogenic advantage of combined puerarin and zinc administration may not merely be due to the expression of osteocalcin. Alternatively, we concluded that the combined administration resulted in better osteogenesis due to increased BMSC proliferation and expression of ALP in BMSCs, which was consistent with the research of Wang PP et al. [7]. However, further investigations should be performed.

Considering adiposity in the bone marrow, our results showed that the OVX-ZP group had decreased serological levels of adiponectin and quantities of lipid droplets from adipocytes in bone marrow. Of note, co-administrated puerarin and zinc suppressed the serological level of adiponectin more effectively than the other five groups, which is an important improvement compared with estrogen treatment of osteoporosis. Puerarin administration alone decreased the levels of adiponectin in serum slightly, which was consistent with our previous study [10]. However, the quantities of lipid droplets from the adipocytes in bone marrow were significantly decreased in the OVX-Z group, which was same as the conclusions of Wang et al. [32]. We speculate that other mechanisms that we did not detect in this study might be involved in the suppression of adiposity induced by zinc in osteoporosis; further investigations are necessary.

5. Conclusions

In this study, we demonstrate that coadministration of puerarin (low dose) and zinc attenuated bone loss and biomechanical decrease in tibia, which was more effectively compared with alternative drug treatment in OVX rats. BMSC proliferation and the expressions of ALP and osteocalcin play a key part in bone regeneration. Moreover, combined administration suppressed adiposity in bone marrow by decreasing serological levels of adiponectin. The results of our study shed light on the potential use of puerarin and zinc in the osteoporotic treatment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank Dr. Hong Lin for her help with the biomechanical testing. This work was supported by the National Natural Science Foundation of China [grant numbers: 30901671].

References

[1] R. Burge, B. Dawson-Hughes, D.H. Solomon, J.B. Wong, A. King, A. Tosteson, Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025, J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res. 22 (2007) 465–475.
[2] D.C. Bauer, Review: human parathyroid hormone reduces fractures and increases bone mineral density in severe osteoporosis, ACP J. Club 145 (2006) 71.
[3] E.F. Eriksen, A. Diez-Perez, S. Boonen, Update on long-term treatment with bisphosphonates for postmenopausal osteoporosis: a systematic review, Bone 58 (2014) 126–135.
[4] P.D. Miller, E.F. McCarthy, Bisphosphonate-associated atypical sub-trochanteric femur fractures: paired bone biopsy quantitative histomorphometry before and after teriparatide administration, Semin. Arthritis Rheum. 44 (2015) 477–482.
[5] W. Max, P. Sinnot, C. Kao, H.Y. Sung, D.P. Rice, The burden of osteoporosis in California, 1998, Osteoporosis international: a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 13 (2002) 493–500.
[6] L. Bao, Y. Zhang, G. Wei, Y. Wang, R. Ma, R. Cheng, et al., The Anti-atherosclerotic Effects of Puerarin on Induced-atherosclerosis in Rabbits, 159, Biomedical Papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia, 2015 53–59.
[7] P.P. Wang, X.F. Zhu, L. Yang, H. Liang, S.W. Feng, R.H. Zhang, Puerarin stimulates osteoblasts differentiation and bone formation through estrogen receptor, p38 MAPK, and Wnt/beta-catenin pathways, J. Asian Nat. Prod. Res. 14 (2012) 897–905.
[8] F. Yang, R. Zhang, F. He, X.X. Wang, S. Zhao, G. Yang, Osteoblast response to puerarin-loaded porous titanium surfaces: an in vitro study, J. Biomed. Mater. Res. A 100 (2012) 1419–1426.
[9] S.Y. Sheu, C.C. Tsai, J.S. Sun, M.H. Chen, M.H. Liu, M.G. Sun, 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. 125 (2012) 3646–3653.
[10] B. Li, H. Liu, S. Jia, Puerarin enhances bone mass by promoting osteoblastogenesis and slightly lowering bone marrow adiposity in ovariectomized rats, Biol. Pharm. Bull. 37 (2014) 1919–1925.
[11] D. Rachon, T. Vortherms, D. Seidlova-Wuttke, W. Wuttke, Dietary daidzein and puerarin do not affect pituitary LH expression but exert uterotropic effects in ovariectomized rats, Maturitas 57 (2007) 161–170.
[12] G. Zheng, X. Zhang, J. Zheng, Q. Meng, D. Zheng, Estrogen-like effects of puerarin and total isoflavones from *Pueraria lobata*, Zhong Yao Cai 25 (2002) 566–568.
[13] W. Cherdshewasart, S. Sriwatcharakul, S. Malaivijitnond, Variance of estrogenic activity of the phytoestrogen-rich plant, Maturitas 61 (2008) 350–357.
[14] J. Zheng, X. Mao, J. Ling, Q. He, J. Quan, Low serum levels of zinc, copper, and iron as risk factors for osteoporosis: a meta-analysis, Biol. Trace Elem. Res. 160 (2014) 15–23.
[15] B. Li, H. Liu, S. Jia, Zinc enhances bone metabolism in ovariectomized rats and exerts anabolic osteoblastic/adipocytic marrow effects ex vivo, Biol. Trace Elem. Res. 163 (2015) 202–207.
[16] B.B. Li, S.F. Yu, Effect of extracellular zinc on osteoclastic resorption in dental mineralized tissues, J. Peking Univ. Health Sci. 38 (2006) 644–647.
[17] S. Uchiyama, M. Yamaguchi, Genistein and zinc synergistically enhance gene expression and mineralization in osteoblastic MC3T3-E1 cells, Int. J. Mol. Med. 19 (2007) 213–220.
[18] S. Uchiyama, M. Yamaguchi, Genistein and zinc synergistically stimulate apoptotic cell death and suppress RANKL signaling-related gene expression in osteoclastic cells, J. Cell. Biochem. 101 (2007) 529–542.
[19] I. Bruchim, R. Sarfstein, H. Werner, The IGF hormonal network in endometrial cancer: functions, regulation, and targeting approaches, Front. Endocrinol. 5 (2014) 76.
[20] O.O. Adesanya, J. Zhou, C. Samathanam, L. Powell-Braxton, C.A. Bondy, Insulin-like growth factor 1 is required for G2 progression in the estradiol-induced mitotic cycle, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 3287–3291.
[21] H. Liu, W. Li, Y. Liu, X. Zhang, Y. Zhou, 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 (2015) 200.
[22] M.L. Bouxsein, S.K. Boyd, B.A. Christiansen, R.E. Guldberg, K.J. Jepsen, R. Muller, Guidelines for assessment of bone microstructure in rodents using micro-computed tomography, J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res. 25 (2010) 1468–1486.
[23] H. Liu, K. Gao, H. Lin, Y. Zhang, B. Li, Relative skeletal effects in different sites of the mandible with the proximal tibia during ovariectomy and the subsequent estrogen treatment, J. Oral Implantol. 41 (2015) 386–390 Spec No.
[24] Y.S. Liu, M.E. Ou, H. Liu, M. Gu, L.W. Lv, C. Fan, et al., The effect of simvastatin on chemotactic capability of SDF-1alpha and the promotion of bone regeneration, Biomaterials 35 (2014) 4489–4498.
[25] D. Yu, S. Mu, D. Zhao, G. Wang, Z. Chen, H. Ren, et al., Puerarin attenuates glucocorticoid-induced apoptosis of hFOB1.19 cells through the JNK- and Akt-mediated mitochondrial apoptotic pathways, Int. J. Mol. Med. 36 (2015) 345–354.
[26] H. Lv, T. Che, X. Tang, L. Liu, J. Cheng, Puerarin enhances proliferation and osteoblastic differentiation of human bone marrow stromal cells via a nitric oxide/cyclic guanosine monophosphate signaling pathway, Mol. Med. Rep. 12 (2015) 2283–2290.
[27] Y. Wang, C. Yang, W.L. Xie, Y.W. Zhao, Z.M. Li, W.J. Sun, et al., 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 (2014) 1032–1036.
[28] T. Tanaka, H. Tang, F. Yu, S. Michihara, Y. Uzawa, N. Zaima, et al., Kudzu (*Pueraria lobata*) vine ethanol extracts improve ovariectomy-induced bone loss in female mice, J. Agric. Food Chem. 59 (2011) 13230–13237.
[29] H.J. Cho, H.J. Jun, J.H. Lee, Y. Jia, M.H. Hoang, J.H. Shim, et al., Acute effect of high-dose isoflavones from *Pueraria lobata* (Willd.) Ohwi on lipid and bone metabolism in ovariectomized mice, Phytother. Res. 26 (2012) 1864–1871.
[30] P. Bhardwaj, D.V. Rai, M.L. Garg, Zinc as a nutritional approach to bone loss prevention in an ovariectomized rat model, Menopause 20 (2013) 1184–1193.
[31] E. Gonzalez-Reimers, M.C. Duran-Castellon, R. Martin-Olivera, A. Lopez-Lirola, F. Santolaria-Fernandez, M.J. De la Vega-Prieto, et al., Effect of zinc supplementation on ethanol-mediated bone alterations, Food Chem. Toxicol. 43 (2005) 1497–1505.
[32] T. Wang, J.C. Zhang, Y. Chen, P.G. Xiao, M.S. Yang, Effect of zinc ion on the osteogenic and adipogenic differentiation of mouse primary bone marrow stromal cells and the adipocytic trans-differentiation of mouse primary osteoblasts, J. Trace Elem. Med. Biol. 21 (2007) 84–91.