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ORIGINAL ARTICLE

Osthole Promote Differentiation and Inhibit Proliferation of Osteoblast by Activating Wnt Signaling and Endoplasmic Reticulum Stress

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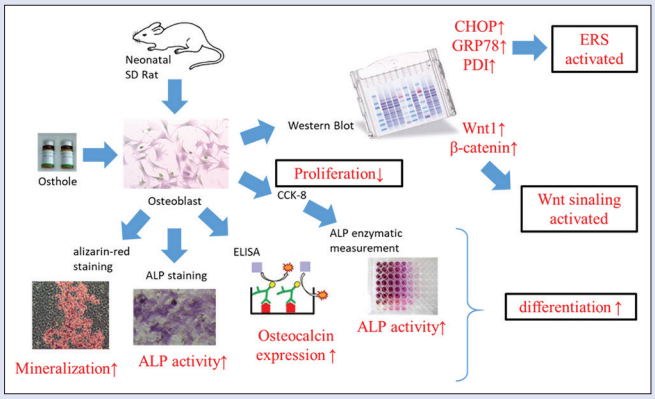
ABSTRACT

Background: Osthole is extracted from Fructus Cnidii and is proved to be effective in the treatment of osteoporosis in rats. However, data are still scarce and the mechanism remains elusive. **Objective:** To investigate the effect of Osthole on proliferation and differentiation of osteoblast. **Materials and Methods:** Cells were divided into five groups: control group, β-estradiol group (10^{-8} M), and Osthole groups (10^{-6} M, 10^{-5} M, and 10^{-4} M). Osteoblast proliferation was evaluated by Cell Counting Kit-8 (CCK-8) assay. Alkaline phosphatase (ALP) activity was detected by ALP staining and enzymatic measurement. Mineralization was detected by alizarin-red staining. The level of osteocalcin was measured by enzyme-linked immunosorbent assay (ELISA). The expression of key proteins of Wnt/β-catenin signaling pathway and endoplasmic reticulum stress (ERS) was analyzed by Western blot. **Results:** Cell proliferation was retarded in moderate-dose and high-dose Osthole group at 1d, 2d and 3d and in low-dose Osthole group at 1d and 2d ($P < 0.05$). ALP activity was enhanced in high-dose Osthole group from 1d to 3d and in moderate-dose Osthole group at 2d ($P < 0.05$). Mineralization of bone matrix was promoted in high-dose Osthole group at 21d ($P < 0.05$). The secretion of osteocalcin was promoted in Osthole groups at 21d ($P < 0.05$). Expression of CHOP, GRP78, PDI, Wnt1, and β-catenin was upregulated in high-dose Osthole group at 2d, indicating that both of ERS and Wnt/β-catenin signaling pathway were activated. **Conclusion:** It can be concluded that the effect of Osthole on inhibition of proliferation is relevant with activation of ERS, and activation of Wnt/β-catenin signaling pathway is one of the mechanisms how Osthole promotes osteoblast differentiation. In summary, this study provided more evidence for Osthole as a potential anti-osteoporosis medicine.

Key words: Differentiation, endoplasmic reticulum stress, Osthole, proliferation, Wnt/β-catenin signaling

SUMMARY

- Osthole was extracted from effective Chinese medicine for osteoporosis treatment.
- Osthole inhibited proliferation and promoted differentiation of rat osteoblast *in vitro*.
- Osthole activated classical Wnt signaling and ERS in rat osteoblast.



Abbreviations used: ALP: Alkaline phosphatase; ELISA: Enzyme-linked immunosorbent assay; CCK-8: Cell Counting Kit-8; ERS: endoplasmic reticulum stress; DMEM: Dulbecco's Modified Eagle Medium; α-MEM: α-minimum essential medium; EDTA: trypsin/ethylenediaminetetraacetic acid; DMF: N,N-dimethylformamide; TBS-T: Tris-buffered saline-Tween 20; RIPA: Radio Immuno Precipitation Assay; HRP: Horseradish peroxidase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; BCA: Bicinchoninic acid; ANOVA: Analysis of variance.

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INTRODUCTION

Osteoporosis is one of the most common diseases in the elderly, threatening life quality of patients. Although various kinds of drugs have been developed, side effects[1,2] become the biggest problem in the treatment of osteoporosis. Therefore, researchers are eager to find the alternatives to classical anti-osteoporosis medicine.[3] Recently, Osthole, extracted from Fructus Cnidii,[4] a Chinese herb often used to treat osteoporosis, has been proved to be effective in the treatment of osteoporosis in mice and rats.[5,6] Studies showed that Osthole can enhance osteogenesis by promoting osteoblast differentiation.[5,7-12] However, data are still scarce to understand how Osthole treats osteoporosis and the results of previous researches vary with each other. Besides, in existing studies, the source of osteoblast varies from each other. Further studies are still essential to confirm the pro-osteogenesis effect of Osthole. The research on mechanism may also help illuminate how Osthole treats osteoporosis by acting on osteoblast isolated from the neonatal rats.

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This paper provides the results of proliferation and differentiation data of osteoblast. Furthermore, we will also discuss how Osthole influences the proliferation and differentiation of osteoblast. We preliminarily study the effect of Osthole on osteoblast and tentatively focus on the Wnt/β-catenin signaling pathway and ERS to discuss the pro-differentiation effect and proliferation inhibition effect of Osthole, respectively. Further studies on osteoclast and *in vivo* will be summarized in our next study.

MATERIALS AND METHODS

Reagents and antibodies

Osthole was obtained from the National Institutes for Food and Drug Control (Beijing, China). Fetal bovine serum (FBS), L-glutamine, Dulbecco's Modified Eagle Medium (DMEM), α-minimum essential medium (α-MEM), and trypsin/ethylenediaminetetraacetic acid (EDTA) were obtained from Gibco (Carlsbad, CA, USA). CCK-8 was obtained from Dojindo (Kumamoto, Japan). ALP assay kit was purchased from Beyotime (Beyotime Biotechnology, Nantong, China). Osteocalcin ELISA kit was obtained from JianCheng (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Bicinchoninic acid (BCA) protein assay kit was obtained from Bio-Rad (Hercules, California, USA). Naphthol AS-MX phosphate, N, N-dimethylformamide (DMF), fast blue BB salt, and Tris base were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Protease inhibitor cocktails and Collagenase I were obtained from BioSharp (BioSharp Biotechnology, Hefei, China). The antibodies for β-catenin, CHOP, and PDI were purchased from Cell Signaling (Cell Signaling Technology, Inc., Danvers, MA, USA). Wnt1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies, and anti-goat secondary antibody were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., CA, USA); GRP78 antibody and anti-rabbit and anti-mouse secondary antibodies were purchased from Abcam (Cambridge, MA, USA).

Cell isolation and culture

Primary osteoblastic cells were prepared from neonatal (1 day old) Sprague-Dawley rats (Qinglongshan, Nanjing, China). The rats were executed by decapitation after being immersed in 75% ethanol for 5 min. Then, excise the rat calvaria, peel off soft tissue, and rinse 3 times with phosphate-buffered saline (PBS). Cut the calvarias into small pieces (about 1 mm^2), trypsinize with D-Hanks' solution containing 0.25% trypsin and 0.02% EDTA for 30 min. Centrifuge at 200 × g for 5 min at room temperature, resuspend the small pieces with 0.1% collagenase I, and incubate in an oscillation incubator for 4 h. Isolate the cells with a cell strainer, centrifuge at 200 ×g for 5 min, and wash the cells twice with PBS. Resuspend the cells with low-glucose DMEM containing 10% FBS and 1% penicillin–streptomycin, count the cells, and seed on culture bottles in a 37°C, 5% CO_2 humidified incubator. Exchange the culture media every 2–3 days.

Grouping

Five groups were set up in this study: control group, β-estradiol group, low-dose Osthole group (10^{-6} M), moderate-dose Osthole group (10^{-5} M), and high-dose Osthole group (10^{-4} M). Each group contains 0.25% dimethyl sulfoxide as cosolvent for water-insoluble β-estradiol and Osthole. β-Estradiol was chosen as positive control not only for its clinical use[13] as anti-osteoporosis medicine but also for its similarity with Osthole as anabolic hormones.[14]

Cell proliferation assay

The cell proliferation was evaluated with CCK-8 assay. In brief, 4 × 10^3 cells were seeded on 96-well plate and cultured with low-glucose DMEM containing Osthole or β-estradiol. 10 μL CCK-8 reagent was added into each well and incubated for 4 h. Absorbance was detected at 450 nm with Enspire 2300 (Perkin Elmer, Waltham, Massachusetts, USA).

Alkaline phosphatase staining and enzymatic activity measurement

ALP staining was performed with diazotization-coupling method. In brief, fix the cells with 95% ethanol for 10 min, decant the ethanol, and wash 3 times with ddH_2O. Then, stain the cells with ALP dye liquor (containing 0.5% DMF, 0.6% Tris base, 0.1% Naphthol AS-MX Phosphate, and 0.6% Fast blue BB salt, pH = 8.5) on a shaker for 1 h, avoiding light. Finally, decant the staining solution and wash the cells with ddH_2O and take photographs under a light microscope (Leica, Germany).

For the ALP activity measurement, 4 × 10^3 cells were seeded on 96-well plate and cultured with low-glucose DMEM for 1d, 2d, and 3d, respectively. After CCK-8 assay, suck out incubation medium and rinse with PBS twice. Add 50 μL lysate (diethanolamine buffer containing 1% triton, pH = 9.8) each well and lyse for 15 min at 4°C. Follow the protocol of ALP assay kit and detect the OD value at 405 nm. Data of ALP activity were normalized with cell proliferation data.

Enzyme-linked immunosorbent assay

Osteoblastic cells were cultured in a 24-well plate with osteoblast-inducing conditional media (α-MEM containing 10% FBS, 50 μM ascorbic acid, and 10 mM β-glycerophosphate) containing Osthole or β-estradiol for 21d. Replace the osteoblast-inducing conditional media with MEM (without phenol red) for 12 h, collect the medium, and centrifuge at 10,000 × g for 5 min. Osteocalcin was detected with rat Osteocalcin ELISA kit following the manufacture's protocol.

Mineralized nodule staining

Alizarin-red method was applied to stain mineralized nodules. Briefly, 1 × 10^5 cells per well were seeded into a 24-well plate. After cultured with low-glucose DMEM containing Osthole or β-estradiol for 21d, when the mineralized nodules showed up in all groups, the cells were fixed with 95% ethanol for 10 min and then washed three times with ddH_2O. Alizarin-red dye liquor (0.1% alizarin-red S dissolved in 0.1 M Tris-Hcl, pH = 8.3) was added into each well and incubated at 37°C for 30 min. Finally, decant the staining solution and wash the cells with ddH_2O and take photographs with a light microscope (Leica, Germany) and a camera (Canon, Japan). Densitometric analysis was carried out with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blotting

Total cytoplasmic protein was isolated with RIPA lysis buffer containing protease inhibitor cocktail and measured with BCA protein assay kit. Run the proteins on polyacrylamide gel and transblot them to PVDF membranes which were then blocked for 2 h at room temperature and incubated overnight at 4°C in buffer (TBS-T with 5% nonfat milk powder) containing diluted primary antibodies. Horseradish peroxidase (HRP)-conjugated secondary antibodies were applied to detect primary antibodies. Chemiluminescent substrate was applied to visualize the bands. Finally, perform densitometric analysis with ImageJ software.

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). All statistical analyses were performed with GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by the SNK-q test or LSD-t test for multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Morphologic observation

To identify the cells isolated from calvaria of neonatal Sprague-Dawley rats, they were observed under a light microscope. ALP is a by-product of osteoblast and mineralized nodules are specific product of osteoblast. Therefore, ALP staining and alizarin-red staining were performed to identify the osteoblast.[15,16] The cells were anchorage-dependent cells with one or two elliptical nuclei [Figure 1d] and typical shapes such as triangle, spindle, and polygon [Figure 1a]. After cultured with conditioned medium (α-MEM + 10% FBS + 50 μM ascorbic acid + 10 mM β-glycerophosphate) for 3d, the cells were dyed purple by diazotization-coupling method [Figure 1c]. After continuous culture with conditioned medium described above for another 7d, mineralized nodules showed up and they could be dyed red with alizarin-red S [Figure 1b].

Osthole inhibits proliferation of osteoblast

CCK-8 assay was carried out to evaluate the effect of Osthole on the proliferation of osteoblast. Compared with control group, all three Osthole groups showed retarded cell proliferation at 1d and 2d. Moderate and high dose of Osthole could also inhibit the proliferation of osteoblasts at 3d. The cell proliferation of osteoblast was negatively associated with the concentration of Osthole [Figure 2a-c].

Osthole enhances alkaline phosphatase activity of osteoblast

ALP activity represents the functional differential of osteoblast. Thus, detecting the activity of ALP can be a way to realize the osteogenesis function of osteoblast.[17] Compared with control group, the ALP activity of osteoblasts in high-dose Osthole group was higher at 1d, 2d, and 3d. In addition, the osteoblasts in moderate-dose Osthole group showed a higher ALP activity at 2d as well. The result also showed that ALP activity of osteoblasts is positively correlated with the concentration of Osthole [Figure 2d-f].

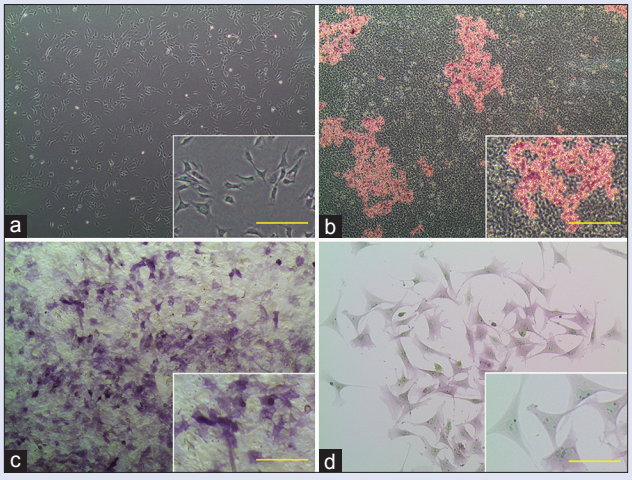

Figure 1: Morphological observation on osteoblast. (a) The second passage of cells isolated from calvarias of neonatal (1 day old) Sprague-Dawley rats. Scale bar: 200 μm. (b) Alizarin-red staining of mineralized nodules produced by mature osteoblasts, which were induced with conditioned medium (α-minimum essential medium + 10% fetal bovine serum + 50 μM ascorbic acid + 10 mM β-glycerophosphate) for 7d. Scale bar: 200 μm. (c) Alkaline phosphatase staining of osteoblasts by diazotization-coupling method. Alkaline phosphatase-positive cells were dyed purple. Scale bar: 80 μm. (d) Alkaline phosphatase staining by diazotization-coupling method and nuclear counterstaining with methyl green. Alkaline phosphatase-positive cytoplasm was light purple while nuclears were green. Scale bar: 40 μm

Osthole promotes the secretion of osteocalcin

Osteocalcin is synthesized and excreted by osteoblast and irrelevant with bone resorption factors. Thus, osteocalcin is an ideal marker evaluating the osteogenesis function of osteoblast.[18] The concentration of osteocalcin was measured with an ELISA kit. The result showed that osteocalcin levels in all three Osthole groups were higher than control group after cultured for 21d [Figure 2g], indicating that Osthole enhanced osteogenesis.

Osthole promotes the mineralization of bone matrix

Mineralized nodule is the most specific characteristic of osteoblast. The amount and area of mineralized nodules are positively correlated with osteogenesis function of osteoblast. Densitometric analysis of mineralized nodules showed stronger mineralization in high-dose Osthole group than control group. However, mineralization of β-estradiol group, medium-dose Osthole group and low-dose Osthole group were not stronger than control group [Figure 2h and i].

Osthole activates Wnt/β-catenin signaling pathway in osteoblast

Wnt/β-catenin signaling pathway regulates not only the osteoblast proliferation[19] but also the differentiation.[20] To realize how Osthole regulates osteogenesis, key proteins in Wnt/β-catenin signaling pathway were detected. The results showed that Wnt1 expression in moderate-dose Osthole and high-dose Osthole group was higher than control group [Figure 3a and e]. The expression of β-catenin had also been upregulated in high-dose Osthole group [Figure 3a and f]. Upregulation of Wnt1 and β-catenin means the activation of Wnt/β-catenin signaling pathway, indicating that Osthole could promote proliferation and differentiation of osteoblast.

Osthole induces endoplasmic reticular stress in osteoblast

Activation of Wnt/β-catenin signaling pathway should have promoted proliferation of osteoblast; however, the CCK-8 assay result showed a retarded cell proliferation of osteoblast. To explain the paradox, the key proteins of endoplasmic reticular stress were detected. CHOP[21] is a specific protein of endoplasmic reticular stress which barely not expressed in normal cells. As is shown in Figure 3a and b, CHOP expression in high-dose Osthole group was extremely high, outclassing other groups, indicating the activation of endoplasmic reticular stress in high-dose Osthole group. Another two proteins related to endoplasmic reticular stress are GRP78 and PDI. The level of these two proteins was upregulated in high-dose Osthole group, corroborating the activation of endoplasmic reticular stress [Figure 3a, c, and d].

DISCUSSION

Osteoporosis is one of the most common metabolic disorders of old age and severely lowers the quality of life of patients. Osthole, a natural coumarin-like derivative extracted from Fructus Cnidii, has been proved to be effective in the treatment of osteoporosis in mice and rats.[5,6] However, how does Osthole works remains elusive.

Zhang *et al.*[22] advocated it for the first time that 10^{-5} M and 10^{-6} M of Osthole could promote the proliferation of osteoblast and enhance the ALP activity. Then, Ming *et al.*[9] further demonstrated

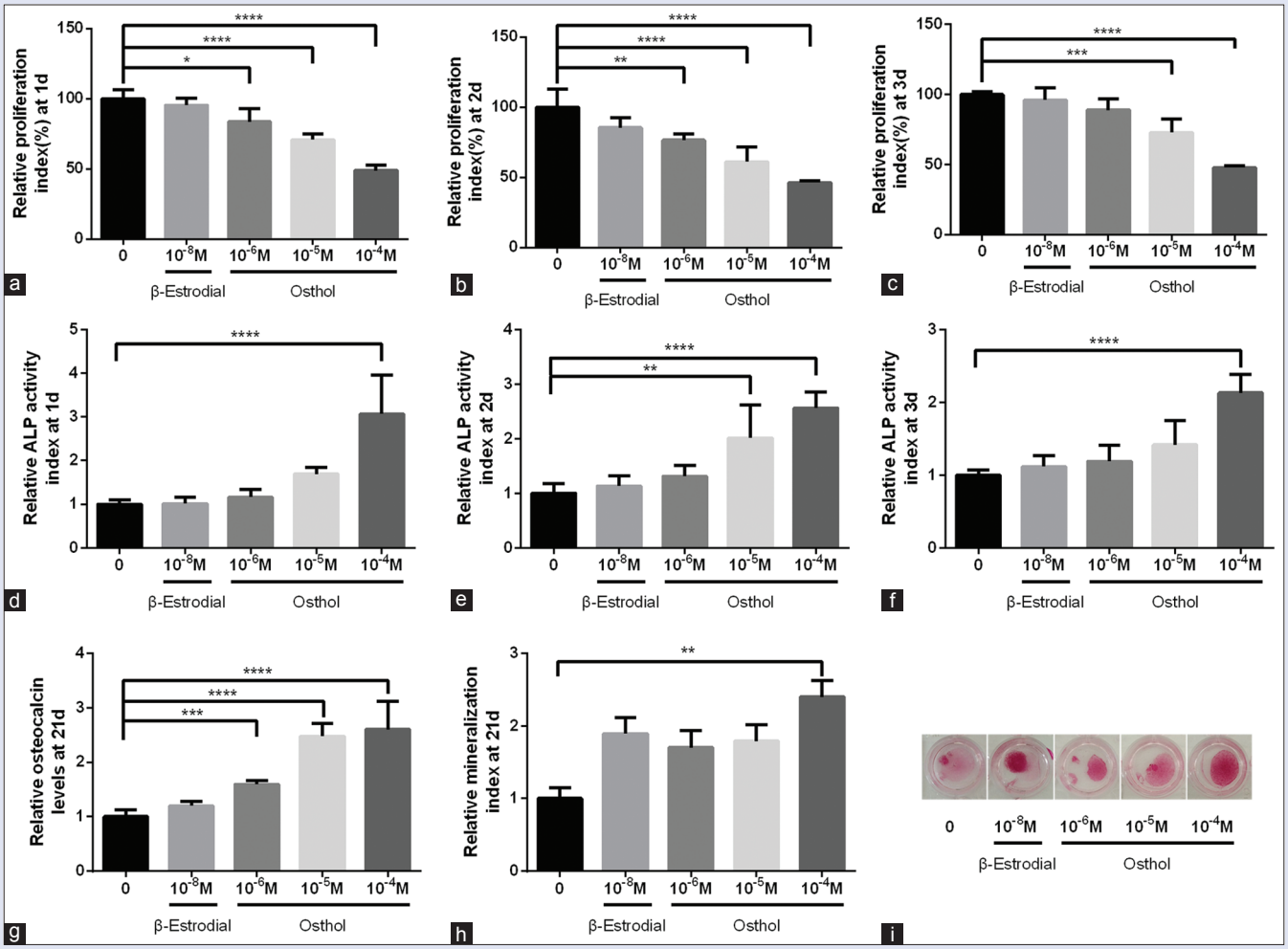

Figure 2: Effect of Osthole on proliferation and differentiation of osteoblast. (a-c) Proliferation of osteoblast was inhibited by Osthole at 1d (10^{-4} M, 10^{-5} M, and 10^{-6} M), 2d (10^{-4} M, 10^{-5} M, and 10^{-6} M), and 3d (10^{-4} M and 10^{-5} M). Data were expressed as mean ± standard deviation ($n = 4$, one-way ANOVA and SNK-q test). (d-f) Alkaline phosphatase activity of osteoblast was enhanced by Osthole at 1d (10^{-4} M), 2d (10^{-4} M and 10^{-5} M), and 3d (10^{-4} M). Data were expressed as mean ± standard deviation ($n = 4$, one-way ANOVA and SNK-q test). (g) The secretion of osteocalcin was promoted by Osthole at 21d (10^{-4} M, 10^{-5} M, and 10^{-6} M). Data were expressed as mean ± standard deviation ($n = 8$, one-way ANOVA and SNK-q test). (h and i) Mineralization of bone matrix was promoted by Osthole at 21d (10^{-4} M). Data were expressed as mean ± standard deviation ($n = 4$, one-way ANOVA and SNK-q test). *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. ****$P < 0.0001$

that 10^{-5} M of Osthole could increase expression of some genes relevant with osteoblast differentiation including Runx 2, bFGF, IGF-1, and Osterix, thus promoting the secretion of osteocalcin and mineralization of bone matrix. Afterward, Kuo *et al.*[12] found that 10^{-5} M of Osthole promotes osteoblast differentiation by regulating bone morphogenetic protein 2/p38 pathway and extracellular signal-regulated kinase 1/2 pathway. More recent study by Jia *et al.*[7] revealed that estrogen receptor is one of the targets of Osthole. However, Jia *et al.*'s experiments were carried out with Saos-2 cells, isolated from osteosarcoma, instead of normal osteoblasts, in which the conclusion was uncertain.

Although accumulated researches proved the osteogenesis effect of Osthole, different results on the proliferation of osteoblast have come out from researchers. Some found no significance in cell proliferation between Osthole group and control group,[12] while others believe that Osthole can promote the proliferation of osteoblast.[8,10,22] The different results may be due to the impurity of Osthole and different sources of osteoblast. Our results showed an inhibitory effect of Osthole on proliferation of osteoblast. As to the effect of Osthole on osteoblast differentiation, we confirmed that Osthole can enhance ALP activity, increase the secretion of osteocalcin, and promote the mineralization of bone matrix.

To explain the results showed above, we put forward a hypothesis: Osthole induces endoplasmic reticular stress due to its strong promotion effect on osteoblast differentiation. Western blot results showed that both Wnt/β-catenin signaling pathway and endoplasmic reticular stress were activated by Osthole. Moreover, the effect was positively relevant with the concentration of Osthole. Wnt/β-catenin signaling pathway is a well-known pathway regulating cell proliferation by promoting transcription of cycline D1, c-Myc, and Bcl-2.[23] In addition, Run × 2 and OPG are targets of β-catenin as well.[24,25] Theoretically, once Wnt/β-catenin signaling pathway was activated in osteoblast, both proliferation and differentiation will be promoted as reported by some researchers. However, we found that Osthole not only activated Wnt/β-catenin signaling pathway but also activated endoplasmic reticular stress. Endoplasmic reticular stress is a highly conserved signaling activated by endoplasmic reticular function disorder induced by overload of secretion protein or shortage of energy.[26] There are two outcomes

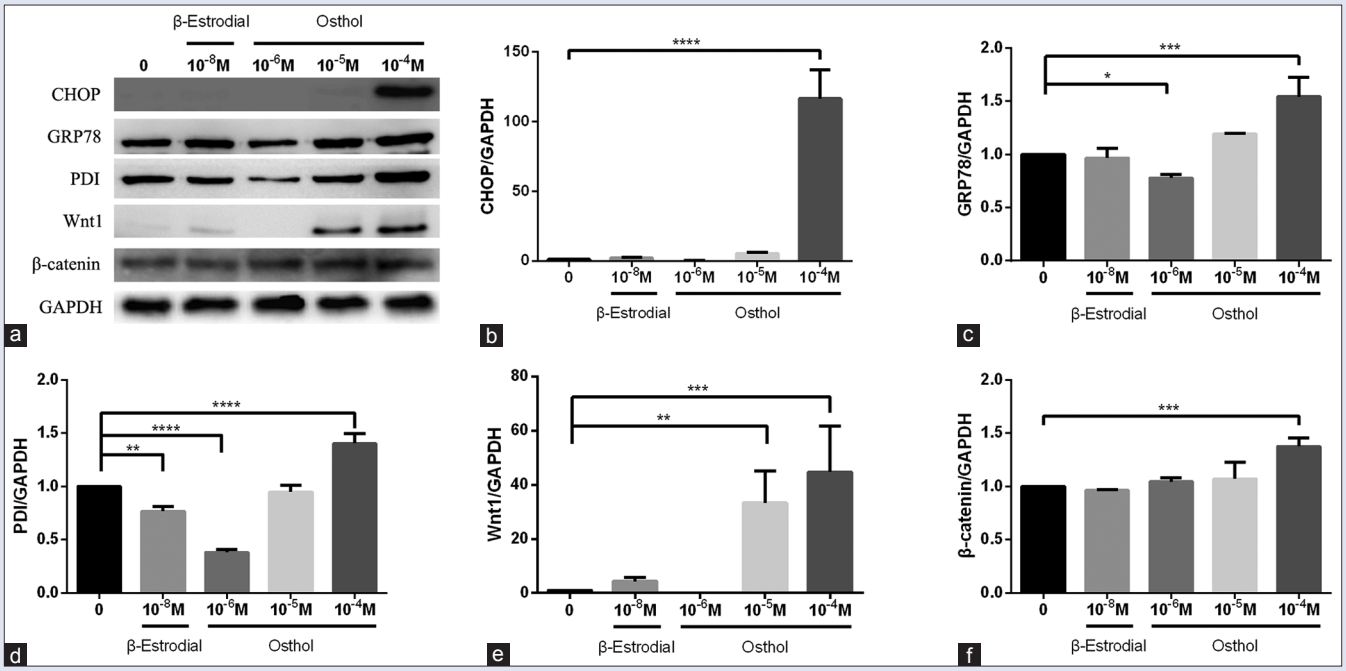

Figure 3: Effect of Osthole on the expression of key proteins in Wnt/β-catenin signaling pathway and endoplasmic reticular stress at 2d. (a) Western blot assay of key proteins in Wnt/β-catenin signaling pathway and endoplasmic reticular stress. (b-f) Quantification of CHOP, GRP78, PDI, Wnt1, and β-catenin protein densitometry. The expression of CHOP, GRP78, PDI, Wnt1, and β-catenin protein was upregulated by Osthole at 2d (10^{-4} M). The expression of Wnt1 was also upregulated by Osthole at 2d (10^{-5} M). Data shown were expressed as mean ± standard deviation ($n = 3$, one-way ANOVA and LSD-t test). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

of endoplasmic reticular stress: adaption or apoptosis. Long-lasting or strong endoplasmic reticular stress leads to cell apoptosis while short or mild leads to cell survival.[27] Osthole is proved similar with estrogen, which can promote protein synthesis.[14] We believe that overpromotion of protein synthesis by Osthole induces endoplasmic reticulum stress, slowing down cell proliferation, thus decreasing the amount of osteoblasts detected by CCK-8 assay. The significantly upregulated ALP activity and osteocalcin could partly corroborate this viewpoint.

Although we have revealed how can Osthole regulate osteogenesis, it is still hard to illuminate how Osthole treat osteoporosis. This problem could be solved if we consider osteoclast, osteocyte, and the vascular epithelial cell, which can be studied *in vivo* or coculture system *in vitro*. Furthermore, apoptosis-related experiments will be conducted, such as apoptosis rate assay with flow cytometer and analysis of apoptosis-related protein and gene expression.

CONCLUSION

In summary, this study demonstrated that Osthole promotes secretion of osteocalcin, enhances ALP activity and mineralization of bone matrix by activating Wnt/β-catenin signaling pathway, but inhibits osteoblast proliferation by activating endoplasmic reticulum stress.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Hagino H, Kishimoto H, Ohishi H, Horii S, Nakamura T. Efficacy, tolerability and safety of once-monthly administration of 75mg risedronate in Japanese patients with involutional osteoporosis: A comparison with a 2.5mg once-daily dosage regimen. Bone 2014;59:44-52.
2. Khosla S, Hofbauer LC. Osteoporosis treatment: Recent developments and ongoing challenges. Lancet Diabetes Endocrinol 2017;5:898-907.
3. Costa CR, Amorim BR, de Magalhães P, De Luca Canto G, Acevedo AC, Guerra EN, *et al.* Effects of plants on osteogenic differentiation and mineralization of periodontal ligament cells: A systematic review. Phytother Res 2016;30:519-31.
4. Gao F, Hu Y, Ye X, Li J, Chen Z, Fan G, *et al.* Optimal extraction and fingerprint analysis of Cnidii fructus by accelerated solvent extraction and high performance liquid chromatographic analysis with photodiode array and mass spectrometry detections. Food Chem 2013;141:1962-71.
5. Zhang ZR, Leung WN, Li G, Kong SK, Lu X, Wong YM, *et al.* Osthole enhances osteogenesis in osteoblasts by elevating transcription factor osterix via cAMP/CREB signaling *in vitro* and *in vivo*. Nutrients 2017;9. pii: E588.
6. Li XX, Hara I, Matsumiya T. Effects of osthole on postmenopausal osteoporosis using ovariectomized rats; comparison to the effects of estradiol. Biol Pharm Bull 2002;25:738-42.
7. Jia M, Li Y, Xin HL, Hou TT, Zhang ND, Xu HT, *et al.* Estrogenic activity of osthole and imperatorin in MCF-7 cells and their osteoblastic effects in saos-2 cells. Chin J Nat Med 2016;14:413-20.
8. Zhai YK, Pan YL, Niu YB, Li CR, Wu XL, Fan WT, *et al.* The importance of the prenyl group in the activities of osthole in enhancing bone formation and inhibiting bone resorption *in vitro*. Int J Endocrinol 2014;2014:921954.
9. Ming LG, Zhou J, Cheng GZ, Ma HP, Chen KM. Osthol, a coumarin isolated from common Cnidium fruit, enhances the differentiation and maturation of osteoblasts *in vitro*. Pharmacology 2011;88:33-43.
10. Zhang W, Ma D, Zhao Q, Ishida T. The effect of the major components of Fructus cnidii on osteoblasts *in vitro*. J Acupunct Meridian Stud 2010;3:32-7.
11. Tang DZ, Hou W, Zhou Q, Zhang M, Holz J, Sheu TJ, *et al.* Osthole stimulates osteoblast differentiation and bone formation by activation of beta-catenin-BMP signaling. J Bone Miner Res 2010;25:1234-45.

12. Kuo PL, Hsu YL, Chang CH, Chang JK. Osthole-mediated cell differentiation through bone morphogenetic protein-2/p38 and extracellular signal-regulated kinase 1/2 pathway in human osteoblast cells. J Pharmacol Exp Ther 2005;314:1290-9.
13. de Villiers TJ. The role of menopausal hormone therapy in the management of osteoporosis. Climacteric 2015;18 Suppl 2:19-21.
14. Pan Z, Fang Z, Lu W, Liu X, Zhang Y. Osthole, a coumadin analog from *Cnidium monnieri* (L.) cusson, stimulates corticosterone secretion by increasing steroidogenic enzyme expression in mouse Y1 adrenocortical tumor cells. J Ethnopharmacol 2015;175:456-62.
15. Vukicevic S, Luyten FP, Kleinman HK, Reddi AH. Differentiation of canalicular cell processes in bone cells by basement membrane matrix components: Regulation by discrete domains of laminin. Cell 1990;63:437-45.
16. Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, *et al*. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. Proc Natl Acad Sci U S A 2002;99:9445-9.
17. Eghbali-Fatourechi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosla S, *et al*. Circulating osteoblast-lineage cells in humans. N Engl J Med 2005;352:1959-66.
18. Stein GS, Lian JB, Stein JL, Van Wijnen AJ, Montecino M. Transcriptional control of osteoblast growth and differentiation. Physiol Rev 1996;76:593-629.
19. Davidson G, Niehrs C. Emerging links between CDK cell cycle regulators and Wnt signaling. Trends Cell Biol 2010;20:453-60.
20. Rauner M, Rachner TD, Hofbauer LC. Bone formation and the Wnt signaling pathway. N Engl J Med 2016;375:1902.
21. Yang Y, Liu L, Naik I, Braunstein Z, Zhong J, Ren B, *et al*. Transcription factor C/EBP homologous protein in health and diseases. Front Immunol 2017;8:1612.
22. Zhang Q, Qin L, He W, Van Puyvelde L, Maes D, Adams A, *et al*. Coumarins from *Cnidium monnieri* and their antiosteoporotic activity. Planta Med 2007;73:13-9.
23. Clevers H, Nusse R. Wnt/β-catenin signaling and disease. Cell 2012;149:1192-205.
24. Yuan Z, Li Q, Luo S, Liu Z, Luo D, Zhang B, *et al*. PPARγ and Wnt signaling in adipogenic and osteogenic differentiation of mesenchymal stem cells. Curr Stem Cell Res Ther 2016;11:216-25.
25. Kobayashi Y, Uehara S, Udagawa N, Takahashi N. Regulation of bone metabolism by Wnt signals. J Biochem 2016;159:387-92.
26. Walter P, Ron D. The unfolded protein response: From stress pathway to homeostatic regulation. Science 2011;334:1081-6.
27. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, *et al*. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev 2004;18:3066-77.