Network Pharmacology-Based Analysis on the Action Mechanism of Oleanolic Acid to Alleviate Osteoporosis

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ABSTRACT: Oleanolic acid (OA) is a triterpenoid commonly found in plants and has shown extensive pharmaceutical activities. This study aimed to investigate the underlying mechanism of anti-osteoporosis (OP) action of OA by utilizing the network pharmacology approach and molecular docking methods. First, the targets of OA were identified using the GeneCards, Stitch, and SwissTarget databases, and the targets related to OP were mined using the NCBI, Genecards, and DisGeNet databases. The overlapped targets of OA and OP were regarded as candidate targets, and the String database was used to obtain the protein-protein interactions among the targets. Then, Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway enrichment pathways of the candidate targets were performed using the DAVID database. In addition, the top 16 targets in the protein interaction network were used for molecular docking. Finally, an animal model constructed using D-galactose-induced oxidative stress and a low-calcium diet with accelerated bone loss was used to verify the in vivo effects of OA on osteoporotic mice. A total of 42 candidate targets for OA to treat OP were obtained. According to the protein-protein interaction network, MAPK1 showed the highest connectivity with other proteins. Additionally, GO analysis identified the top 20 biological processes, 9 cellular components, and top 20 molecular functions. Moreover, the candidate targets were mainly involved in 13 signaling pathways such as TNF signaling pathway, insulin resistance, MAPK signaling pathway, apoptosis, and PI3K-Akt signaling pathways. Furthermore, molecular docking revealed that OA has a high degree of connections with 16 key proteins. In addition, the anti-OP effects of OA are further validated through the in vivo model. Altogether, our study elucidated the candidate targets for OA to alleviate OP, explored the protein-protein interactions and related signaling pathways of the targets, and validated the anti-OP effects of OA. It could provide a better understanding of the action mechanism in OA to treat OP.

1. INTRODUCTION

Osteoporosis (OP) is a systematic bone disease characterized by the reduction in bone mineral density, as well as the deterioration of microstructure in bone tissue due to calcium and protein consumption, leading to bone fragility and vulnerability to fracture. With the rapid growth of the aging population, OP has become a health concern worldwide. To date, common treatments for OP have focused on anti-bone resorption drugs that slow bone loss such as bisphosphonates, calcitonin, denosumab (RANKL), and raloxifene (a selective estrogen receptor modulator). However, these therapies have some adverse effects, such as auricular fibrillation, osteonecrosis of the jaw, and ovarian cancer. Therefore, exploring more drugs with fewer adverse effects and multitargeted actions is an important direction in the current research on OP.

Oleanolic acid (OA) is a representative compound of the pentacyclic triterpenoids, which is widely found in food and herbs in the form of free acid or triterpene saponin glycosides.
the systemic network action mechanism of certain drugs and multiple targets of the drug to treat certain diseases.\textsuperscript{16,17} So far as we know, a disease is commonly affected by multiple genes, proteins, pathways, and so on, rather than a unique genetic variation.\textsuperscript{78} Therefore, the network target and multicomponent concept of network pharmacology are the most suitable tools to explore the therapeutic effects of drugs at the molecular level.

In this study, we aimed to employ network pharmacology and molecular docking to explore whether OA possesses anti-OP effects and delineate the underlying mechanism of action.

2. RESULTS

2.1. Identify the Candidate Targets. As shown in Figure 1B, after removing the redundant information, we obtained 4598 OP-related therapeutic targets and 50 OA targets, among which the 42 targets are overlapped, suggesting that these 42 intersection targets are candidate molecular targets for mediating the anti-OP effects of OA.

2.2. Proteins Interaction Among the Candidate Targets for OA to Treat OP. In order to reveal the potential pharmacological mechanisms of OA in alleviating OP, a protein interaction network was constructed based on 42 candidate protein targets of OA. Details of the 42 target proteins are shown in Table 1.

As shown in Figure 2A, the protein interaction network contains 36 nodes (proteins) and 124 edges (protein interactions). The nodes with the highest degree of connection to other gene symbols represent hubs in the entire network, which are the most potential targets in the pharmacological process. Figure 2B shows the top 20 molecular targets for the anti-OP effects of OA in the protein interaction network. Notably, the highest connected target is MAPK1, which is linked with 20 other proteins. TP53, CASP3, IL6, and JUN are linked with 18, 16, 16, and 15 other proteins, respectively.

2.3. Enrichment Analysis of the Candidate Targets.

2.3.1. GO analysis. Biological process analysis is shown in Figure 3. The top 20 processes were positive regulation of transcription from the RNA polymerase II promoter, positive regulation of transcription (DNA-templated), negative regulation of the apoptotic process, response to drug, aging, inflammatory response, negative regulation of cell proliferation, positive regulation of smooth muscle cell proliferation, response to estradiol, response to ethanol, positive regulation of gene expression, steroid hormone mediated signaling pathway, transcription initiation from the RNA polymerase II promoter, response to lipopolysaccharides, positive regulation of the nitric oxide biosynthetic process, response to glucocorticoid, response to antibiotics, response to hydrogen peroxide, regulation of blood pressure, and cell aging, and most of them had high correlation with the pathogenesis of OP.

Additionally, the results indicated that nine cellular components were involved in the anti-OP effects of OA when corrected by P-value ≤ 0.01 (Figure 4), including the cytosol, nucleoplasm, death-inducing signaling complex, nucleus, cytoplasm, neuronal cell body, protein complex, caveola, and endoplasmic reticulum.

As shown in Figure 5, a total of 20 molecular functions were identified by GO analysis, of which the top 10 were steroid hormone receptor activity, sequence-specific DNA binding, enzyme binding, transcription factor binding, drug binding, RNA polymerase II transcription factor activity (ligand-activated sequence-specific DNA binding), identical protein binding, protein phosphatase 2A binding, protein complex binding, and protein binding.

Altogether, based on the GO analysis, we found that positive regulation of transcription from the RNA polymerase II promoter had the most targets among the biological processes. Nucleus had the most targets among cellular components. In terms of molecular functions, protein binding showed many more targets.

2.3.2. KEGG pathway analysis. To further identify the potential pathways involved in the anti-OP effects of OA, KEGG pathway enrichment analysis was performed. As a result, we obtained 13 pathways associated with the candidate targets (Figure 6), including TNF signaling pathway, apoptosis, insulin resistance, NOD-like receptor signaling pathway, toll-like receptor signaling pathway, MAPK signaling pathway, p53 signaling pathway, HIF-1 signaling pathway, serotonergic synapse, thyroid hormone signaling pathway, sphingolipid signaling pathway, osteoclast differentiation, and PI3K-Akt signaling pathway. Particularly, the TNF signaling pathway, insulin resistance, and MAPK signaling pathway are the top three of enrichment, suggesting that these pathways may mediate the anti-OP effects of OA.

2.4. Docking Mode of Interactions between OA and the Key Targets. To investigate the possible modes of binding and the degree of it, the docking targets selected of
Table 1. Target Proteins of OA against OP in the Protein Interaction Network

| no. | protein names                  | gene symbol | number of neighboring proteins | no. | protein names                  | gene symbol | number of neighboring proteins |
|-----|--------------------------------|-------------|--------------------------------|-----|--------------------------------|-------------|--------------------------------|
| 1   | mitogen-activated protein kinase 1 | MAPK1       | 20                             | 22  | prostaglandin-endoperoxide synthase 1 | PTGS1       | 3                             |
| 2   | tumor protein PS3               | TP53        | 18                             | 23  | superoxide dismutase 1          | SOD1        | 3                             |
| 3   | caspase 3                       | CASP3       | 16                             | 24  | topoisomerase I                 | TOP1        | 3                             |
| 4   | interleukin-6                   | IL6         | 16                             | 25  | arachidonate-5-lipoxygenase     | ALOX5       | 2                             |
| 5   | Jun proto-oncogene              | JUN         | 15                             | 26  | apolipoprotein B                | APOB        | 2                             |
| 6   | tumor necrosis factor           | TNF         | 14                             | 27  | nicotinamide                    | NAMPT       | 2                             |
| 7   | prostaglandin-endoperoxide synthase 2 | PTGS2      | 12                             | 28  | protein tyrosine phosphatase nonreceptor type 1 | PTPN1 | 2                             |
| 8   | myelocytomatosis oncogene       | MYC         | 11                             | 29  | protein tyrosine phosphatase receptor type F | PTPRF       | 2                             |
| 9   | nitric oxide synthase 3         | NOS3        | 11                             | 30  | retinoic acid receptor alpha    | RARA        | 2                             |
| 10  | interleukin 1 beta              | IL1B        | 10                             | 31  | retinoic acid receptor beta     | RARB        | 2                             |
| 11  | peroxisome proliferator-activated receptor gamma | PPARG     | 10                             | 32  | G protein-coupled bile acid receptor 1 | GPBAR1 | 1                             |
| 12  | catalase                        | CAT         | 9                              | 33  | 3-hydroxy-3-methylglutaryl-CoA reductase | HMGCR | 1                             |
| 13  | estrogen receptor 1             | ESR1        | 9                              | 34  | hydroxysteroid 11-beta dehydrogenase 1 | HSD11B1 | 1                             |
| 14  | caspase 8                       | Casp8       | 8                              | 35  | prostaglandin 12 receptor       | PTGIR       | 1                             |
| 15  | cytochrome C, somatic           | CYCS        | 8                              | 36  | RAR-related orphan receptor C   | RORC        | 1                             |
| 16  | mechanistic target of rapamycin kinase | Mtor      | 7                              | 37  | nuclear receptor subfamily 1 group H member 2 | NRIH2 | 0                             |
| 17  | nuclear factor (erythroid-derived 2)-like 2 | Nfe2l2 | 7                              | 38  | DNA polymerase beta             | POLB        | 0                             |
| 18  | fatty acid synthetase           | FAS         | 4                              | 39  | phosphodiesterase 4D            | PDE4D       | 0                             |
| 19  | peroxisome proliferator-activated receptor alpha | Ppara | 4                              | 40  | protein tyrosine phosphatase nonreceptor type 2 | PTPN2 | 0                             |
| 20  | NADPH quinone oxidoreductase 1  | NQO1        | 3                              | 41  | acid phosphatase 1              | ACP1        | 0                             |

Table 2, and the affinity of the 16 target proteins with OA were all less than −5 kcal/mol, which suggested that all bindings were significant. Notably, NOS3 had the lowest affinity for OA, indicating that it had the strongest binding capacity. Overall, docking results successfully predicted docking between OA and the binding pocket of all 16 tested target proteins.

2.5. Effects of OA on the Bone Index and Bone Dry–Wet Ratio in Osteoporotic Mice. To validate the therapeutic effects, in vivo studies were carried out in this work. The bone index and bone dry–wet ratio partially reflected the bone quality of mice. Compared with the control group, the dry–wet ratio of femur and humerus in the model group was significantly decreased (Figure 8B, P < 0.01). Conversely, the bone dry–wet ratio of mice was significantly increased after treatment with different concentrations of OA (Figure 8B, P < 0.01 or P < 0.05 vs model), with the results of the OA–H group being very similar to the control group. In addition, there was also a trend toward dose-dependent improvement in the femoral and humeral index after OA treatment, although the difference was not statistically significant (Figure 8A).

2.6. Effects of OA on the Bone Microarchitecture in Osteoporotic Mice. The bone microstructure of the right femurs in mice was measured by micro-CT. As shown in Figure 9, the 3-D reconstruction of the bone microarchitecture exhibited the typical osteoporosis in the model group. Compared with the control mice, which had a dense and regular meshwork of bone trabecula, the model mice showed a significant bone loss, as evidenced by a substantial reduction in the number and density of trabeculae bone and the presence of large cavities. Notably, all treatment groups of OA evidently improved the microstructure of bone trabeculae in mice after modeling, among which OA–M and OA–H had more remarkable improvement effects. After treatment with OA, an increased number and better connectivity of bone trabeculae were observed in mice, as well as a dose-dependent alleviation of bone loss. The antosteoporotic effect of high
concentrations of OA treatment even made the bone status of mice similar to that of normal mice.

3. DISCUSSION

OA is a naturally occurring pentacyclic triterpenoid that can be extracted from various herbs and plants. It has been testified that OA not only exerts anti-inflammatory, antitumor and antidiabetic effects, but also has potential efficacy against diseases associated with calcium imbalance and bone loss. Moreover, OA modulated RANKL-mediated late osteoclastogenesis and stimulated osteoblastic differentiation of bMSCs, further suggesting that OA could exert osteoprotective effects in osteoporosis. The emerging potential methods of pretreatment and extraction of active components may provide guarantee for the sustainable supply of OA in a large scale. Nowadays, osteoporosis (OP) has become a widespread concern for chronic disease in the development of ageing society, with several limitations in its treatment and medication. The discovery of new drugs is currently an important direction in the study of OP treatment, of which OA has promising potential.

The network pharmacology approach, which reflects the interactions between biological macromolecules and chemical components, is a novel research paradigm to facilitate the discovery of new drugs. Utilizing this approach, we obtained the candidate targets involved in the OA treatment of OP, constructed target networks, and conducted the enrichment analysis to reveal the possible action mechanism. As a result, 42 candidate targets of OA against OP were obtained. Furthermore, 36 proteins were screened in the protein interaction network, which represents key molecular targets that mediate the anti-OP effects of OA. The majority of networks were involved in the cell cycle and DNA repair (e.g., TP53, CASP3, CASP8, and BCL2), immune and inflammatory responses (e.g., IL6, TNF, JUN, and PTGS2), cell-to-cell signaling and interactions (e.g.,

Figure 2. (A) Protein interaction network among the candidate targets for OA to treat OP. (B) Top 20 molecular targets for the anti-OP effects of OA in the protein interaction network. The target protein (Y-axis) and the number of neighboring proteins of the target one (X-axis).
MAPK, IL1B, and PPARA), the catabolism of lipids (e.g., PPARG and MTOR), intracellular antioxidant function (e.g., CAT and NFE2L2), mediation of estrogenic responses (e.g., ESR1), and vasodilatation and regulation of blood flow (e.g., NOS3). Within these targets, MAPK1 showed the highest connectivity with other proteins, followed by TP53, CASP3, IL6, JUN, and TNF. Interestingly, TNF and MAPK were also high enrichment pathways in the results of KEGG analysis, suggesting their importance in mediating the anti-OP effects of OA.

Furthermore, GO analysis found that the action of OA in OP mainly involves biological processes including inflammatory response, aging, response to estradiol, response to glucocorticoid, response to hydrogen peroxide, and so forth, which engage core targets in the network. Recent studies have shown a direct link between OP and the inflammatory response due to a malfunctioning immune system.

**Figure 3.** GO analysis of biological processes. Biological processes (Y-axis), gene number (X-axis), and P-value (color change).

**Figure 4.** GO analysis of cellular components. Cellular components (Y-axis), gene number (X-axis), and P-value (color change).

**Figure 5.** GO analysis of molecular functions. Molecular functions (Y-axis), gene number (X-axis), and P-value (color change).
Inflammatory cytokines such as IL1 and TNF, which play a crucial role in the inflammatory response, are involved in the OP pathological process by increasing bone resorption in osteoclasts or decreasing bone formation in osteoblasts, thereby disrupting the bone turnover balance.\(^{32,42}\) Similarly, aging and oxidative stress are also important factors in accelerating OP.\(^{38}\) Oxidative stress inhibits the differentiation of bone marrow stem cells into osteoblasts and activates osteoclasts, leading to a decrease in bone mass and bone strength.\(^{43}\) In addition, aging increases intracellular calcium levels and reduces the calcium transmembrane distribution gradient, leading to a reduction in absorption of calcium, and furthermore, long-term calcium deficiency exacerbates bone loss.\(^{44}\) The results of GO analysis suggest that OA may regulate these key protein targets in the network to regulate pathological processes associated with OP, such as diabetes, estrogen deficiency, inflammation, oxidative stress, and so forth.

Next, the data obtained from KEGG pathway analysis of target proteins uncovered that a majority of the enrichment pathways were associated with OP, such as TNF signaling pathway, MAPK signaling pathway, toll-like receptor signaling pathway, p53 signaling pathway, HIF-1 signaling pathway, osteoclast differentiation, apoptosis, insulin resistance, and PI3K-Akt signaling pathway.

To our knowledge, MAPK is an essential signal transduction pathway in living organisms, which is involved in physiological processes such as cell growth, differentiation, and proliferation, as well as regulating osteoclast differentiation and thus affecting the development of OP.\(^{33}\) Studies have confirmed that MAPK directly participated in the differentiation of osteoblast precursor cells into mature osteoblasts upon activation.\(^{45,46}\) The TNF signaling pathway, which mediates the chronic inflammation-related bone remodeling process, can regulate the bone resorption–bone remodeling balance by participating in the RANK/RANKL/OPG pathway; thus, it is an important part of ameliorating OP.\(^{42}\) The PI3K/Akt pathway regulates the survival and differentiation of osteoblasts and osteoclasts to maintain bone mass and bone turnover homeostasis.\(^{47}\) Specifically, it promotes the expression of osteoblastic differentiation markers such as alkaline phosphatase (ALP) and BMP-2 to regulate osteoblasts.\(^{47}\) Toll-like receptors are a class of nonspecific immune receptors that mediate toll-like signaling pathways, which affect osteoblast differentiation, proliferation, mineralization, and apoptosis via activating ERK, p38, JNK, NF-κB, and other osteogenic-related pathways.\(^{48}\) The HIF-1 signaling pathway inhibits the activation of osteoclasts and attenuates the development of OP.\(^{49}\) Thus, the pharmacological mechanism of OA in the pathogenesis of OP might be mainly benefited through regulating these pathways, among which MAPK is the most significantly enriched pathway. Besides, there were interactions among the crucial pathways, which could synergically regulate the related biological processes of OP.

Molecular docking studies further provided a visual explanation of the interaction between OA and its predicted protein targets related to OP. Docking analysis provided evidence that all 16 key proteins act as targets for OA in osteoporosis. In addition, results also showed that hydrophobic interaction, hydrogen bonding, and salt bridge were the main forms of interaction, which suggested the molecular mechanisms of OA in OP. Future studies may explore whether a certain method to measure the water content in OA prior to the experiment would contribute to more accurate results.\(^{50}\)

The experimental validation in this study utilized d-galactose-induced oxidative stress combined with a low-calcium diet to construct an animal model of low-conversion osteoporosis.\(^{20}\) As shown in micro-CT images, OA significantly alleviated osteoporosis in mice in a dose-dependent manner. Moreover, OA could enhance the bone dry to wet ratio by
promoting bone salt deposition and accelerating calcification, thereby improving the bone microarchitecture. Our previous in vitro studies have demonstrated that OA could enhance the cell viability and upregulate the OPG mRNA expression in osteoblasts and regulate the phosphorylation of ERK, JNK, p38, and AKT, which further verified that the

Figure 7. Molecular docking models of OA binding to its potential target proteins (A) MAPK1, (B) TP53, (C) CASP3, (D) IL6, (E) JUN, (F) TNF, (G) PTGS2, (H) MYC, (I) NOS3, (J) IL1B, (K) PPARG, (L) CAT, (M) ESR1, (N) BCL2, (O) CASP8, and (P) CYCS. The yellow stick models represent the OA molecule, and the blue sticks represent residues in the protein binding site. The gray dashed lines indicate hydrophobic interaction, solid blue lines indicate hydrogen bonds, and yellow dashed lines indicate salt bridge, with the interaction distances indicated next to the bonds.
osteogenic effects of OA are related to MAPK and AKT signaling pathways. All of these results validated the potential role of OA on antiosteoporosis from in vivo and in vitro perspectives.

4. CONCLUSIONS

In summary, this study focused on screening and analyzing the key targets and pathways of OA’s anti-OP action through network pharmacology to further clarify the mechanism of its therapeutic effects. Our results predict that OA could alleviate OP via multiple targets and multiple pathways, suggesting possibilities for the treatment of OP and providing a reference for in vitro and in vivo validation trials of OA based on these targets.

5. MATERIALS AND METHODS

5.1. Obtaining the Targets Associated with OA and OP. The chemical structure of OA was retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov) (Figure 1A). The genes related to OP were searched from the National Center for Biotechnology Information (NCBI) Gene Database (https://www.ncbi.nlm.nih.gov/), GeneCards (https://www.genecards.org/) and DisGeNet (https://www.disgenet.org/) with the search term “osteoporosis” and the species Homo sapiens. The target proteins of OA were searched from the Genecards (relevance score > 30), Stitch (combined_score > 0.7), and Swisstarget (probability > 0.4). Then, we used Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) to obtain the candidate targets for OA to alleviate OP.

5.2. Analyzing the Protein–Protein Interactions Among the Obtained Targets. To analyze the protein interaction network of the candidate targets, the target symbols were uploaded into the String database (https://string-db.org/) with the species option set as Homo sapiens and the confidence score set greater than 0.7.

5.3. Enrichment Analysis of the Candidate Targets. The abovementioned candidate targets were entered into the DAVID (https://david.ncifcrf.gov/home.jsp) for Gene Ontology (GO) functional enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway enrichment analysis on the targets (\(P < 0.05\)). The species option was set as Homo sapiens, and the analysis about the cell components, molecular functions, biological process, and signaling pathways involving the targets was conducted. We then used the Bioinformatics website (http://www.bioinformatics.com.cn/) for the graphs.

5.4. Docking Analysis. The precision of docking between OA and the abovementioned candidate target proteins was tested using Autodock Tolls 4.2 software (La Jolla, CA, US). The X-ray crystal structures of predicted targets were obtained from the RCSB Protein Data Bank (http://www.pdb.org/).

Table 2. Information on Molecular Docking

| no. | proteins | RCSB PDB ID | affinity (kcal/mol) |
|-----|----------|-------------|---------------------|
| A   | MAPK1    | 6g9m        | −9.5                |
| B   | TP53     | 1gzh        | −7.7                |
| C   | CASP3    | 3del       | −8.2                |
| D   | IL6      | 1aln       | −7.9                |
| E   | JUN      | 6y3v        | −8.1                |
| F   | TNF      | 6op0        | −7.1                |
| G   | PTGS2    | 5019        | −8.8                |
| H   | MYC      | 4y7r        | −9.3                |
| I   | NOS3     | 4d1p        | −9.7                |
| J   | IL1B     | 5r8j        | −8.4                |
| K   | PPARG    | 3boq        | −8.4                |
| L   | CAT      | 1dgg        | −9.3                |
| M   | ESR1     | 1xp1        | −7.3                |
| N   | BCL2     | 6o0k        | −8.7                |
| O   | CASP8    | 4prz        | −8.9                |
| P   | CYCS     | 3cef        | −6.9                |

Figure 8. Effects of OA on the bone index (A) and bone dry–wet ratio (B) in mice of each group. The left femur and humerus of all mice were taken, and the soft tissue was removed. Then, the bones were weighed wet and placed in an 80 °C incubator. The bone dry weight was weighted after 72 h. Bone index (mg/g) = bone wet weight/body weight × 1000; bone dry–wet ratio (%) = bone dry weight/bone wet weight × 100%. Compared to the control group, *\(P < 0.05\) and **\(P < 0.01\); compared with the model group, ▲\(P < 0.05\) and ▲▲\(P < 0.01\).
Moreover, the 3D structure of OA was generated with ChemBioDraw 3D. Then, Pymol software (Portland, OR, US) and Autodock software were used to modify the structures by removing waters, removing ligands, adding hydrogen, and optimizing and patching amino acids. The Autodock vina in Autodock Tolls was used to calculate the score of docking between OA and the candidate targets. The best docking poses models with RMSD ≤ 2 were considered accurate. Visualization of the docking results was conducted using the protein−ligand interaction profiler (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index) and Pymol software.56

5.5. Experimental Validation.

5.5.1. Animals. Forty 10 week-old female Kunming mice (body weight = 40 ± 5 g) were purchased from Beijing Vital River Laboratory Animal, Inc. (Beijing, China) and housed with daylight as the only source of illumination and with facilities to maintain temperature (22 ± 1 °C) and humidity (55 ± 5%). All animals had free access to food and water. After acclimation for 7 days, all mice were randomly divided into five groups (n = 8 per group): control group, model group, and OA-treated groups (20, 40, and 80 mg/kg). Except for the control group, which was injected with normal saline and fed with a standard diet (the exact percentage of calcium in the diet was 1.1%), the mice in other groups were administered with D-galactose injection (10 mg/kg/d) and fed with a low-calcium diet (the exact percentage of calcium was 0.1%). The OA-treated mice were simultaneously gavaged with low concentration OA (OA-L), middle concentration OA (OA-M), and high concentration OA (OA-H). Conversely, the control group and model group were daily administered with vehicle (corn oil) by gavage. The experiment was conducted for 20 days, and the Animal Care Committee of China Agricultural University, Beijing (AW41401202-2-1), approved all procedures.

5.5.2. Analysis of the Bone Index and Bone Dry−Wet Ratio. After sacrifice, the humerus and femurs of all mice were harvested with soft tissues removed and stored at ~80 °C for further analysis. The left humerus and femurs of mice were weighed, and the results obtained were recorded as wet weight. They were then dried in an incubator at 80 °C. After 72 h, the bones were weighed and recorded as dry weight. Bone index (mg/g) = bone wet weight/body weight × 1000; bone dry−wet ratio (%) = bone dry weight/bone wet weight × 100%.

5.5.3. Analysis of the Bone Microarchitecture by Micro-computed Tomography (Micro-CT). The trabecular bone of right femoral metaphysis of mice was scanned with micro-CT (SIEMENS, Munich, Germany). The micro-CT analysis was performed with a total rotation of 360°, an exposure time of 1500 ms, an effective pixel size of 9.21 μm, and a high system magnification. The raw data were generated using the micro-CT system’s scan reconstruction software COBRA_Excim (EXXIM Computing Corp., Livermore, CA). The imaging analysis software Inveon Research Workplace (IRW, SIEMENS, Munich, Germany) and the Mimics (Materialise Inc., Leuven, Belgium) were then used to generate the medical digital imaging and communications (Dicom) format files and to perform image reconstruction and screenshots.

5.5.4. Statistical Analysis. SPSS software (Version 20.0, SPSS Inc., Chicago, IL, USA) was used to analyze the in vivo data. One-way analysis of variance (ANOVA) followed by Dunnett's test was used to determine the statistical difference between multiple groups. Results were expressed as mean ± SD where n = 8; P < 0.05 was considered significant.

Figure 9. Micro-CT images of the femurs in each group of mice (A−E). D-galactose (10 mg/kg/d) was administrated by subcutaneous injection, combined with a low-calcium diet (0.1% Ca) to accelerate bone loss. Meanwhile, the mice were orally administrated with OA (20, 40, and 80 mg/kg) for 20 days. After euthanasia, the right femurs of all mice were imaged with a micro-CT machine.

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Notes
The authors declare no competing financial interest.

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