Osteoking downregulates Mgp in an osteoporotic fracture rat model

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Abstract

OBJECTIVE: To investigate the effectiveness of osteoking, a Traditional Chinese Medicine originating from Yi nationality, against osteoporosis (OP) and osteoporotic fracture (OPF), and to elucidate its mechanism of action.

METHODS: An osteoporotic fracture rat model was established; animals were divided into three treatment groups: parathyroid hormone, osteoking and 0.9%NaCl. After 4, 8 and 12 weeks of treatment, serum and bone tissues were collected. Enzyme-linked immunosorbent assay, x-ray, histopathological evaluation and proteomics were used. Proteomics and GO annotation were performed based on identified peptides. The relative network was obtained from the STRING database and verified by polymerase chain reaction and Western blotting.

RESULTS: After osteoking treatment, the bone mineral density (BMD) increased with time in the osteoking group. At week 12, the BMD and bone mineral salt content of the osteoking group were 4.5% and 20.6% higher than those of the negative control group, respectively. Furthermore, the body
weight followed the order of positive control group > osteoking group > negative control group, with significant differences among the groups (P < 0.05). Micro-CT analysis of femur sections revealed that the bone surface/volume ratio was significantly higher in the osteoking group than that in the negative control group. X-ray images demonstrated that the osteoking group showed clear callus. Moreover, high-voltage micro-CT demonstrated a massive cortical bone accumulation in the osteoking group. The gray values of callus in the osteoking group were higher than those in the negative group. From week 4 to 12, the serum bone alkaline phosphatase level increased by 49.6% in the osteoking group and the serum propeptide of type I procollagen level decreased by 80.6%. Alizarin red staining demonstrated that the calcium deposition in the osteoking group was higher than that in the negative control group. Notably, the expression of Mgp, a key osteogenesis inhibitor, was lower in the osteoking group compared with the negative control group. Moreover, Sparc, bone morphogenetic protein-2 and Bglap expression was higher in the osteoking group through activation of the transforming growth factor-receptor activator of nuclear factor kB Ligand pathway.

CONCLUSION: Osteoking treatment increased bone quality and promoted calcium deposition. The results suggest that osteoking inhibits Mgp through the TGF-β/RANKL pathway to improve OP/OPF.

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Keywords: Osteoporosis; Osteoporotic fractures; Matrix Gla protein; Osteoking

INTRODUCTION

Osteoking is a Traditional Chinese Medicine (TCM). According to TCM theory, osteoking mainly regulates the kidneys and spleen to nourish the bone. It consists of seven herbal sources and turtle shell. It is used in the clinic to heal fractures and treat femoral head necrosis.12 Briefly, it is prescribed to accelerate blood circulation, ease pain and exert anti-inflammatory effects. Osteoporosis (OP) is characterized by decreased bone mass and degeneration of the bone microstructure.1 OP is a systemic metabolic bone disease that increases skeletal brittleness and the risk of fractures. It is estimated that almost 200 million people worldwide suffer from OP.2 With the increasing pace of population ageing, the number of people in China over 60 years old increased from 178 million to 221 million from 2011 to 2015. People withOP suffer from pain, physical activity and functional limitations, and psychological pressure. In addition, OP may cause osteoporotic fracture (OPF), a more serious disease.3 The high incidence of OPF is associated with high mortality (OPF-related mortality is more common than mortality associated with breast cancer, cervical cancer and uterine cancer), high morbidity and high treatment costs.4,5

Currently, treatments for OP include phosphate, oestrogen replacement, and selective oestrogen receptor modulators. Most of these therapeutics are inconvenient, must be taken long term and some have side effects.6-10 Interestingly, TCM has potential for OP treatment. For example, corydices, dried tangerine and Epimedium have been shown to prevent and treat OP.11-15 Osteoking may improve OP/OPF. Preliminary studies have indicated that it had a positive therapeutic effect on OP rats and improved the prognosis of fracture patients in the clinic.16-19 In this study, we investigated the effectiveness of osteoking on OP and OPF, and attempted to elucidate its mechanism of action.

MATERIALS AND METHODS

This study was approved by the Animal Experimental Ethical Committee of Kunming Medical University (No. KMMU 20150007).

Drugs and reagents

Osteoking was prepared according to the Chinese Pharmacopeia (China Pharmacopeia Committee, 2002) and was supplied by Crystal Natural Pharmaceutical Co. (No. 20160506, Kunming, China). Its formula is as follows: Chenpi (Pericarpium Citri Reticulatae), Honghua (Flos Carthami), Sanqi (Radix Notoginseng), Duzhong (Cortex Eucommiae), Renshen (Radix Ginseng), Huangqi (Radix Astragali Mongolici) and Biejeia (Carapa Trionycis). The above materials were ground into a coarse powder and immersed in 10-fold distilled water for 12 h at room temperature, and then boiled using a distillation apparatus for 1 h. This process was repeated twice for the second and third extractions, the residue from the previous extraction was filtered and the same extraction procedures were applied. Thereafter, the combined extracts were filtrated and evaporated using a rotary evaporator at 50 °C to a relative density of 1.03 g/cm³, centrifuged for 30 min at 1450 × g and the obtained supernatant was centrifuged once again after precipitation for 12 h. Subsequently, distilled water was added to a total volume of 1000 mL as the clinical use concentration. The parathyroid hormone (1-34 PTH) was supplied by Karebay Biochem Inc. (Purity 95%; Shanghai, China). The antibodies used were: anti-β-actin clone AC-15 (A1978; Sigma, St Louis, MO, USA), anti-Mgp (10734-1-AP), anti-bone morphogenetic protein-2 (BMP-2) (18933-1-AP), an-
ti-Sparc (15274-1-AP), anti-Bglap (23418-1-AP), anti-TGF-β/(21898-1-AP; Proteintech, Rosemont, IL, USA).^{14}

**Animals**

All animal experiments were approved by the Animal Study Committee of Kunming Medical University and were conducted according to the requirements of the National Institutes of Health Guidelines for care and use of laboratory animals. Three-month-old Sprague Dawley (SD) female rats [(270 ± 15) g; Dossy Co., Chengdu, China] were maintained in standard conditions with a controlled temperature (21-23 °C) and a strict 12-h light/dark cycle. All the rats were fed with standard rat chow and allowed free access to distilled water ad libitum at all times during acclimatization and experimental treatment periods.

**Rat OP and OPF models**

After 7 d of adaptation, animals were randomly divided into bilateral ovariectomy (OVX) group and sham-surgery group. Briefly, eight rats underwent bilateral laparotomy (sham rats) and 62 rats were subjected to OVX (OVX rats). Preoperative, all animals were fasted for 12 h. Benzylpenicillin sodium (60 000 IU/kg; Harbin Pharmaceutical Co., Harbin, China) was administered for 3 consecutive days after operation. However, eight rats from the OVX group died following surgery. After 23 weeks, rats in the OVX group and sham group were inspected with dual-energy X-ray. The bone mineral density (BMD) of the OVX group was (0.177 ± 0.006) g/cm² and that of the sham group was (0.188 ± 0.008) g/cm² (P < 0.05), indicating that the rat OP model was successfully duplicated. All rats in the OVX group underwent right femur internal fixation under general anaesthesia with an intraperitoneal injection of sodium pentobarbital (30 mg/kg; Shanghai Westang Biotech Co., Shanghai, China). When the right femur was exposed, the middle of the femur was broken with a dental sander (Saeshin Co., Daegu, South Korea). Then, a no. 12 Kirschner wire was inserted in an antidromic manner into the bone stumps to fix the bone. The OPF rat model was established.

**Experimental protocol**

Fifty-four OPF model rats were randomly divided into three groups (n = 18 per group): a positive control group treated with 0.33 g·kg⁻¹·2d⁻¹ subcutaneous (s.c.) 1-34 PTH, a negative control group treated with 0.59 mL·kg⁻¹·2d⁻¹ intragastric (i.g.) 0.9%NaCl (Baxter Co., Shanghai, China), and an osteoking group treated with 0.59 mL·kg⁻¹·2d⁻¹ i.g. osteoking. The dosage is equivalent to human clinical usage, which depends on the rat’s weight and surface area, as well as the conversion coefficient of rat vs human. The formula is 6.3 × weight × the clinic dosage × 0.9 (6.3 is the rat-human conversion coefficient; 0.9 is the weight and surface area coefficient) according to a reported method.^{15}

**BMD analysis**

Whole-body BMD was measured using dual-energy X-ray absorptiometry (DXA; Lunar Prodigy Advance; GE Lunar, New York, USA). Specific software for small animals (GE Medical Systems, New York, USA; enCORE 2004 software, version 8. 80. 001) was used.

**X-ray evaluation**

All rats were checked with diagnostic X-ray machines (RWD Animal Experiment Instrument Co., Shenzhen, China) after internal femur fixation at 4, 8 and 12 weeks after treatment. Both sides of the femurs were imaged from the back.

**Biochemical serum analysis**

Blood samples were allowed to clot for 2 h at room temperature before centrifugation for 15 min at 1000 × g. The serum was promptly separated and stored at −80 °C prior to the assay. The serum concentrations of bone alkaline phosphatase (BALP), propeptide of type I procollagen (PINP), tartrate-resistant acid phosphatase 5b (TRACP-5b) and C-telopeptide of type II collagen (CTX-Ⅱ) (Casbio Co., Wuhan, China) were measured using rat enzyme-linked immunosorbent assay (ELISA) kits in an M200 PRO apparatus (TECAN Ltd., Mannedorf, Switzerland). All data were plotted and analyzed by Curve Expert software (Version 1.4, Chicago, IL, USA). All samples were run in the same assay unless an individual value required repeating.

**Histopathological evaluation**

At weeks 4, 8 and 12 after treatment, two sides of the femur and the third lumbar spine vertebrae were harvested and stored at −20 °C. Next, samples were fixed in 10% paraformaldehyde (Gefan Biotechnology Co., Shanghai, China) for 14 d, dehydrated and gradually decalcified. Five-micrometre-thick sections were prepared using a Leica RM2245 microtome and were stained with hematoxylin and eosin (HE; Servicebio Co., Wuhan, China) and Alizarin red (Sigma Co., Shanghai, China).

**Micro-computed tomography (Micro-CT) analysis**

Micro-CT analysis was performed according to recent guidelines^{16} using a SkyScan 1176 micro-CT imaging system (SkyScan, Antwerp, Belgium) with a spatial resolution of 17.75 μm (X-ray source 70 kV/357 μA, 90 kV/270 μA; exposure time 250 ms/360 ms; magnification ×15; 1.0 mm aluminium/0.1 mm copper filter). Volumetric reconstructions and analysis were performed using the built-in software, NRecon 1.6 and CTAn 1.8 (SkyScan). For bone regeneration analysis, the volume of interest was measured by the average grayscale at the specific bone position (minimum to maximum degree: 0-255).

**Proteome and enrichment analysis**

After 12 weeks of osteoking treatment, the protein expression in the osteoking group and the negative control group was measured by label-free proteome analy-
sis (Genecreate Co., Wuhan, China). The basic principle was based on the extraction peak area (XIC) of the peptide segment mother ion, then the peptide segment and protein in the sample were identified, and the identified peptide fragment/protein were quantitatively analyzed. Two groups were designed as the osteoking group and negative control group. After redundancy, the samples contained 36728 protein sequences from the Uniprot database. The protein was quantitatively analyzed by Skyline software (Beijing, China) and the difference multiples \[ |F| \geq 2 \] (The STRING database: https://string-db.org/). The results of Go and KEGG enrichment were analyzed by DAVID (https://david.ncifcrf.gov/) and figures were manufactured by Omicshare (https://www.omicshare.com/tools/).

**Quantitative PCR**

Total RNA from cells and tissue samples was separately isolated using TRIzol Reagent (Roche Co., Basel, Switzerland) according to the manufacturer’s instructions. The primers were designed according to NCBI database sequences and checked by Oligo Calc. (http://biotools.nubic.northwestern.edu/OligoCalc.html). For quantitive RT-PCR, cDNA was prepared from 2 g of RNA using a prime script RT reagent kit and analyzed with SYBR GreenMaster Mix (TaKaRa, Japan) using an LC480 real-time PCR system (Roche). Data were quantified using the relative quantitative \[ 2^{-\Delta\Delta CT} \] method and were normalized by \( \beta \)-actin expression.

**Statistical analysis**

All data were processed with SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA) and the values were expressed as the mean ± standard deviation (\( \bar{x} \pm s \)). One-way analysis of variance followed by Tukey’s post hoc test was conducted to test differences between groups. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

### Osteoking increases bone quality in OPF rat model

First, we examined the effects of osteoking on bone formation by administering 0.59 mL/kg osteoking to OPF rats (35 weeks old; right femur internal fixation) for 12 weeks. A positive control group treated with 1-34 PTH and a negative control group treated with 0.9% NaCl were included for comparison. The BMD increased from week 4 to 12 in the osteoking group. At week 12, compared with the negative control group, the BMD in the osteoking group was significantly increased by 4.5%, and the BMC in the osteoking group was increased by 20.6% (Table 1 and Figure 1A). After 12 weeks of osteoking treatment, the rats’ body weight followed the order: positive control group > osteoking group > negative control group, and the differences among the groups were significant \( (P < 0.05) \). Particularly, compared with the negative control group, the osteoking group showed a significantly higher body weight at week 12 (Table 2). Moreover, there were more trabecular bone structures in the osteoking group (Figure 1B, 1C). Micro-CT analysis of femur sections revealed that the osteoking group had better bone quality and the BV/TV was significantly higher in the osteoking group than in the negative control group (Figure 1D).

**Osteoking promotes fracture healing**

After osteoking treatment, compared with the negative control group, X-ray images demonstrated that the osteeking group had clearer osteotylus (Figure 2A). After 12 weeks of osteoking treatment, high-voltage micro-CT revealed that there was a massive accumulation of cortical bone in the osteoking group. The gray values of the callus in the osteoking group were higher than those in the negative group (Figure 2B, 2C). ELISA was performed to assess serum markers of bone turnover. From week 4 to 12, the serum level of BALP, a marker of bone formation, increased by 49.6% in the osteoking group. Furthermore, the BALP levels were higher in the osteoking group than in the negative control group. In contrast, the serum level of PINP, a marker of bone resorption, decreased by 80.6% (Table 3 and Figure 2D). Serum markers of osteoclasts were at low levels, below the sensitivity of the kits (0.078 ng/mL).

### Table 1 BMD of OPF rats (\( \bar{x} \pm s \))

<table>
<thead>
<tr>
<th>Week</th>
<th>Group</th>
<th>Positive control</th>
<th>Negative control</th>
<th>Osteoking</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>BMD (g/cm^2)</td>
<td>0.177±0.006</td>
<td>0.177±0.006</td>
<td>0.177±0.006</td>
</tr>
<tr>
<td></td>
<td>BMC (mg)</td>
<td>7.54±0.523</td>
<td>7.54±0.523</td>
<td>7.54±0.523</td>
</tr>
<tr>
<td>4</td>
<td>BMD (g/cm^2)</td>
<td>0.205±0.010*</td>
<td>0.178±0.004</td>
<td>0.182±0.010</td>
</tr>
<tr>
<td></td>
<td>BMC (mg)</td>
<td>9.23±0.586*</td>
<td>8.25±0.569*</td>
<td>7.67±0.866</td>
</tr>
<tr>
<td>8</td>
<td>BMD (g/cm^2)</td>
<td>0.205±0.008*</td>
<td>0.177±0.004*</td>
<td>0.186±0.007</td>
</tr>
<tr>
<td></td>
<td>BMC (mg)</td>
<td>8.92±0.680*</td>
<td>7.75±0.947*</td>
<td>7.93±1.380</td>
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<tr>
<td>12</td>
<td>BMD (g/cm^2)</td>
<td>0.205±0.011*</td>
<td>0.179±0.009*</td>
<td>0.187±0.002</td>
</tr>
<tr>
<td></td>
<td>BMC (mg)</td>
<td>11.26±2.050*</td>
<td>7.85±1.103*</td>
<td>9.46±1.850</td>
</tr>
</tbody>
</table>

Notes: positive control group was treated with 0.33 g/kg \( \cdot 2 \text{d} \) PTH; Negative control group was treated with 0.59 mL \( \cdot \) kg \( \cdot 2 \text{d} \) \( 0.9\% \text{NaCl} \); Osteoking group was treated with 0.59 mL \( \cdot \) kg \( \cdot 2 \text{d} \) osteoking. BMD: bone mineral density; BMC: bone mineral salt content; OPF: osteoporotic fracture. Values are expressed as the mean ± standard deviation (\( n = 6 \)). *\( P < 0.05 \) vs the osteoking group.
Figure 1: Osteoking increased the bone quality of rats with an osteoporotic fracture. A: the bone mineral density results which analyzed by Dual energy x-ray absorptiometry. B1-B3: the decalcified sections of the third lumbar which stained by hematoxylin-eosin (HE) after 12 weeks of administration. Scale bar, ×100, 1 mm. C1: positive control; C2: negative control; C3: osteoking. C, D: the representative images of femur which analyzed by Micro-CT. Scale bar, ×1 mm. Micro-CT: microX-ray computed tomography. The values were expressed as the mean ± standard deviation (n = 6). *P < 0.05 vs the osteoking group.

Table 2: Body weight of osteoporotic fracture rats (g)

<table>
<thead>
<tr>
<th>Week</th>
<th>Positive control</th>
<th>Negative control</th>
<th>Osteoking</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>447±60</td>
<td>447±60</td>
<td>447±60</td>
</tr>
<tr>
<td>4</td>
<td>422±35</td>
<td>436±41</td>
<td>430±99</td>
</tr>
<tr>
<td>8</td>
<td>453±63</td>
<td>426±32</td>
<td>455±53</td>
</tr>
<tr>
<td>12</td>
<td>470±66</td>
<td>421±49</td>
<td>440±57</td>
</tr>
</tbody>
</table>

Notes: positive control group was treated with 0.33 g·kg⁻¹·2d⁻¹ PTH; Negative control group was treated with 0.59 mL·kg⁻¹·2d⁻¹ 0.9% NaCl; Osteoking group was treated with 0.59 mL·kg⁻¹·2d⁻¹ osteoking. Values are expressed as the mean ± standard deviation (n = 6). *P<0.05 vs the osteoking group.

Osteoking inhibits Mgp and promotes calcium deposition

Alizarin red staining of bone sections demonstrated that calcification in the osteoking group was higher than that in the negative control group (Figure 3A). Proteomics analysis of rat femoral head specimens from the osteoking and negative control groups showed an obvious difference in matrix Gla protein (Mgp) expression. GO annotation and relative pathway enrichment analysis were performed based on identified peptides and proteins (Figure 3B). The results indicated that the osteoking effect on OP/OPF was enriched in the TGF-β/signaling pathway. A relative network was obtained from the STRING database (Figure 3C) and the key Mgp-regulated proteins were BMP-2, Sparc, and Bglap. The differential expression of Mgp was verified by PCR and western blot analyses. Mgp, a vitamin K-dependent protein, can inhibit osteogenic calcification. Mgp expression was significantly lower in the osteoking group than in the negative control group (Figure 3D). The expression of its regulated proteins, such as Sparc, BMP-2 and Bglap, was higher in the osteoking group through activation of the TGF-β/RANKL pathway (Figure 3E). Mgp suppression improved OP/OPF.
micro-CT examination revealed that osteoking in the osteoking treatment. Microscopy observations and BMD and bone mineral salt accumulation increased after osteoking treatment. Classical double-energy X-rays demonstrated that the tree shrew model have confirmed the results in rats. Osteoking improved the bone mass in rats lent to the human routine application was used on rat models. Osteoking had a significant positive effect on OPF in rats. Moreover, we simulated fracture of the right femur in OPF rats with an intramedullary nail. The X-rays results indicated better recovery of OPF rats after administration of osteoking. High-voltage micro-CT scanning was used to reduce interference by the intramedullary metal nails. The results showed more calluses in the osteoking group than in the negative control group. Several studies have examined the efficacy of components of osteoking, such as turtle shell, Ginseng and Astragalus, in treating OP. It was increased the trabecular number and bone volume fraction. Osteoking had a significant positive effect on OPF in rats. Moreover, we simulated fracture of the right femur in OPF rats with an intramedullary nail. The X-rays results indicated better recovery of OPF rats after administration of osteoking. High-voltage micro-CT scanning was used to reduce interference by the intramedullary metal nails. The results showed more calluses in the osteoking group than in the negative control group. Several studies have examined the efficacy of components of osteoking, such as turtle shell, Ginseng and Astragalus, in treating OP. It was

DISCUSSION

In the current study on osteoking, to elucidate the mechanism behind its action, a single dosage equivalent to the human routine application was used on rat models. Osteoking improved the bone mass in rats with OP, and the results obtained from the biological tree shrew model have confirmed the results in rats. Classical double-energy X-rays demonstrated that the BMD and bone mineral salt accumulation increased after osteoking treatment. Microscopy observations and micro-CT examination revealed that osteoking increased the trabecular number and bone volume fraction. Osteoking had a significant positive effect on OPF in rats. Moreover, we simulated fracture of the right femur in OPF rats with an intramedullary nail. The X-rays results indicated better recovery of OPF rats after administration of osteoking. High-voltage micro-CT scanning was used to reduce interference by the intramedullary metal nails. The results showed more calluses in the osteoking group than in the negative control group. Several studies have examined the efficacy of components of osteoking, such as turtle shell, Ginseng and Astragalus, in treating OP. It was
concluded that other components of osteoking improved OP/OPF.

The proteome analysis revealed Mgp as a significantly changed protein. A relative network of the mechanism of osteoking was obtained from the STRING database and the upstream and downstream targets of Mgp were showed.

Mgp is a vitamin K-dependent protein that belongs to the mineral-binding Gla protein family. Mgp is a mineralization inhibitor in the bone. Osteocalcin (OC or bone Gla protein, Bgp) and Mgp have similar protein domains and gene organization and control tissue mineralization. Mgp acts as a co-enzyme of γ-glutamyl carboxylase (GGCX), which carboxylates undercarboxylated vitamin K-dependent proteins. In vivo, Mgp binds calcium ions and hydroxyapatite via its five γ-carboxylglutamic acid (Gla) residues, leading to the inhibition of matrix mineralization.

Mgp has been shown to modulate the biological activity of members of the TGF-β super family, such as BMPs. Related studies have shown that Mgp exerts a dose-dependent inhibitory effect on osteoblast differentiation through interference with the binding of BMP-2/4 to their receptors. Other studies have shown Mgp expression in other tissues, such as the trachea, lungs, kidneys, brain, skin and eyes, and that it has potential roles in tumour pathology.

In our study, there was lower Mgp expression in the osteoking group, and OPF rats had increased bone mineral salt deposition. Lower Mgp expression stimulates osteogenesis and has a beneficial effect on OP. Bglap and Sparc are also important proteins downstream of Mgp. Bglap has a relationship with Tnfsf11b and Runx-2 in the RANKL pathway, which promotes osteoblast maturation and inhibits osteoclast activity. Sparc regulates PTH, which has been proven to ameliorate OP in vivo. These proteins’ relationships with Mgp were confirmed in the current study.

Greater bone mineral salt deposition and better recovery of OPF rats was observed after osteoking treatment. Osteoking accelerates fracture healing and improves OP. Osteoking is a potential medication for treating OPF in the clinic.

Osteoking is a multi-component TCM and its components are natural and common. According to the pharmacological analysis of single component therapies, it is important to find single-component targets. However, TCM has its unique pharmacological theory, and in the case of osteoking, it is hard to explain which component works and whether these components interact with each other.

In conclusion, osteoking had positive effects on OP/OPF in rat models. It improved OP/OPF by inhibiting Mgp and promoted calcium deposition through the TGF-β/RANKL signaling pathway.

ACKNOWLEDGEMENTS

Thank everyone of our team for their works.

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Table 3 BALP, PINP, TRACP-5b and CTX-II of osteoporotic fracture rats

<table>
<thead>
<tr>
<th>Week</th>
<th>Group</th>
<th>BALP (U/L)</th>
<th>PINP (pg/mL)</th>
<th>TRACP-5b (mU/mL)</th>
<th>CTX-II (ng/mL)</th>
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<td>4</td>
<td>Positive control</td>
<td>25.510±4.650‘</td>
<td>128.130±16.900‘</td>
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<td>0.710±0.010‘</td>
<td>0.120±0.010</td>
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<td>16.300±1.730‘</td>
<td>213.220±26.940‘</td>
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<td>8</td>
<td>Positive control</td>
<td>12.490±2.620‘</td>
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<td>1.340±0.030</td>
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<td>-</td>
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<td>22.370±8.590‘</td>
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<td>41.360±17.780</td>
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</tbody>
</table>

Notes: positive control group was treated with 0.33 g·kg^-1·2d^-1 PTH; negative control group was treated with 0.59 mL·kg^-1·2d^-1 0.9% NaCl; osteoking group was treated with 0.59 mL·kg^-1·2d^-1 osteoking. BALP: bone alkaline phosphatase; PINP: propeptide of type I procollagen; TRACP-5b: tartrate-resistant acid phosphatase 5b; CTX-II: C-telopeptide of type II collagen. Values are expressed as the mean ± standard deviation (n = 6). *P < 0.05 vs the osteoking group.
Figure 3 Osteoking inhibited Mgp and promoted calcium deposition
A1-A3: the decalcified sections of the third lumbar which stained by alizarin red. Scale bar, 200×, 1 mm. B1: positive control; B2: negative control; B3: osteoking. B1, B2: gene ontology and kyoto encyclopedia of genes and genomes enrichment analysis results. The results analyzed by the hypergeometric test. C: the network related to osteoking was analyzed by STRING database. D, E: the expression of Mgp and its relative targets which analyzed by quantitative polymerase chain reaction and Western blotting. Positive control group was treated with 0.33 g·kg⁻¹·2d⁻¹ PTH; negative control group was treated with 0.59 mL·kg⁻¹·2d⁻¹ 0.9% NaCl; osteoking group was treated with 0.59 mL·kg⁻¹·2d⁻¹ osteoking. PTH: parathyroid hormone. The values were expressed as the mean±standard deviation (n = 6).


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