Original Article

Ginsenoside Rc alleviates osteoporosis by the TGF-β/Smad signaling pathway

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Article Info

Abstract

Osteoporosis is a common chronic bone disorder in postmenopausal women. Ginsenosides are primary active components in ginseng and the effects of various ginsenoside variants in osteoporosis treatment have been widely revealed. We planned to explore the impact of ginsenoside Rc on bone resorption in an osteoporosis rat model. We used ovariectomized rats to assess the potential impact of ginsenoside Rc on osteoporosis. μ-CT was implemented for analyzing the microstructure of the distal left femur in rats. H&E staining together with Masson staining were applied for bone histomorphometry evaluation. ELISA kits were implemented to detect serum concentrations of TRACP-5b, OCN, CTX, as well as PINP. Ginsenoside Rc treatment lessened the serum levels of TRACP-5b as well as CTX, while increasing serum levels of OCN, and PINP of OVX rats. Moreover, we found that ginsenoside Rc contributed to the synthesis of type I collagen via increasing Col1a1 and Col1a2 levels in femur tissues of ovariectomized rats. Our findings also revealed that ginsenoside Rc activated the TGF-β/Smad pathway by increasing TGF-β as well as phosphorylated Smad2/3 protein levels. Ginsenoside Rc alleviates osteoporosis in rats through promoting the TGF-β/Smad pathway.

Keywords: Ginsenoside Rc, Type I collagen, Osteoblast, The TGF-β/Smad pathway, Osteoporosis.

1. Introduction

Osteoporosis is a prevalent chronic disease that results in an elevated risk of bone fractures in postmenopausal women [1]. Osteoporosis has features of decreased bone mass, injured bone structure and calcium malabsorption [2, 3]. The risk factors for osteoporosis include malnutrition, hormone fluctuation, inflammation, and mechanical stress [4]. Currently, most therapeutic agents for osteoporosis are based on inhibition of bone resorption [5, 6].

A previous study has revealed that the coupled dynamic balance between bone formation and bone resorption belongs to the basis for keeping the stability of bone morphology [5]. Type I collagen is composed of 2 α1 chains as well as 1 α2 chain, accounting for approximately 80-90% of the bone organic matter, which promotes bone homeostasis [7]. In addition, type I collagen belongs to the central part of bone extracellular matrix [8]. Increasing studies have revealed that the decreased synthesis of type I collagen along with the change in its morphology and stability will aggravate osteoporosis [9, 10].

Recently, the vital functions of Chinese herbs in osteoporosis treatment have been revealed. Guilu erxian glue comprises Testudinis Plastrum, Ginseng root, Cornu Cervi and Lycii Fructus, and has been indicated to exert favorable effects on degenerative joint diseases and perimenopausal syndrome [11]. Ginseng is one of the most valuable medicinal herbs. The primary active components in ginseng are ginsenosides containing two parts: the scaffold (sapogenin) and the sugar side chain(s). More than 600 ginsenosides have been identified, and the most common sapogenins are protopanaxadiols as well as protopanaxatriols [12]. Many variants of ginsenosides have been identified to exert protective roles in osteoporosis, for example, ginsenoside Rb2 reduces oxidative damage as well as bone-resorbing cytokines during osteogenesis to exert anti-osteoporotic effects [13]. Ginsenoside Rg3 promotes osteogenic differentiation by facilitating autophagy to attenuate ovariectomy-induced osteoporosis [14]. Ginsenoside Rh2 suppresses osteogenic differentiation from bone marrow macrophages by downregulation of NF-κB [15].

Ginsenoside Rc (molecular formula: C_{58}H_{90}O_{32}; CAS: 11021-14-0) exerts an anti-inflammatory effect on synovial cells in rheumatoid arthritis patients [16], a systemic autoimmune disease that can lead to cartilage destruction and systemic osteoporosis, which indicates the putative association of ginsenoside Rc with osteoporosis. We hypothesized that ginsenoside Rc can alleviate osteoporosis and investigated the impacts of ginsenoside Rc on bone microstructure as well as serum bone turnover markers of ovariectomized (OVX) rats. Moreover, influence of ginsenoside Rc on expression of osteoblast markers in primary
human osteoblasts as well as the downstream pathway of ginsenoside Rc were investigated.

2. Materials and methods

2.1. OVX rat model

Fifty Sprague-Dawley rats (about 220 g; 3-month-old) could be acquired from Vital River (Beijing, China) and fed at 20-24°C, 45-60% humidity, as well as 12 h light/dark cycle. Rats were separated into 5 groups: sham group, OVX group, OVX+estrogen group, OVX+ginsenoside Rc group, and as well as OVX+ginsenoside Rc+SB431542 group, with 10 rats in each group. The rats received fast for 12 h before surgery. 10% chloral hydrate solution (0.3 mL/100 g) was intraperitoneally injected into the rats for anesthesia. In addition to sham operation group (n=10), all the other experimental rats underwent lower abdominal incisions to remove their ovaries (n=40). Some rats in the positive control group were daily administered with 17β-estradiol (2 µg/kg) [17]. Ten weeks after operation, rats in the OVX group received intraperitoneal treatment with ginsenoside Rc (20 mg/kg, purity ≥ 98%, Aladdin, n=20) or 10% DMSO (n=10) every 2 days for 4 weeks. Rats in OVX+ginsenoside Rc+SB431542 group (n=10) received an intraperitoneal administration of ginsenoside Rc (20 mg/kg) and SB431542 (2.5 mg/kg), the inhibitor of TGF-β [18], every 2 days for 4 weeks. By the end of the trial, rats received sacrifice using thiopental (100 mg/kg). Abdominal aorta puncture was used to collect blood, and serum was separated for the evaluation of bone turnover markers. Femur tissues were collected for detection of bone mineral density (BMD). Samples of femur tissues and human osteoblasts were used for extracting proteins. The protein concentrations were assessed by a bicinchoninic acid protein assay kit (Beyotime, China), and then received reduction to 10% sodium dodecyl sulfate, polyacrylamide gel electrophoresis gel and shifted onto polyvinylidene fluoride membranes (Millipore, USA). Membranes were then sealed with 5% nonfat milk, followed by treatment with the primary antibodies at 4°C overnight. The primary antibodies were: COL1A1 (sc-293182, Santa Cruz Biotechnology), COL1A2 (sc-393573, Santa Cruz Biotechnology), ALP (ab224335, Abcam), OCN (ab133612, Abcam), p-Smad2 (ab188334, Abcam), BMP2 (ab214812, Abcam), TGF-β (ab215715, Abcam), Smad3 (ab40854, Abcam), and β-actin (ab6276, Abcam). Next, the membranes were cultured with secondary antibodies at room temperature for 1 h. β-actin acted to be the internal control. An enhanced chemiluminescence detection kit (Thermo Fisher Scientific) was adopted for visualizing protein bands and the ImageJ software was used for densitometry analysis of the band intensity.

2.4. Western blot analysis

RNA immunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) was used for extracting proteins from femur tissues and human osteoblasts. The protein concentrations were assessed by a bicinchoninic acid protein assay kit (Beyotime, China), and then received reduction to 10% sodium dodecyl sulfate, polyacrylamide gel electrophoresis gel and shifted onto polyvinylidene fluoride membranes (Millipore, USA). Membranes were then sealed with 5% nonfat milk, followed by treatment with the primary antibodies at 4°C overnight. The primary antibodies were: COL1A1 (sc-293182, Santa Cruz Biotechnology), COL1A2 (sc-393573, Santa Cruz Biotechnology), ALP (ab224335, Abcam), OCN (ab133612, Abcam), p-Smad2 (ab215715, Abcam), Smad3 (ab40854, Abcam), p-Smad3 (ab51177, Abcam), Smad2 (ab40855, Abcam), BMP2 (ab214812, Abcam), and β-actin (ab6276, Abcam). Next, the membranes were cultured with secondary antibodies at room temperature for 1 h. β-actin acted to be the internal control. An enhanced chemiluminescence detection kit (Thermo Fisher Scientific) was adopted for visualizing protein bands and the ImageJ software was used for densitometry analysis of the band intensity.

2.5. Bone histomorphometry evaluation

The proximal femur was immobilized in 10% methanol for 48 hours and received decalcification for four weeks, followed by being dehydrated and embedded in paraffin. Next, paraffin sections received deparaffinization and were subjected to hematoxylin and eosin (H&E) staining as well as Masson staining.
Ginsenoside Rc alleviates osteoporosis.

2.7. ALP activity assay
Rat femur tissues were harvested followed by culture for 72 h. After washing, an alkaline phosphatase assay kit (Nanjing Jiancheng Bioengineering Institute, China) could be adopted for measuring relative ALP activity using chemical colorimetry. The absorbance at 405 nm from each group was detected by virtue of a spectrophotometer.

2.8. Enzyme-linked immunosorbent assay (ELISA)
The blood was gathered, followed by receiving centrifugation to obtain the serum. The serum levels of tartrate-resistant acid phosphatase-5b (TRACP-5b), osteocalcin (OCN), C-terminal telopeptide of type 1 collagen (CTX), as well as procollagen I N-terminal propeptide (PINP) could be quantified using corresponding ELISA kits.

2.9. Statistical analysis
Statistical analysis was implemented by SPSS 19.0 software (IBM, USA). Data were exhibited as mean ± standard deviation. Differences were analyzed by t-test or one-way analysis of variance followed by Tukey’s post hoc test. P < 0.05 meant statistically significant.

3. Results
3.1. Ginsenoside Rc increases serum levels of OCN, and PINP levels, and reduces TRACP-5b together with CTX levels in OVX rats
Relative to sham group, the distal femurs of OVX rats exhibited less osteoid staining, indicating the successful establishment of OVX rodents. Estrogen or ginsenoside Rc treatment alleviates OP symptoms (Fig 1A). To determine whether ginsenoside Rc exerts effects on osteoporosis development, we measured the serum levels of bone turnover markers. OCN and PINP are bone formation markers, and TRACP-5b and CTX are bone absorption markers. As presented in Fig 1B-E, the decreased serum contents of OCN, PINP, and increased serum contents of TRACP-5b and CTX in OVX group were rescued by estrogen or ginsenoside Rc treatment. Furthermore, H&E staining illustrated that relative to the sham group, the trabecular region presented smaller, thinner, and sparser in the OVX group. Estrogen or ginsenoside Rc treatment rescued the effects of OVX surgery on histomorphology of femurs (Fig 1F). The BMD, BV/TV, COD, Tb.N, together with Tb.Th in the ROI in OVX rats presented apparently reduced relative to sham rats, while the BS/BV, SMI, and Tb.Sp presented significantly higher (Table 1). Treatment of estrogen rescued the effects of ovariectomy on these parameters in rats. Interestingly, ginsenoside Rc had rescue effects on these parameters except Tb.Th.

3.2. Ginsenoside Rc promotes the synthesis of collagen I
Fig 2A indicated that ginsenoside Rc induced upregulation of Alp mRNA in femurs of OVX rats. As presented in Fig 2B, the ALP activity was decreased in femur tissues of OVX rats, while ginsenoside Rc treatment reversed such effect. Coll1a1 and Coll1a2 mRNA as well as protein levels presented reduced in OVX rats but were increased after treatment of ginsenoside Rc (Fig 2C-E).

3.3. Effects of ginsenoside Rc on the TGF-β/Smad pathway
The TGF-β/Smad pathway is activated during osteogenic differentiation [20] and is inhibited during osteoporotic progression [21]. As indicated in Fig 3A-B, TGF-β, BMP2, p-Smad2, along with p-Smad3 protein levels were

**Table1.** Microarchitectural quantitative analysis results of an ROI in the femur trabecular bone.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OVX</th>
<th>OVX+estrogen</th>
<th>OVX+ginsenoside Rc</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD (mgHA/ccm)</td>
<td>655.17±19.4</td>
<td>489.79±17.5</td>
<td>612.87±15.1</td>
<td>598.67±14.9</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>0.29±0.03</td>
<td>0.04±0.01***</td>
<td>0.13±0.02**</td>
<td>0.08±0.01**</td>
</tr>
<tr>
<td>SMI</td>
<td>1.15±0.18</td>
<td>2.81±0.26**</td>
<td>1.78±0.19*</td>
<td>2.04±0.23*</td>
</tr>
<tr>
<td>COD (mm⁻³)</td>
<td>168.42±14.47</td>
<td>14.11±1.45***</td>
<td>82.33±5.99**</td>
<td>47.17±3.72**</td>
</tr>
<tr>
<td>BS/BV (mm⁻³)</td>
<td>34.53±1.18</td>
<td>58.21±3.75*</td>
<td>42.51±2.17**</td>
<td>45.81±2.81**</td>
</tr>
<tr>
<td>Tb.N (-1)</td>
<td>4.95±0.51</td>
<td>0.99±0.08***</td>
<td>3.58±0.28**</td>
<td>2.04±0.16**</td>
</tr>
<tr>
<td>Tb.Sp (mm)</td>
<td>0.20±0.014</td>
<td>1.03±0.072***</td>
<td>0.27±0.019**</td>
<td>0.49±0.032**</td>
</tr>
<tr>
<td>Tb.Th (mm)</td>
<td>0.071±0.006</td>
<td>0.055±0.004***</td>
<td>0.065±0.005**</td>
<td>0.059±0.004</td>
</tr>
</tbody>
</table>

Student’s t-test was conducted for comparisons between 2 groups. ROI: region of interest, OVX: ovariectomy, BMD: bone mineral density, BV/TV: bone volume over total volume, SMI: structural model index, COD: connectivity density, BS/BV: bone surface to volume ratio, Tb.N: trabecular number, Tb.Sp: trabecular separation, Tb.Th: trabecular thickness. *P<0.05, **P<0.01, ***P<0.001 vs. the control group, ^P<0.05, ^P<0.01, ^***P<0.001 vs. the OVX group.
Ginsenoside Rc alleviates osteoporosis.

3.4. Repression of TGF-β/Smad pathway rescues the impacts of ginsenoside Rc

The elevated mRNA expression and activity of ALP caused by ginsenoside Rc were rescued by SB431542 (Fig 4A-B). Increased Col1a1 and Col1a2 mRNA levels stimulated by ginsenoside Rc were rescued by SB431542 (Fig 4C-D). Similarly, COL1A1 and COL1A2 protein levels were elevated in OVX+ginsenoside Rc group. However, up-regulation of COL1A1 and COL1A2 was rescued by SB431542 (Fig 4E). Figure 4F revealed that SB431542 successfully rescued the ginsenoside Rc-induced activation of the TGF-β/Smad pathway.

3.5. Ginsenoside Rc promoted osteoblast markers expression by the TGF-β pathway

Reactive activity of ALP was increased by ginsenoside Rc treatment and was decreased by SB431542 treatment in human osteoblasts (Fig 5A). Fig 5B-C displayed that the mRNA together with protein levels of osteoblast markers including ALP, OCN, COL1A1 as well as COL1A2 were increased by ginsenoside Rc and were decreased by SB431542 in human osteoblasts. Figure 5D revealed that ginsenoside Rc or SB431542 possessed no influence on the viability of human osteoblasts.

4. Discussion

Bone remodeling includes bone resorption modulated by osteoclasts together with bone formation modulated by osteoblasts [22]. Excessive bone resorption is the main cause of osteoporosis. Osteoclasts originate from hematopoietic precursor cells, which can differentiate into multinucleated cells [23]. When osteoclasts adsorb on the bone surface, hydrolytic enzymes, synthesized as well as secreted by osteoclasts, degrade bone minerals along with collagen matrices. In addition, the serum levels of bone resorption markers reflect the degradation degree of bone mineral as well as collagen matrices [24]. In the current research, serum OCN and PINP levels presented decreased in OVX group, whereas ginsenoside Rc treatment restored the decrease of OCN and PINP. Serum levels of bone absorption markers, TRACP-5b and CTX, were increased in OVX group, and ginsenoside Rc treatment rescued the increase of TRACP-5b and CTX.

Increasingly studies have revealed that type I collagen has a vital function in bone homeostasis and belongs to the major part of bone extracellular matrix [7, 8]. The alterations in synthesis, morphology, and stability of type I collagen are closely associated with the progression of osteoporosis [9, 10]. TGF-β affects the synthesis of type I collagen, and TGF-β/Smad pathway is essential for osteoblast differentiation and function.
Ginsenoside Rc alleviates osteoporosis.

Moreover, ginsenoside Rc has been revealed to upregulate ACE2 [28], and upregulation of ACE2 had a therapeutic value for ovariectomy-induced osteoporosis in rats [30]. Ginsenoside Rc can suppress the activation of TBK1 [16], and inhibitor of TBK1 can be used to suppress osteoclastogenesis and to prevent OVX-induced bone loss [31]. Oxidative stress is implicated in postmenopausal osteoporosis and catalase, an oxidative stress marker, is decreased in postmenopausal osteoporosis [32]. Ginsenoside Rc can induce the upregulation of catalase [33]. ACE2 and catalase were associated with the TGF-β pathway [34, 35]. It can be inferred that ACE2, TBK1 and catalase are involved in the therapeutic effect of ginsenoside Rc on osteoporosis. Nevertheless, this study had several limitations. Firstly, clinical data was lacking. Secondly, more pathways underlying the protective effect of ginsenoside Rc on osteoporosis need investigation.

5. Conclusions

This study revealed the protective role of ginsenoside Rc on osteoporosis via increasing the synthesis of type I collagen in a TGF-β/Smad3 pathway-dependent way. These findings may provide theoretical basis for finding novel strategies for osteoporosis.

Abbreviations

TRACP-5b: tartrate-resistant acid phosphatase-5b; OCN: osteocalcin; CTX: C-terminal telopeptide of type 1 collagen; PINP: procollagen I N-terminal propeptide; ROI: region of interest; BMD: bone mineral density; SMI: structural model index; COD: connectivity density; BS/BV: bone surface to volume ratio; BV/TV: bone volume over total volume; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation.

Declarations

Ethics declarations

Ethics approval and consent to participate

The animal study was approved by the ethics committee of Wuxi Hospital Affiliated to Nanjing University of Chinese Medicine (Jiangsu, China). The study was carried out in compliance with the ARRIVE guidelines. All the study was conducted in accordance with all the national/institutional/international guidelines.

Consent for publication

Not applicable.

Availability of data and materials

All data from this study are available in this published article.

Competing interests

The authors declare that they have no competing interests.

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