Original Full Length Article

Ginsenoside-Rb2 displays anti-osteoporosis effects through reducing oxidative damage and bone-resorbing cytokines during osteogenesis

Qiang Huang, Bo Gao, Qiang Jie, Bo-Yuan Wei, Jing Fan, Hong-Yang Zhang, Jin-Kang Zhang, Xiao-Jie Li, Jun Shi, Zhuo-Jing Luo, Liu Yang, Jian Liu

Institute of Orthopedic Surgery, Xijing Hospital, Fourth Military Medical University, Xi’an 710032, People’s Republic of China

A R T I C L E  I N F O

Article history:
Received 27 February 2014
Revised 5 June 2014
Accepted 6 June 2014
Available online 13 June 2014
Edited by J. Aubin

Keywords:
Ginsenoside-Rb2
Oxidative damage
Osteogenesis
Osteoporosis
Ovariectomized mouse
Bone-resorbing cytokine

A B S T R A C T

Reactive oxygen species (ROS) are a significant pathogenic factor of osteoporosis. Ginsenoside-Rb2 (Rb2), a 20(S)-protopanaxadiol glycoside extracted from ginseng, is a potent antioxidant that generates interest regarding the bone metabolism area. We tested the potential anti-osteoporosis effects of Rb2 and its underlying mechanism in this study. We produced an oxidative damage model induced by hydrogen peroxide (H2O2) in osteoblastic MC3T3-E1 cells to test the essential anti-osteoporosis effects of Rb2 in vitro. The results indicated that treatment of 0.1 to 10 μM Rb2 promoted the proliferation of MC3T3-E1 cells, improved alkaline phosphatase (ALP) expression, elevated calcium mineralization and mRNA expressions of Alp, Col1a1, osteocalcin (Ocn) and osteopontin (Opn) against oxidative damage induced by H2O2. Importantly, Rb2 reduced the expression levels of receptor activator of nuclear factor kappa-B ligand (RANKL) and IL-6 and inhibited the H2O2-induced production of ROS. The in vivo study indicated that the Rb2 administered for 12 weeks partially decreased blood malondialdehyde (MDA) activity and elevated the activity of reduced glutathione (GSH) in ovariectomized (O VX) mice. Moreover, Rb2 improved the micro-architecture of trabecular bones and increased bone mineral density (BMD) of the 4th lumbar vertebrae (L4) and the distal femur. Altogether, these results demonstrated that the potential anti-osteoporosis effects of Rb2 were linked to a reduction of oxidative damage and bone-resorbing cytokines, which suggests that Rb2 might be effective in preventing and alleviating osteoporosis.

© 2014 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-SA license (http://creativecommons.org/licenses/by-nc-sa/3.0/).

Introduction

Oxidative stress is described as an imbalance between an overproduction of reactive oxygen species (ROS) and an insufficient defense of antioxidants [1]. Oxidative stress can lead to oxidative damage that affects all of the cellular components, including proteins, lipids and nucleic acids. Osteoporosis is defined as a systemic degenerative disease, which is characterized by decreased bone mass and progressive bone micro-architectural deterioration and results in increasing bone fragility and susceptibility to fractures [2]. Currently, many studies have demonstrated a relationship between oxidative damage and osteoporosis or aging. Sendur and colleagues found that the decreased BMD of postmenopausal osteoporotic women was related to higher oxidation of plasma lipid [3] and lowered superoxide dismutase (SOD), catalase and glutathione peroxidase efficacy [4–6]. Ovariectomy has been shown to induce oxidative damage and to decrease the efficacy of antioxidant defense mechanisms, thus leading to osteoporosis. The activities of lipid peroxidation and H2O2 were increased, and enzymatic antioxidants, such as superoxide dismutase, glutathione peroxidase, and glutathione S transferase, were decreased in ovariectomized animals [7]. Moreover, many studies have demonstrated that superoxide and H2O2 were highly deleterious to cell survival and that they played a major causative role in the aging process [8]. These research findings provided a paradigm shift of osteoporosis pathogenesis from the “estrogen-centric” concept to one in which age-related mechanisms intrinsic to bone and oxidative stress were protagonists. Moreover, the age-related changes in other organs and tissues, such as the ovaries, accentuated these alterations [9].

Bone remodeling is a process that occurs throughout one’s entire life and involves two types of cells: osteogenic cells and monocyte-derived osteoclasts [10–12]. The osteogenic cells participate in bone formation, and osteoclasts are responsible for bone resorption [13,14]. The balance between bone formation and bone resorption is essential for maintaining bone homeostasis. Bone turnover changes significantly in postmenopausal osteoporosis: bone resorption is maintained or increases, but bone formation decreases, thereby leading to a net bone loss [15].

Emerging evidence has shown that ROS increased bone resorption by enhancing osteoclastic development and activity [16]. Indeed, in vivo bone resorption occurs preferentially in sites where ROS and
hence oxidative stress levels are high [17,18], ROS also led to osteoblast apoptosis, as well as reducing their activity, which led to decreased osteogenesis [19]. Oxidative stress decreases the life span of osteoblasts in the bone, as highlighted by the observation that the administration of antioxidants abrogates osteoblast apoptosis in ovariectomized or aged mice [20,21]. Therefore, we recommend substances that contain antioxidants because they may ameliorate the dysfunction of these two cell types by maintaining bone hemostasis. This suggestion might be implemented by acting as a latent method for the prevention and new treatment for osteoporosis or other related bone metabolic diseases.

Because of fewer side-effects and longer term usage than Western medicine, traditional Chinese medicines with anti-oxidative properties have recently attracted more attention. The ginseng root, which is a highly effective phyto medicinal remedy, is a well-recognized, traditional Chinese medicine that is widely used. The primary components found in ginseng are ginsenosides, which contribute to the most active properties. Ginsenoside-Rb2 (Fig. 1) is the most quantitative saponin that is contained in Panax ginseng [22]. It was reported that Rb2 possessed anti-diabetic, anti-adipocyte, anti-carcinogenic and anti-oxidative properties [23–26]. More importantly, recent studies showed that H2O2, which is known as a cancer promoter, inhibited the gap junctional intercellular communication (GJIC) of epithelial cells. Rb2 supplementation might inhibit the occurrence of cancer through the up-regulation of GJIC in the cancer-accelerating phase [27]. SOD1 is considered to be one of the antioxidant enzymes. A previous study [28] has shown that ginsenoside-Rb2 could induce the transcriptional expressions of Cu, Zn-superoxide dismutase gene (SOD1). The mutated AP2 binding site in the promoter of SOD1 gene abrogated this effect, which suggests that the SOD1 gene was highly activated by ginsenoside-Rb2 through the AP2 binding site [28]. Kang et al. studied the hydroxyl radical (•OH) clearing capacity change of Rb2 through heat procedure using an electron spin resonance spectrometer. Specially, ginsenoside-Rb2 was heat processed using the same amount of glycine, which was an amino acid commonly employed in the Maillard response model system [26].

Whether ginsenoside-Rb2 might provide the potential anti-osteoporosis effects and whether these effects are produced by reducing oxidative damage and bone-resorbing cytokines have not been established. Therefore, in the present study, we tested the anti-osteoporosis effects of Rb2 and the underlying mechanisms in vitro and in vivo using an H2O2-induced oxidative damage model of osteoblastic MC3T3-E1 cells and a murine ovariectomized model.

Materials and methods

Materials

Rb2 extracted from ginseng (molecular weight, 1079; purity, >98.0%; dissolved in distilled water) was purchased from Shanghai Tauto Biotech Co., LTD (China). The culture flasks and plates were obtained from Nunc (Denmark). The ALP activity assay kit was obtained from GENMED Scientific Inc. (USA). RANKL and IL-6 ELISA assay kits were obtained from R&D system Inc. (Minneapolis, MN, USA). The total RNA kit was obtained from OMEGA. Prime Script RT reagent kit and SYBR Premix Ex Taq were obtained from TaKaRa Biotechnology (Dalian, China). The oligonucleotide primers were synthesized by TaKaRa Biotechnology. BCP/NBT alkaline phosphatase color development kit was obtained from Gibco Life Technologies (Grand Island, USA). The reactive oxygen species assay kit, GSH and MDA assay kits were obtained from the Beyotime Institute of Biotechnology (Shanghai, China). All other reagents were of analytical grade.

Cell cultures

Murine MC3T3-E1 cells were purchased from the Center Laboratory for Tissue Engineering, College of Stomatology, Fourth Military Medical University, Xi’an, China [29,30]. The cells were cultured in α-MEM using 10% heat-inactivated FBS and 100 U/ml penicillin and 100 mg/ml streptomycin in a condition of 5% CO2 and 37 °C. H2O2 acted as exogenous ROS treatment, whereas N-acetyl-L-cysteine (NAC) acted as an ROS cleaner. When the cells reached confluence, the serum-free medium containing Rb2 was dissolved in distilled water and cultured for 24 h before the administration of 0.3 mM H2O2 for 24 h. For each experiment, Rb2 administration continued after the pretreatment. All of the experiments were performed in duplicate wells and repeated three times.

Assays of cell survival

For this experiment, the murine MC3T3-E1 cells were administered using different consistencies of Rb2 (0 μM, 0.1 μM, 1 μM, and 10 μM) for 24 h and 72 h to test the toxicity of Rb2. Subsequently, the cells were incubated using the serum-free regular culture medium, which contained Rb2 (0 μM, 0.1 μM, 1 μM, and 10 μM) for 24 h followed by the administration of 300 μM H2O2 for 24 h. The MTT assays were used for measuring cell survival. The absorbances of all of the wells were recorded using a micro-plate reader at 492 nm wavelength. The cell survival of the control group, which was not exposed to either H2O2 or Rb2, was defined as 100%.

Alkaline phosphatase (ALP) staining and activity assay

After a 6-day osteogenic induction, the murine MC3T3-E1 cells were incubated using serum-free medium, which contained Rb2 and/or H2O2 for 2 days. The cells were stained using the BCP/NBT alkaline phosphatase color development kit according to the manufacturer’s instructions. To evaluate the ALP activity, the cell monolayer was lysed using the cell lysis buffer. Subsequently, the lysate was centrifuged at 10,000 g for 5 min. The clear supernatant was used to measure the ALP activity, which was determined employing the ALP activity assay kit. The total protein consistencies were determined using the Bradford protein assay method. The ALP activity was normalized to total protein, which was measured using the Bradford protein assay method.

Calcium mineralization assay

After a 21-day osteogenic induction, the murine MC3T3-E1 cells were incubated using a serum-free medium, which contained Rb2.

Fig. 1. Chemical structure of Rb2 (from National Center of Biotechnology Information, Pubchem CID: 5458674).
and/or H₂O₂ for 2 days. After the cells were collected, the cells were fixed with formalin for 20 min and stained with Alizarin Red S for 45 min under room temperature. To measure matrix calcification, unbound alizarin red was washed off using PBS. Subsequently, the stain was solubilized using 10% cetylpyridinium chloride by shaking for 15 min. The absorbances of the released Alizarin Red S were recorded using a micro-plate reader at 562 nm wavelength.

**Quantitative real-time PCR**

After a 6-day osteogenic induction, murine MC3T3-E1 cells were incubated using a serum-free medium, which contained Rb₂ and/or H₂O₂ for 2 days. The total cellular RNA was extracted from these cells using Trizol reagent. Single strand cDNA synthesis was determined using the Prime Script RT reagent kit (TaKaRa). The RT-PCR was performed using the CFX96 (Bio-RAD) instrument, and individual PCRs were conducted in 96-well optical reaction plates using SYBR Green-I (TaKaRa) according to the manufacturer’s instructions. Target gene (A-In, ColIα1, Ocn and Opn) expressions were normalized to the reference gene β-actin. The 2^−ΔΔCt method was applied to calculate the relative gene expression. These PCR products were subjected to a melting curve analysis and a standard curve to confirm the correct amplification. All of the PCRs were performed in triplicate, and the primers used for PCR are shown in Table 1.

**RANKL and IL-6 measurements**

After a 6-day osteogenic induction, the murine MC3T3-E1 cells were incubated using serum-free medium, which contained Rb₂ and/or H₂O₂ for 2 days. The expressions of RANKL and IL-6 in culture medium were tested using the sandwich ELISA assay kit according to the manufacturer’s instructions. The total protein consistencies were measured using the Bradford protein assay method.

**Intracellular reactive oxygen species (ROS) measurement**

After a 6-day osteogenic induction, the murine MC3T3-E1 cells were incubated using serum-free medium, which contained Rb₂ and/or H₂O₂ for 2 days. The intracellular ROS expression level was determined using the ROS assay kit. DCFH-DA can be oxidized by ROS in viable cells to 2′,7′-dichlorofluorescein (DCF), which is highly fluorescent at 530 nm. These cells were washed three times with PBS. DCFH-DA, which was diluted to a final consistency of 10 μM, was added and cultured for 30 min at 37 °C in the dark. When washed three times by PBS, the relative expression of fluorescence was quantified using a multi-detection micro-plate reader (485 nm excitation and 535 nm emission).

**Animals and ginsenoside-Rb₂ intervention**

Forty 8-week-old, BALB/c female mice, weighing 20.84 ± 1.21 g, were obtained from the Experimental Animal Center of The Fourth Military Medical University (Xi’an, China). There was no significant difference in the initial body weights of the mice among all 4 groups in this experiment. The mice were allowed to adapt to the laboratory environment (a well-ventilated controlled room at 20 °C on a 12-h light/dark cycle; the animals were given free access to water and food) for 1 week before the surgery. Subsequently, the mice experienced sham-operation (n = 10) or were surgically ovariectomized (OVX) (n = 30) under anesthesia using pentobarbital sodium (50 mg/kg body weight, i.p.). The ovariectomy operation was performed according to Steven K. Boyd’s procedure [31]. A total of 30 BALB/c female mice were randomly divided into three groups: 1) OVX group, administered intraperitoneally with distilled water (n = 10); 2) OVX group, administered intraperitoneally with Rb₂ (body weight, 4.6 μmol/kg; n = 10) daily; and 3) OVX group, administered intraperitoneally with Rb₂ (body weight, 18.5 μmol/kg; n = 10) daily. Rb₂ was dissolved in distilled water. One week after the operation, the treatments commenced and continued for 12 weeks. Blood samples were obtained from the hearts in anesthetized mice and serum samples were prepared by centrifugation. The left femurs and 4th lumbar vertebrae (L4) of the mice were collected and the adherent tissue was discarded. All of the experimental procedures were officially approval by the Ethics in the Animal Research Committee of the Fourth Military Medical University (permission code 2010C00843).

**Measurements of serum malondialdehyde (MDA) and reduced glutathione (GSH)**

The activity of MDA in whole blood samples was determined using a lipid peroxidation MDA assay kit according to the manufacturer’s instructions. The binding of thiobarbituric acid to malondialdehyde, which was formed during lipid peroxidation, results in a chromogenic complex. In the spectrophotometer, the change of absorption peak was detected at 532 nm. Colorimetry was used to detect the

---

**Table 1**

Real-time PCR primers for amplification of specific MC3T3-E1 mRNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′−3′)</th>
<th>Reverse (5′−3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-In</td>
<td>GCCATGTGAAGTATCGGAAACAC</td>
<td>ATGGCCGTCGTCATCTCCAC</td>
</tr>
<tr>
<td>ColIα1</td>
<td>GCCACCTTCAGCTCCTGGACCTC</td>
<td>GGGACCTCTAGGGCCTGTTGA</td>
</tr>
<tr>
<td>Ocn</td>
<td>AACTCTCTGCCTCAGTGTCTCTC</td>
<td>CCTTGAGTCTCCTCCTGCTT</td>
</tr>
<tr>
<td>Opn</td>
<td>TAGAGTATGGATTTGCGGACAGA</td>
<td>TATAGAGATCCTGCTCAGGAGCAT</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CATCCGTAAGACACCTCTTGCCAA</td>
<td>ATGAGCCACCCAGATTCACA</td>
</tr>
</tbody>
</table>

**Fig. 2.** Protection by Rb₂ on H₂O₂-induced cytotoxicity in MC3T3-E1 cells. A: MC3T3-E1 cells were cultured in different concentrations of Rb₂; B: MC3T3-E1 cells were administered with Rb₂ for 24 h before 0.3 mM H₂O₂ administration for 24 h. The control values for cell survival were 0.61 ± 0.02 OD. *P < 0.05 compared with untreated control cells; **P < 0.01 compared with untreated control cells; ***P < 0.01 in contrast to the group treated with H₂O₂ alone.
malondialdehyde activity in the whole blood samples. Additionally, the activity of GSH was determined using the GSH assay kit. The GSH activity was determined by the reaction of GSH with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce a product that could be measured using a spectrophotometer at 412 nm.

Assessment of bone micro-architecture and bone mass by micro-computed tomography

The 4th lumbar vertebrae (L4) and the distal femur were scanned using explore Locus SP Pre-Clinical Specimen micro-CT (GE Healthcare,

Fig. 3. Protection by Rb2 on MC3T3-E1 cell dysfunction induced by H2O2. After induced for 6 days or 21 days, MC3T3-E1 cells were administered with Rb2 for 24 h before 0.3 mM H2O2 administration for 24 h. A and B: Protection by Rb2 on ALP staining and ALP activity in MC3T3-E1 cells after H2O2 treatment. The control value for ALP activity was 0.36 ± 0.03 unit/mg protein. C and D: Protective effects of Rb2 on calcium deposition in MC3T3-E1 cells by Alizarin Red S staining after H2O2 treatment. The control values for mineralization were 0.73 ± 0.02 OD. ① Control group; ② H2O2; ③ H2O2 + Rb2 (0.1 μM); ④ H2O2 + Rb2 (1 μM); ⑤ H2O2 + Rb2 (10 μM); ⑥ H2O2 + NAC (1 mM). **P < 0.01 compared with untreated control cells; *P < 0.05 and **P < 0.01 in contrast to the group treated with H2O2 alone.

Fig. 4. Protection by Rb2 on the transcriptional expressions of Alp, Col1a1, Ocn and Opn after H2O2 treatment. When induced for 6 days, MC3T3-E1 cells were administered with Rb2 for 24 h before 0.3 mM H2O2 administration for 24 h. *P < 0.05 and **P < 0.01 compared with untreated control cells; *P < 0.05 and **P < 0.01 in contrast to the group treated with H2O2 alone.
Histological examination by Van Gieson (VG) staining

The 4th lumbar vertebrae and the left femur of all of the mice were collected and fixed in 4% paraformaldehyde for 48 h. After dehydration and embedding, the 4th lumbar vertebrae and the distal femur were embedded in polymethyl-methacrylate (PMMA) and processed into 240-mm-thick sections in the coronal plane using a rotation microtome. Subsequently, all of the sections were hand-grounded to a thickness of 20 mm for VG staining, which was used for staining collagen fiber [32].

Fig. 5. Rb2 inhibited the expressions of RANKL and IL-6 after H2O2 treatment. After osteogenic induction for 6 days, MC3T3-E1 cells were administered with Rb2 for 24 h before 0.3 mM H2O2 administration for 24 h. A: Expression of RANKL with the presence of Rb2 and/or H2O2. The control values for RANKL were 2.27 ± 0.18 ng/mg. B: Expression of IL-6 with the presence of Rb2 and/or H2O2. The control values for IL-6 were 0.72 ± 0.17 ng/mg. **P < 0.01 compared with untreated control cells; *P < 0.05 and ***P < 0.01 in contrast to the group treated with H2O2 alone.

USA) with 8-mm resolution, 50-kV tube voltage and 0.1-mA tube current. The reconstruction and 3D quantitative analyses were determined using software provided by a desktop micro-CT system (GE Healthcare, USA). Similar settings for scans and analyses were used for all of the samples. In the femur, the scanning regions were confined to the distal metaphysis, extending proximally 2.0 mm from the proximal tip of the primary spongiosa. The trabecular bone region from the vertebral body was outlined for each micro-CT slice, excluding both the cranial and caudal endplate regions. The following 3D indices in the defined region of interest (ROI) were analyzed: bone mineral density (BMD), connectivity density (Conn.D), structure model index (SMI), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and relative bone volume over total volume (BV/TV, %). The operator who conducted the scan analysis was blinded to the procedure associated with the specimens.

Statistical analysis

The data represented the mean ± SD values of multiple repeats of the same experiment (n = 5). The data for all of the measurements were analyzed using a one-way analysis of variance (ANOVA) with subsequent post hoc multiple comparison by Dunnett’s test. Statistically significant values were defined as P < 0.05.

Results

Rb2 inhibited H2O2-induced cytotoxicity in MC3T3-E1 cells

Before the anti-cytotoxicity effect of Rb2 was tested, the toxicity of Rb2 on MC3T3-E1 was observed. The results showed that Rb2 treatment alone did not affect cell survival at the consistencies tested in this experiment (Fig. 2A). Subsequently, we tested the survival of MC3T3-E1 cells to observe the defensive effects by Rb2 on the repercussion of the cells after H2O2-induced oxidative damage. We implemented oxidative damage on MC3T3-E1 cells using 0.3 mM H2O2 for 24 h. When different concentrations of Rb2 (0.1, 1, 10 μM) were administered to MC3T3-E1 cells for 24 h before H2O2 treatment, the viability of the cells increased compared to the group without Rb2 treatment, which indicates that Rb2, in part, inhibited H2O2-induced cytotoxicity (Fig. 2B). N-acetylcysteine represented the positive control, which significantly suppressed cytotoxicity induced by H2O2 at 1 mM.

Protection by Rb2 on H2O2-induced dysfunction of MC3T3-E1 cells

We tested the protective effect of Rb2 on H2O2-induced dysfunction of MC3T3-E1 cells by evaluating osteoblast differentiation and mineralization with ALP staining, ALP activity and Alizarin Red S staining. Additionally, we detected the expression of osteogenic genes including Alp, ColIa1, Ocn and Opn. Compared with the control group, all markers of osteogenic differentiation, such as ALP expression, calcium deposition and osteogenic genes (Figs. 3 and 4), decreased after treatment of H2O2. However, when we pretreated MC3T3-E1 cells with Rb2 (0.1 μM, 1 μM, and 10 μM) increased in a dose-dependent manner. As shown in Fig. 3C and D, Rb2 (0.1 μM, 1 μM, and 10 μM) exhibited a good recovery effect on calcium deposition, which was suppressed by H2O2. Furthermore, pretreatment with Rb2 partially elevated the expression of osteogenic genes compared with the group of H2O2 treated alone (Fig. 4). Our data indicated that Rb2, in part, attenuated H2O2-induced dysfunction of MC3T3-E1 cells.

Rb2 inhibited RANKL and IL-6 expression in MC3T3-E1 cells

Osteoblasts always express and secrete several cytokines, which bind to receptors in osteoclasts and affect osteoclast activity in bone remodeling. RANKL and IL-6 are two essential bone-resorbing factors expressed in osteoblasts. After the addition of 0.3 mM H2O2, the expressions of RANKL and IL-6 increased. However, when pretreated with Rb2 of 0.1 μM, 1 μM and 10 μM, the elevated expressions of RANKL and IL-6 were, in part, inhibited (Fig. 5).

Rb2 inhibited the production of reactive oxygen species induced by H2O2 in MC3T3-E1 cells

To clarify whether the cell-protective capacity of Rb2 was associated with its antioxidant property, we tested ROS production using a fluorescent probe DCFH-DA. The results in Fig. 6 showed that when MC3T3-E1 cells were treated with 0.3 mM H2O2, ROS increased. These data indicated that H2O2 stimulated the generation of oxidants and resulted in oxidative stress to MC3T3-E1 cells. However, when pretreated with Rb2 of different concentrations, ROS production was partially suppressed
These results suggested that the protection by Rb2 might be related to its antioxidant activity.

Rb2 inhibited serum oxidative damage of ovariectomized mice

To test whether Rb2 can inhibit serum oxidative damage caused by estrogen insufficiency, the activities of serum malondialdehyde (MDA) and reduced glutathione (GSH) were observed in ovariectomized mice with or without Rb2 intervention. The results in Fig. 7 showed that in contrast to the sham-operated group, the activity of MDA in serum increased, whereas the GSH activity in the ovariectomized group decreased. However, Rb2 treatment partly rescued the serum activities of MDA and GSH.

Rb2 improved bone mass and bone structure of ovariectomized mice

To evaluate Rb2 acting on trabecular bone mass and micro-architecture, different concentrations of Rb2 were supplemented to the ovariectomized (OVX) mice. When all of the mice were collected at 13 weeks after operation, there were no significant differences in body weights of all four groups. The body weights of the sham-operated group, OVX group, OVX + Rb2 (4.6 μmol/kg) group and OVX + Rb2 (18.5 μmol/kg) group were 24.26 ± 0.99 g, 24.12 ± 1.06 g, 24.03 ± 1.09 g and 24.07 ± 0.98 g, respectively. The micro-architectures of distal femurs are shown in Fig. 8A and B. The analyses of the trabecular bone of the 4th lumbar vertebrae (L4) and the distal femur showed that ovariectomy reduced bone mass and deteriorated bone micro-architecture, which was indicated through decreases in BMD, Conn.D, Tb.N, Tb.Th and BV/TV (Fig. 8C and D) (P < 0.05). SMI and Tb.Sp exhibited increases that contributed to ovariectomy (P < 0.05), as shown in Fig. 8E and F. However, the treatment of ovariectomized mice with 4.6 μmol/kg or 18.5 μmol/kg Rb2, in part, rescued these bone parameters and improved the micro-architecture of the trabecular bone in the 4th lumbar vertebrae and the distal femur. Moreover, we evaluated the changes of bone micro-architecture by VG staining. As shown in Fig. 8G and H, compared with the sham-operated group, the number of trabeculae decreased and the trabecular space became broader in the OVX group. The supplement of Rb2 reversed these changes by an elevated number of trabecular bone and a reduction of trabecular bone space (Fig. 8G and H). These results were consistent with the micro-CT data (Fig. 8A–F).

Discussion

The reactive oxygen species are highly reactive oxygen free radicals or non-radical molecules, which include hydrogen peroxide (H₂O₂),
Several pathways and cytokines couple the link between osteoblasts and osteoclasts. RANKL and IL-6 are generated by osteoblasts and function as bone-resorbing cytokines by stimulating osteoclast activity. RANKL belongs to the superfamily of tumor necrosis factor [38]. By binding to RANK, RANKL leads to osteoclastic differentiation, prolongs osteoclastic activity and increases bone resorption [11]. Several studies have shown that reactive oxygen species could elevate the expression of RANKL in osteoblasts [39]. IL-6 is also generated by osteoblasts when it is motivated by IL-1, TNF-α, and lipopolysaccharide [40]. IL-6 could affect the expression of RANKL, improve osteoclast development and be a significant pathogenic factor of estrogen deficiency osteoporosis [41,42]. It was reported that reactive oxygen species might indirectly influence osteoclasts by increasing the expression of bone-resorptive cytokines, which are highly involved in estrogen deficiency osteoporosis [43]. In our study, Rb2 was noted to inhibit, in part, the H2O2-induced generations of RANKL and IL-6 in MC3T3-E1 cells. The reductions of RANKL and IL-6 might contribute to the anti-resorbing property, which Rb2 exhibited.

Ginsenoside-Rb2 (Rb2), which is extracted from Panax ginseng, belongs to traditional Chinese medicine. Recently, its anti-oxidative property has been reported [16]. In this study, we could reverse, in part, H2O2-induced production of reactive oxygen species by pretreatment with Rb2 for 24 h. Our data indicated that Rb2 might be a useful antioxidant to protect MC3T3-E1 cells from cytotoxicity induced by oxidative damage. Consequently, the protection by Rb2 to MC3T3-E1 cells could be mediated via its antioxidant property. The beneficial effect of Rb2 might be linked to reduced oxidative damage and bone-resorbing cytokines. However, the molecular mechanism has not been established. Compared with other growth factors, reactive oxygen species result in the retention of FoxO in the nucleus and the activation of their transcription [44]. FoxO protein family is characterized by a common winged-helix DNA binding domain called Forkhead box [45]. Animal studies have demonstrated that in response to oxidative damage, c-jun kinase (JNK) and mammalian sterile 20-related kinase-1 (Mst1) bond superoxide anion (O2•−) and hydroxyl radical (•OH) [33]. Their over-production induces oxidative stress. A considerable number of studies indicated that increased oxidative stress was involved in the pathogenesis of osteoporosis caused by estrogen deficiency and aging [34,35]. Because of good stability and property to pass through cell membranes, hydrogen peroxide is favorable to serve as both an extra- and an intercellular signal [36]. Therefore, an in vitro H2O2-induced oxidative damage model was used in this study. The study of Zhang et al. showed that the treatment of 0.3 mM H2O2 for 24 h reduced the number of MC3T3-E1 cells to 50% and that 0.3 mM H2O2 was suitable for generating an in vitro oxidative stress model [32]. Therefore, we used 0.3 mM H2O2 for 24 h to make an oxidative damage model in the present study. Our data showed that H2O2 toxicity was reversed to a certain degree by pretreatment with Rb2. This study demonstrated that Rb2, in part, decreased H2O2-induced cytotoxicity on MC3T3-E1 cells.

Oxidative damage not only affects osteoblast survival but also has an impact on its differentiation. H2O2-induced oxidative damage is reported to inhibit osteoblast differentiation in primary murine bone marrow stem cells and to reduce the expression of osteoblast markers in osteoblastic MC3T3-E1 cells [19,37]. Our study also showed that H2O2 supplement was linked to a reduction of cellular alkaline phosphatase activity, a decrease of calcium mineralization and lowered expressions of osteogenic genes, including Alp, Col1a1, Ocn and Opm, which confirms previous results that cytotoxicity of H2O2, in part, results in MC3T3-E1 cell dysfunction. Moreover, our data showed that H2O2-mediated inhibition of osteogenic differentiation could be reversed by Rb2. Rb2 alone could not significantly enhance MC3T3-E1 cell survival and osteogenic differentiation within experimented consistencies. Therefore, we speculated that the antioxidant property of Rb2 contributed to the protective effect. Because Rb2 improves osteoblastic survival and differentiation against oxidative damage, Rb2 might become an anti-osteoporosis agent in the bone metabolism area.
dramatically deteriorate after ovariectomy by both micro-CT scanning and VG staining [32,56]. Nevertheless, these negative effects could be reversed, to some degree, after administering Rb2. These results further confirmed that Rb2 might, in part, ameliorate trabecular micro-architecture and bone mass of OVX mice and that Rb2 might be a good candidate for the prevention and the treatment of estrogen-deficient osteoporosis.

To conclude, the results described in this article indicated that in an in vitro analysis, ginsenoside-Rb2 protected osteoblastic MC3T3-E1 cells from cytotoxicity and osteoblast dysfunction induced by hydrogen peroxide at 0.1 to 10 μM. This activity is linked to a reduction of oxidative damage and bone-resorbing cytokines. More importantly, ginsenoside-Rb2 decreased serum reactive oxygen species to a certain degree and partly reversed estrogen-deficient effects in vivo at doses of 4 to 18 μmol/kg body weight. Ginsenoside-Rb2 may act as a substantive alternative in bone metabolic diseases, especially osteoporosis. Extensive research is needed in the future to explore the intricate molecular mechanisms of ginsenoside-Rb2 on bone metabolism.

Acknowledgments

This work was supported by the Ministry of Science and Technology of the People’s Republic of China (2011CB964703), National High Technology Research and Development Program 863 (2012AA020502) and National Natural Science Foundation of China (30901504), and the Program for Changjiang Scholars and Innovative Research Team in University (no. IRT1053). No benefits in any form have been or will be received from a commercial party directly or indirectly by the authors of this manuscript.

References


