Acta Pharmacologica Sinica (2012) 33: 479–489
 1671-4083/12 $32.00
www.nature.com/aps

Original Article

Strontium fructose 1,6-diphosphate prevents bone loss in a rat model of postmenopausal osteoporosis via the OPG/RANKL/RANK pathway

Bo MA[1], Qi ZHANG[1, 2, *], Di WU[3], Yong-lu WANG[1, 2], Ying-ying HU[1], Yan-ping CHENG[1], Zhen-dong YANG[1], Ya-ya ZHENG[1], Han-jie YING[4, *]

[1]School of Pharmaceutical Sciences, Nanjing University of Technology, Nanjing 210009, China; [2]Jiangsu Provincial Institute of Material Medica, Nanjing University of Technology, Nanjing 210009, China; [3]Laboratory for Applied PK/PD, Division of Clinical Pharmacology and Therapeutics, The Children's Hospital of Philadelphia; Kinetic Modeling and Simulation Core, Institute of Translational Medicine and Therapeutics, University of Pennsylvania, Philadelphia, PA 19104, USA; [4]School of Life Science & Pharmaceutical Engineering, Nanjing University of Technology, Nanjing 210009, China

Aim: To evaluate the protective effects of strontium fructose 1,6-diphosphate (FDP-Sr), a novel strontium salt that combined fructose 1,6-diphosphate (FDP) with strontium, on bone in an ovariectomy-induced model of bone loss.
Methods: Eighty female Sprague-Dawley rats were ovariectomized (OVX) or sham-operated. Three months later, the rats were assigned to six groups (10 for each): sham-operated, OVX control, OVX+FDP-Sr (110, 220, or 440 mg/kg), or OVX+strontium ranelate (SR, 180 mg/kg). Drugs were administered orally for 3 months. When the treatment was terminated, the following parameters were assessed: bone mineral density (BMD), the biomechanical properties of the femur and lumbar vertebrae, trabecular histomorphology, serum phosphorus, calcium, bone-specific alkaline phosphatase (B-ALP), tartrate-resistant acid phosphatase 5b (TRACP5b), N-telopeptide of type I collagen (NTx) and a series of markers for oxidative stress. Receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) levels in serum were measured using ELISA and their gene expression levels in the bone were measured using R-T PCR.
Results: Treatment with FDP-Sr (220 or 440 mg/kg) or SR (180 mg/kg) significantly increased the BMD and improved the bone micro-architecture and bone strength in OVX rats. The treatments also decreased in the levels of H_2O_2 and MDA, restored the CAT level in serum and bone marrow, increased the serum B-ALP and decreased NTx and TRACP 5b in OVX rats. Treatment with FDP-Sr decreased the RANKL level, and increased the OPG level in serum in a dose-dependent manner. It also significantly down-regulated the RANKL expression and up-regulated OPG expression in bone marrow.
Conclusion: FDP-Sr may be an effectve treatment for postmenopausal osteoporosis that acts, in part, via a decrease in osteoclastogenesis through the OPG\RANKL\RANK pathway.

Keywords: osteoporosis; ovariectomy; strontium fructose 1,6-diphosphate; strontium ranelate; osteoprotegerin (OPG); receptor activator of NF-κB ligand (RANKL); bone mineral density; tartrate-resistant acid phosphatase 5 (TRACP5); N-telopeptide of type I collagen (NTx)

Acta Pharmacologica Sinica (2012) 33: 479–489; doi: 10.1038/aps.2011.177; published online 19 Mar 2012

Introduction

Osteoporosis is a major clinical condition in the elderly population and is a skeletal disorder characterized by bone mass reduction, increased bone fracture risk and potential bone architecture alterations[1, 2]. The incidence of osteoporosis is increasing owing to the increasing elderly population. Currently, osteoporosis and its complications affect approximately 200 million people worldwide. Hip fracture is one of the most serious complications of osteoporosis and a measure of osteoporosis severity. By 2050, the incidence of hip fracture is predicted to double in North America and Europe, whereas higher incidences are predicted in other countries, including a five-fold increase in Asia and up to a seven-fold increase in Latin America[3]. Although these estimates are for the general population, women are more prone to bone loss than men, owing to postmenopausal estrogen deficiency, which results in osteoporosis.

* To whom correspondence should be addressed.
E-mail nancyzhang03@hotmail.com (Qi ZHANG);
yinghanjie134@163.com (Han-jie YING)
Received 2011-09-13 Accepted 2011-11-23

The mechanisms through which estrogen deficiency induces bone loss remain unclear. Bone remodeling has been suggested to play a fundamental role in the maintenance of skeletal mechanical integrity via a balance between bone formation and bone resorption[4, 5]. Estrogen deficiency leads to a negative bone remodeling balance, in which bone resorption exceeds bone formation. This imbalance aggravates the loss of bone mass and increases the incidence of osteopenia. Thus, estrogen deficiency is a major risk factor for osteoporosis. Conventional therapeutic agents that aim to stimulate bone formation or inhibit bone resorption fail to re-establish bone turnover equilibrium even after long-term usage. Furthermore, these agents have multiple side effects, including bisphosphonate-induced osteonecrosis of the jaw[6, 7], estrogen replacement therapy (ERT)-induced malignancies[8, 9], and increased stroke rates[10, 11]. Therefore, it is necessary to develop novel drugs with the "dual effect" of stimulating bone formation and inhibiting bone resorption while causing fewer side effects.

Strontium, a significant component of the skeleton, can decrease bone resorption and increase bone formation and bone mass without affecting bone mineralization. Thus, strontium may be of potential benefit for the treatment of osteoporosis[12]. Following the formulation of strontium ranelate (Protelos; Servier Laboratories, France), strontium treatment has been considered to be an important advance in the pharmacotherapy of osteoporosis because strontium has demonstrated both anti-resorptive and bone-forming effects[13–16].

Many strontium salts have been synthesized and studied in the context of osteoporosis. To date, strontium ranelate is the only drug on the market and has been highly evaluated for clinical use. Ranelic acid serves as a carrier for strontium ranelate, but the biologic activity and toxicity of ranelic acid are unclear. Some side effects of ranelic acid, such as toxic epidermal necrolysis, have been reported[17]. Researchers remain uncertain if these side-effects are caused by strontium or ranelic acid. It would be of great interest to couple the Sr^{2+} with a natural, non-toxic acid radical that could increase the safety and efficacy of the strontium compound. Strontium fructose 1,6-diphosphate (FDP-Sr) is designed following the above principle.

FDP-Sr is a novel compound synthesized by combining fructose 1,6-diphosphate (FDP) with strontium (Figure 1). FDP serves as both a glycolytic intermediate and an important adenosine triphosphate supplier in energy metabolism. It exerts protective effects against ischemia[18] or hypoxia[19] and facilitates the recovery of relevant tissue[20]. FDP also has advantages, in terms of safety, over other chemical acid radicals due to its endogenous nature. In addition, FDP induces calcium uptake in bone formation and modulates bone metabolism *in vitro*[21]. Therefore, FDP may be especially suitable for elderly patients. FDP-Sr also showed a favorable pharmacokinetic profile as a promising anti-osteoporosis drug candidate (rapid oral absorption, modest protein-binding, and a significantly higher affinity for and longer retention in the bone than in other organs)[22]. As such, the combination of FDP with strontium may potentially improve the effect of strontium against osteoporosis.

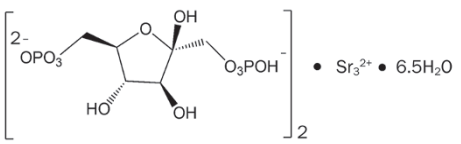

Figure 1. The chemical structure of FDP-Sr.

The aim of this study was to investigate the protective effect of FDP-Sr on bone loss in ovariectomized (OVX) rats. Bone mineral density (BMD), biomechanical testing, bone histomorphology, bone turnover and oxidative stress were examined in OVX rats treated with FDP-Sr. OPG and RANKL mRNA expression in the bone was examined by real-time PCR to determine the mechanism of osteoblasts (OB) coupled with osteoclasts (OC) under FDP-Sr treatment.

Materials and methods

Animals

Eighty specific-pathogen-free (SPF) virgin female Sprague–Dawley rats (6 months old, body weight 280±20.0 g) were obtained from the Animal Center of Nanjing Medical University for this study. Rats were housed under controlled conditions; room temperature was maintained at 22±1 °C, and standard solid food with water *ad libitum* was provided during the experiment. This study was reviewed and approved by the Animal Ethical Committee of Nanjing University of Technology.

Experimental design and treatments

After 7 days of acclimatization, all animals were anesthetized with 30 mg/kg pentobarbital sodium (Fluka, Germany), injected intraperitoneally, and then a bilateral ovariectomy (n=60) or a sham operation (n=20) was performed. Three months later, 20 rats were randomly chosen from sham-operated (n=10) and OVX (n=10) operated animals, and the BMD and biomechanical properties were examined to confirm the occurrence of osteoporosis in the rats. Subsequently, the remaining sham-operated animals (n=10) were treated with vehicle (deionized water), and the remaining OVX rats were randomly assigned to 5 groups (n=10 per group): OVX control (vehicle), OVX+FDP-Sr (FDP-Sr 110, 220 of 440 $mg \cdot kg^{-1} \cdot d^{-1}$), OVX+Strontium Ranelate (SR, 180 $mg \cdot kg^{-1} \cdot d^{-1}$, Osseor, Les Laboratories Servier Industrie, 45520 Gidy, France). The body weights of the rats were measured weekly and used to adjust the quantity of the FDP-Sr or SR treatments. After three months of treatment, all rats were weighed, anesthetized and bled from the carotid arteries. The tibia, femur and lumbar vertebrae were de-fleshed from adjacent tissues, wrapped in saline-soaked gauze bandages to prevent dehydration, and stored frozen at -20 °C in small Ziploc freezer bags until the BMD and biomechanical properties were measured. Blood samples were collected, and plasma was then prepared by centrifugation at 2000×g for 10 min. The plasma samples were

then frozen at -80 °C for the analysis of biochemical markers. The uterus was removed from each rat and immediately weighed.

Bone mineral density assessment

After the rats were sacrificed, the complete left femur and lumbar vertebrae (L1-L5) were removed. BMD was determined by dual-energy X-ray absorptiometry (GE, Lunar Prodigy^{TM}) equipped with the appropriate small animal software for bone density assessment. BMD was calculated from the bone mineral content (BMC) of the measured area.

Biomechanical three-point bending testing and compression measurements

Prior to mechanical testing, the lumbar vertebrae (L3) and left femur were slowly thawed at 37 °C, and then the soft tissue around the bone was removed. Subsequently, the left femur was placed on the lower supports of a three-point bending fixture with the posterior side facing down in an Instron Mechanical Testing Instrument (Instron 5544; Instron, Norwood, MA, USA). The span between the two lower supports was set at 18 mm. The upper loading device was aligned to the center of the femoral shaft. Load was applied at a constant displacement rate of 10 mm/min (temperature: 23°C, humidity: 60%–70%) until the femur broke. Simultaneously, the upper and lower end of the L3 was polished so that they were parallel, and all of the L3 were maintained at the same high degree. They were placed between two platens; the load and displacement were recorded on an Instron Mechanical Testing Instrument until the specimen broke. Maximum load (ultimate strength, F_{max}), stiffness (slope of the linear part of the curve representing elastic deformation), and energy absorption (area under the curve, Wabs) were obtained.

Bone histomorphometric analysis

The fourth lumbar vertebra were removed and fixed with a 10% tetraacetic acid (EDTA) solution (pH 7.4) at 4 °C for 3 weeks. After decalcification, each bone sample was cut along the coronal plane and embedded in paraffin for tissue sectioning and histological staining. A histomorphometric study of the lumbar vertebra was performed using image analysis software (Image Pro Plus 6.1 for Windows; Media Cybernetics, USA). Parameters measured included mean thickness of the trabeculae (Tb.Th, μm), trabecular area percentage (Tb.Ar, %) and trabecular separation (Tb.Sp, mm).

Bone metabolic biochemical markers

Serum calcium (S-Ca) and phosphorus (S-P) concentrations were measured on an automatic biochemical analyzer (Hitachi 7170, Hitachi, Japan). Serum bone-specific alkaline phosphatase (B-ALP, a bone resorption marker and indicator of bone formation), and the bone resorption markers serum tartrate-resistant acid phosphatase 5b (TRACP 5b) and N-telopeptide of type I collagen (NTx) were estimated using an ELISA kit (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. B-ALP has 4.7% intra- and 3.4% inter-assay variabilities, TRACP 5b has 7.3% intra- and 3.5% inter-assay variabilities, and NTx has 4.0% intra- and 6.7% inter-assay variabilities.

Oxidative stress assays

Bone marrow was isolated from lumbar vertebrae, homogenized (100 mg/mL) at 4 °C in 0.1 mol/L Tris-HCl buffer pH 7.4 and centrifuged at 2000×*g* for 10 min. The supernatant was collected and promptly frozen at -80 °C until analysis. Oxidative stress was analyzed in bone marrow and serum. Superoxide dismutase (SOD, an enzyme that repairs cells and tissues as well as reduces superoxide induced damage), hydrogen peroxide (H_2O_2, an oxidizer), malondialdehyde (MDA, a marker for oxidative stress) and catalase (CAT, an enzyme rapidly catalyze the decomposition of hydrogen peroxide) concentrations were determined by colorimetric methods using the appropriate kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Protein levels in the bone marrow were determined by standard Coomassie brilliant blue staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Serum OPG, RANKL, and IGF-I

Serum levels of OPG, IGF-I, and RANKL were assessed by ELISA (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The OPG ELISA had 3.4% intra- and 5.0% inter-assay variabilities, the IGF-I ELISA had 8.2% intra- and 7.9% inter-assay variabilities and the RANKL ELISA had 7.4% intra- and 8.6% inter-assay variabilities. The ratio of OPG to RANKL, expressed as OPG/RANKL, was used to explain the process of bone formation coupled with bone resorption.

Real-time PCR analyses of bone OPG and RANKL

The metaphyseal portion of the tibia was cut and homogenized using a Mixer Mill MM400 (Retsch, Germany) to isolate the mRNA. Total RNA was isolated using TRIzol following the manufacturer's guidelines (Invitrogen, Rockville, MD, USA). The concentration and purity of the RNA were determined by measuring the absorbance at 260 nm and 280 nm. The amount of RANKL and OPG mRNA was determined with ABI Prism 7900HT quantitative real-time PCR (Applied Biosystems, Foster City, CA). The primers were as follows: RANKL (forward, 5′-ACC AGC ATC AAA ATC CCA AG-3′; reverse, 5′-TTT GAA AGC CCC AAA GTA CG-3′) and OPG (forward, 5′-GTT CTT GCA CAG CTT CAC CA-3′; reverse, 5′-AAA CAG CCC AGT GAC CAT TC-3′). PCR amplification was carried out in a 20 μL reaction mixture (2 μL of cDNA and 200 nmol/L primers for OPG and RANKL, respectively, and 1 μL SYBR green). The temperature program was as follows: inactivation of reverse transcriptase at 95 °C for 30 s, followed by 45-cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. The specificity of the PCR results was confirmed by melting curve analysis. The mRNA levels for each gene were calculated using a standard curve generated from 10-fold dilutions

of a control RNA (Roche, Penzberg, Germany). The expression levels of RANKL and OPG were normalized to S18 RNA levels, and all samples were analyzed in triplicate.

Statistical analysis

All data are presented as the mean±SD. One-way ANOVA followed by the least-significant difference (LSD) was used to determine statistical differences. $P<0.05$ was used as the criteria for statistical significance.

Results

Body weight and uterine index

There was no difference in the body weight observed among all groups in the initial experimental period. Following the initial experimental period, the body weights in the OVX-control group and OVX+FDP-Sr/SR group significantly increased compared to the sham-operated group; no adverse effects from FDP-Sr treatment were observed (Figure 2A). Atrophy of the uterine tissue was caused by bilateral ovariectomy after 6 months, and the uterine index (uterine wet weight/body weight) and E_2 levels (Figure 2B, 2C) in the OVX control and treatment groups were markedly decreased compared with the sham-operated group ($P<0.01$). These data indicated the success of the surgical procedure and confirmed that the FDP-Sr treatment did not have estrogenic effects.

Bone mineral density measurements

Ovariectomy caused significant BMD reduction in the rat femur and lumbar vertebrae compared to that in the sham-operated group ($P<0.05$ CV: 5.23% and $P<0.01$, CV: 6.25%, respectively) three months following the operation (Figure 3). At the end of the treatment period, the BMD values for the femur ($P<0.01$) and lumbar vertebrae ($P<0.01$) in the OVX-control group (CV: 8.13% and 7.30%) were significantly lower than those in the sham-operated group. Treatment with 220 or 440 $mg \cdot kg^{-1} \cdot d^{-1}$ of FDP-Sr markedly inhibited OVX-induced decreases (0.173±0.011 CV: 6.36% and 0.175±0.013 CV: 7.43%, respectively) in BMD of the femur ($P<0.05$ for both). All three FDP-Sr doses were shown to significantly inhibit the decrease in lumbar vertebrae BMD in a dose-dependent manner in

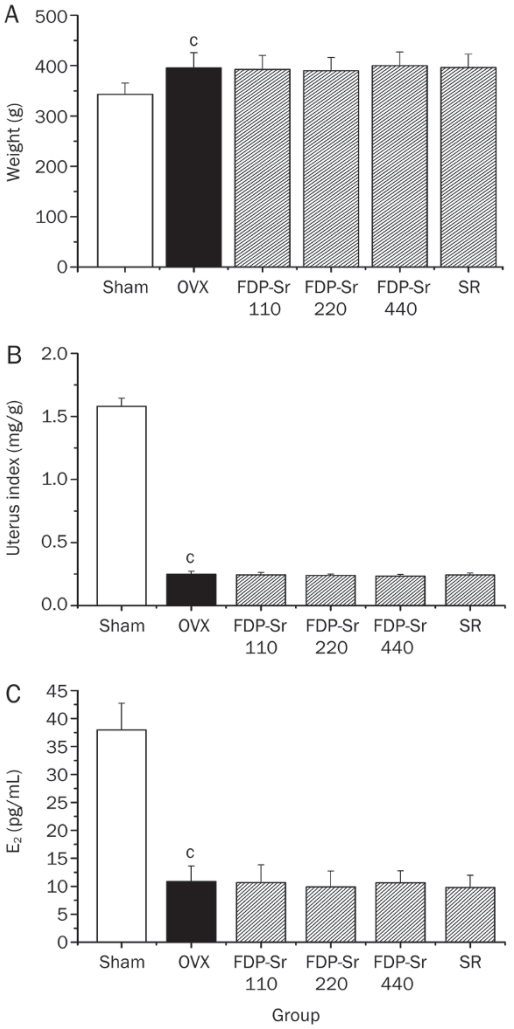

Figure 2. Effect of a 3-week treatment with FDP-Sr or SR on E2, and body and uterus weights of OVX rats. (A) The body weights of the rats were recorded at the end of the experimental period. (B) The uterus index is represented as the uterus weight divided by the body weight. (C) E2 was recorded after 3 weeks of treatment. Values with a superscript are significantly different from those of the sham-operated group ($^bP<0.05$, $^cP<0.01$) or OVX group ($^eP<0.05$, $^fP<0.01$).

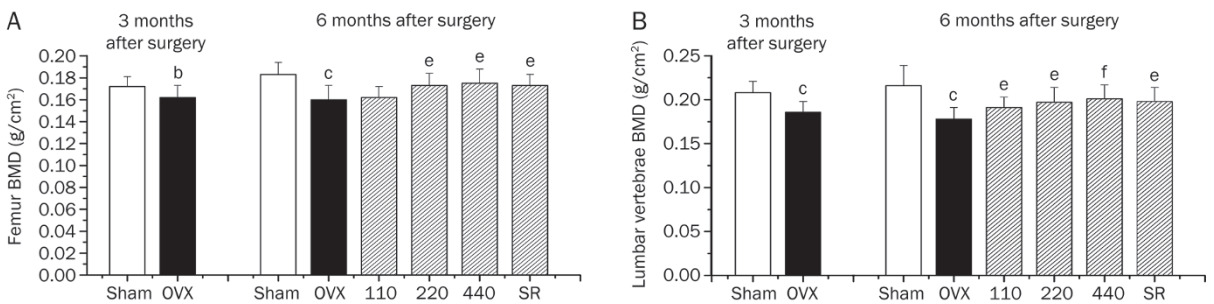

Figure 3. Bone mineral density of the femur and the lumbar vertebrae. (A) Femur. (B) Lumbar vertebrae (L1–L5). Each column and bar represents mean±SD. $n=10$. Values with a superscript are significantly different from those of the sham-operated group ($^bP<0.05$, $^cP<0.01$) or OVX group ($^eP<0.05$, $^fP<0.01$).

comparison with the OVX control group (P<0.05, P<0.05, and P<0.01, respectively). There were no differences in BMD between the FDP-Sr and SR treatment groups.

Mechanical tests of femur and lumbar vertebrae

After three months of estrogen deficiency, maximum load, stiffness and energy were significantly decreased (P<0.05 for all) in the OVX control group compared with the sham-operated group as demonstrated in the femoral three-point bending test (Table 1). Compression testing of the third lumbar vertebra demonstrated that the bone strength of rats in the OVX control group was reduced. Significant decreases were observed in the maximum load (P<0.01), stiffness (P<0.05) and energy to failure (P<0.05) when compared with the sham-operated group.

At the end of the treatment period, the maximum load (P<0.05), stiffness (P<0.05) and energy (P<0.01) of the femoral three-point bending test were significantly decreased in the OVX control group compared with the sham-operated group. This decrease was dose-dependently attenuated by FDP-Sr treatment. Treatment with 220 mg/kg or 440 mg/kg FDP-Sr improves femoral three-point bending test parameters by increasing the maximum load (P<0.05 for both), stiffness (P<0.05 and P<0.01, respectively) and energy to failure (P<0.05 for both) compared with those in the OVX control group. Compression testing of the third lumbar vertebrae showed that the maximum load, stiffness and energy to failure also notably decreased (P<0.01 for all) in the OVX control group compared with the sham-operated group six months after surgery. Treatment with 220 or 440 mg/kg FDP-Sr increased the value of maximum load (P<0.05 and P<0.01, respectively), stiffness (P<0.05 and P<0.01, respectively) and energy to failure (P<0.05 for both) compared with the OVX control group.

Histomorphometry study

As shown in Figure 4 and Table 2, the ovariectomy procedure induced significant reductions in trabecular area percentage (Tb.Ar, Table 2) and trabecular thickness (Tb.Th μm, Table 2) compared to the sham-operated group. These reductions were accompanied by significant increases in trabecular separation (Tb.Sp, Table 2) (P<0.001 for all).

After three months of FDP-Sr treatment, positive effects on the trabecular microarchitecture of lumbar vertebra were observed. Compared to the OVX control group, 220 or 440 $mg \cdot kg^{-1} \cdot d^{-1}$ FDP-Sr can prevent the further loss of Tb.Ar (P<0.05 for 440 mg/kg) and Tb.Th (P<0.05 for both). The increased Tb.Sp in the lumbar vertebrae was significantly inhibited at the end of the 220 (P<0.05) and 440 $mg \cdot kg^{-1} \cdot d^{-1}$

Table 1. Effect of FDP-Sr on bone mechanical strength of lumbar vertebrae (L3) and femur. Mean±SD. n=10. Values with a superscript are significantly different from those in the sham (^bP<0.05, ^cP<0.01) or OVX group (^eP<0.05, ^fP<0.01).

	3 months after surgery		6 months after surgery					
	Sham	OVX	Sham	OVX	FDP-Sr 110	FDP-Sr 220	FDP-Sr 440	SR
Three point bending femur								
Maximum load (N)	119.2±13.2	101.5±14.3^b	137.2±22.4	107.0±18.3^b	122.4±14.7	127.9±21.4^e	127.9±14.2^e	126.6±12.5^e
Stiffness (N/mm)	195.2±19.8	175.8±13.4^b	221.0±32.2	183.2±26.4^b	200.1±37.0	213.2±30.8^e	219.1±24.3^f	216.8±28.4^e
Energy (N×mm)	28.7±4.6	23.0±4.6^b	29.4±4.1	23.8±4.1^c	24.8±4.2	28.2±4.9^e	27.8±3.9^e	28.0±3.2^e
Compression lumbar vertebrae (L3)								
Maximum load (N)	196.2±18.9	171.1±14.8^c	202.8±22.6	152.9±25.4^c	168.1±26.3	184.8±25.3^e	192.5±16.2^f	184.9±20.9^e
Stiffness (N/mm)	761.1±79.1	665.0±88.5^b	814.1±83.1	641.3±90.7^c	717.1±90.6	754.4±91.4^e	764.6±83.6^f	756.6±97.6^e
Energy (N×mm)	31.6±4.9	25.6±4.4^b	33.4±5.0	22.7±5.8^c	25.8±6.9	29.3±6.7^e	29.2±5.1^e	29.0±5.1^e

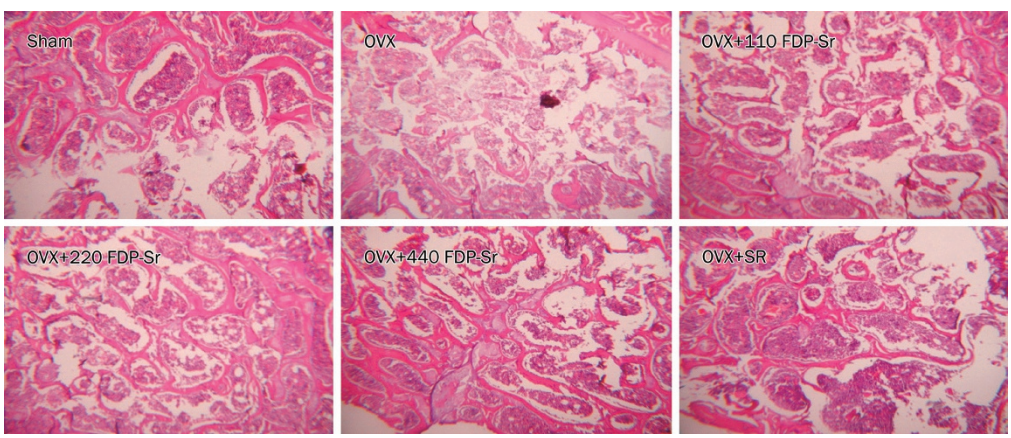

Figure 4. Histological sections [stained with H&E (40×)] of bone trabeculae from the 4th lumbar vertebra in rats from different groups. The ovariectomy caused a loss of trabeculae in the lumbar vertebra of the OVX group, but SR and 220 or 440 $mg \cdot kg^{-1} \cdot d^{-1}$ FDP-Sr significantly protected bone from ovariectomy-induced osteopenia.

Table 2. Histomorphometric analysis of lumbar vertebrae (L4) in groups. Mean±SD. n=10. Values with a superscript are significantly different from those in the sham (^bP<0.05, ^cP<0.01) or OVX group (^eP<0.05, ^fP<0.01).

			FDP-Sr			
	Sham	OVX	OVX+110	OVX+220	OVX+440	OVX+SR
Tb.Th (μm)	76.12±7.59	58.24±5.66^c	60.58±5.44	63.79±5.81^e	64.49±5.54^e	63.61±3.89^e
Tb.Ar (%)	53.37±2.91	44.52±3.87^c	45.46±3.96	46.64±3.73	48.33±4.14^e	48.18±2.91^e
Tb.Sp (mm)	60.68±3.93	80.59±10.66^c	75.72±10.55	71.46±6.95^e	66.61±5.63^f	68.87±6.27^e

(P<0.01) FDP-Sr treatment.

Biochemical parameters

Serum calcium (Ca) levels were significantly decreased in the OVX rats compared with the sham-operated rats (P<0.01). Administration of 220 mg·kg^{-1}·d^{-1} FDP-Sr significantly enhanced the serum Ca levels in OVX rats (P<0.05), whereas the values of serum P (phosphorus) did not show significant differences among the groups.

Serum B-ALP, NTx, and TRACP 5b were significantly increased in the OVX control group compared with the sham-operated group six months after bilateral ovariectomy (P<0.01, P<0.01, and P<0.05, respectively), suggesting that bone turnover was elevated because of estrogen deficiency; this has been previously reported[5]. After three months of treatment, FDP-Sr was able to maintain an increase in serum B-ALP level in a dose-dependent manner (5.7%, 12.6%, and 15.4%, and all three doses resulted in a significant difference compared to those in the OVX control group (P<0.05, P<0.01, and P<0.01, respectively). However, treatment with FDP-Sr can markedly suppress the ovariectomy-induced increase in serum NTx (P<0.05 for 110 and 220 mg·kg^{-1}·d^{-1}, P<0.01 for 440 mg·kg^{-1}·d^{-1}) and TRACP 5b (P<0.05 for 220 mg·kg^{-1}·d^{-1}, P<0.01 for 440 mg·kg^{-1}·d^{-1}) (Table 3).

Oxidative stress assays

Estrogen deficiency induced abnormal changes in free radical parameters, significantly increased levels of H_2O_2 and MDA in serum and bone tissue, respectively (P<0.05 for all), and biomarkers of anti-oxidant such as CAT in serum (P<0.01) and bone tissue (P<0.05) were significantly decreased compared to those in the sham-operated group six months after the surgery. Treatment with FDP-Sr decreased both H_2O_2 and MDA levels in serum and bone tissue in a dose-dependent manner. Additionally, FDP-Sr inhibited the decrease of CAT in serum and bone tissue after three months of treatment, but this anti-oxidant effect was not observed in the strontium ranelate group, which suggests that this effect is due to FDP (Table 4).

Serum OPG, RANKL, and IGF-I

Serum OPG was 38.5% lower (P<0.01) in the OVX animals (506.75±75.826 pg/mL) compared to the sham-operated animals (724.0±42.55 pg/mL). In contrast, estrogen deficiency inncrease serum RANKL (P<0.01), and the ratio of OPG to RANKL (P<0.01) was significantly decreased compared with the sham-operated group. Meanwhile, OVX significantly induced a decrease in serum IGF-I (P<0.05) 6 months after surgery compared to the sham-operated group. Treatment with FDP-Sr reversed the above findings. Treatment with FDP-Sr 220 or 440 mg·kg^{-1}·d^{-1} resulted in a significant OPG increase (P<0.01, 812.75±138.87 pg/mL for 220 mg·kg^{-1}·d^{-1}, P<0.01, 969.05±61.57 pg/mL for 440 mg/kg) and RANKL decrease (P<0.01 49.27±6.85 pg/mL for 220 mg/kg, P<0.01, 43.44±6.51 pg/mL for 440 mg/kg) 3 months after treatment. Thus, the ratio of OPG to RANKL was significantly increased in both the 220 and 440 FDP-Sr mg·kg^{-1}·d^{-1} treatment groups compared to the OVX animals. Additionally, the medium (4.29±0.46 ng/mL) and high (4.18±0.47 ng/mL) doses of FDP-Sr significantly increased serum IGF-I after 3 months compared to the OVX control group (3.34±0.34 ng/mL). Significant differences in OPG and RANKL were also

Table 3. Effects of FDP-Sr or SR on bone biochemical parameters in serum of ovariectomized rats. Mean±SD. n=10. Values with a superscript are significantly different from those in the sham (^bP<0.05, ^cP<0.01) or OVX group (^eP<0.05, ^fP<0.01).

			FDP-Sr			
	Sham	OVX	OVX+110	OVX+220	OVX+440	OVX+SR
S-Ca (mmol/L)	2.26±0.16	2.09±0.13^b	2.06±0.11	2.16±0.12	2.07±0.16	2.08±0.12
S-P (mmol/L)	1.67±0.14	1.69±0.18	1.72±0.21	1.71±0.17	1.79±0.20	1.75±0.13
B-ALP (μg/L)	0.994±0.073	1.33±0.034^c	1.406±0.062^e	1.498±0.045^f	1.535±0.078^f	1.423±0.045^e
NTx (nmol/L)	2.179±0.123	3.036±0.347^c	2.600±0.174^e	2.426±0.235^e	2.261±0.071^f	2.395±0.265^e
TRACP-5b (U/L)	1.877±0.169	2.056±0.184^b	1.982±0.138	1.891±0.089^e	1.835±0.145^f	1.874±0.105^e

Table 4. Effect of FDP-Sr on marker of oxidative stress in serum and tibia metaphysis. Mean±SD. *n*=10. Values with a superscript are significantly different from those in the Sham ($^{b}P<0.05$, $^{c}P<0.01$) or OVX group ($^{e}P<0.05$, $^{f}P<0.01$).

			FDP-Sr			
	Sham	OVX	OVX+110	OVX+220	OVX+440	OVX+SR
Serum						
H_2O_2 (mmol/L)	18.62±2.22	21.59±3.13^{b}	19.20±3.11	18.98±3.29	18.72±2.67^{e}	21.26±1.99
CAT (U/mL)	0.637±0.122	0.444±0.102^{c}	0.485±0.102	0.563±0.127^{e}	0.605±0.100^{f}	0.422±0.101
SOD (U/mL)	141.4±4.89	139.9±5.29	140.5±6.10	140.5±5.68	140.3±5.25	137.9±4.53
MDA (nmol/mL)	8.18±0.807	9.13±0.958^{b}	8.33±1.103	8.57±1.258	8.21±0.770^{e}	8.63±0.900
Metaphysis						
H_2O_2 (mmol/g prot)	4.83±1.93	6.50±1.33^{b}	5.49±0.99	5.13±0.88^{e}	4.82±1.39^{e}	5.99±1.05
CAT (U/mg prot)	1.55±0.28	1.18±0.34^{b}	1.09±0.47	1.41±0.55	1.59±0.49^{e}	1.37±0.50
SOD (U/mg prot)	42.61±12.77	36.55±11.45	36.71±6.38	39.16±14.42	40.25±12.90	35.21±7.28
MDA (nmol/mg prot)	2.14±0.81	3.08±0.74^{b}	2.66±0.37	2.52±0.48	2.34±0.48^{e}	3.04±0.45

observed in the middle- and high-dose FDP-Sr treatment groups compared to the SR group (Figure 5).

Real-time PCR analyses of bone OPG and RANKL

To investigate the role of FDP-Sr in the OPG/RANKL/RANK pathway and to identify the mechanism regulating bone formation and resorption, we performed real-time RT-PCR on bone tissues to examine OPG and RANKL expression. Our results demonstrated that the metaphyseal portion of the tibia bone from the OVX rats produced more RANKL and less OPG than the sham-operated rats and that the OPG/RANKL ratio was lower in OVX rats than in sham-operated rats. All three doses of FDP-Sr significantly increased OPG expression, reduced RANKL and elevated the OPG/RANKL ratio in OVX rats. Additionally, significant increases in OPG expression and reduced RANKL expression were found in middle and high FDP-Sr doses compared to the SR group (Figure 6).

Discussion

Osteoporosis associated with ovarian hormone deficiency following menopause can be caused by inadequate peak bone mass, excessive bone resorption and inadequate bone formation[23, 24]. The ovariectomized rat model is a classic model for conducting osteoporosis studies[25, 26]. In the current study, FDP-Sr counteracted the bone loss induced by ovariectomy, preserved bone microarchitecture and maintained bone strength in OVX rats. Estrogen deficiency leading to endocrine dysfunction significantly increased body weight, although feed consumption was similar among all groups. A recent study by Rogers *et al* indicated that adiposity induced by ovariectomy is associated with decreased energy expenditure, adipose tissue (AT) expansion, and hepatic steatosis[27]. No dose of FDP-Sr prevented the body weight gain induced by estrogen deficiency. Estrogen deficiency was confirmed by atrophy of the uterine tissue, another key marker of estrogen deficiency, on six months after bilateral ovariectomy surgery. FDP-Sr was unable to slow ovariectomy-induced atrophy of the uterine tissue. These results indicate that FDP-Sr differed from estrogen replacement therapy in the regulation of body weight and uterine growth in the OVX rats.

Osteoporosis is a metabolic disorder in which a loss of bone mass and strength leads to fragility fractures[28]. In this study, BMD and biomechanical properties were chosen as indexes to evaluate the establishment of osteoporosis due to ovariectomy[29, 30]. As expected, OVX resulted in a significant BMD decrease in the femur and lumbar vertebra following three months of treatment after bilateral ovariectomy surgery. Estrogen is important for maintaining bone density, and when estrogen levels drop after menopause, bone loss accelerates[5]. Additionally, a significant reduction in biomechanical parameters was observed including the compression strength of the lumbar vertebra and the three-point bending test of the femur compared to values in the sham-operated group. Three months of FDP-Sr therapy dose-dependently increased the BMD of the lumbar vertebrae and femur. Elevated biomechanical properties of the femur and vertebrae further indicated that FDP-Sr was effective at restoring bone strength in OVX rats. Physiological analyses indicated that the turnover rate for cancellous bone is higher than that for cortical bone. Cancellous bone is the major component of vertebrae, and cortical bone is the chief component of the femur. Therefore, the lumbar vertebrae play a critical role in predicting osteoporosis and monitoring the response to pharmacotherapy. The findings in this study are in agreement with previous reports in OVX rats in demonstrating that decreases in BMD and biomechanical properties occur faster in the vertebrae than in the femur. The protective effect of FDP-Sr on bone is more easily observed in the lumbar spine, although this protective effect was observed in the femur after three months of treatment.

Histomorphometric analysis of the trabecular bone in lumbar vertebral bodies was performed at the end of the study. Deterioration of the trabecular architecture has been implicated in decreased bone strength and increased fracture incidence in humans. Thus, the assessment of trabecular architectural properties is necessary to evaluate the treatment impact on the quality of the lumbar vertebrae in addition to BMD

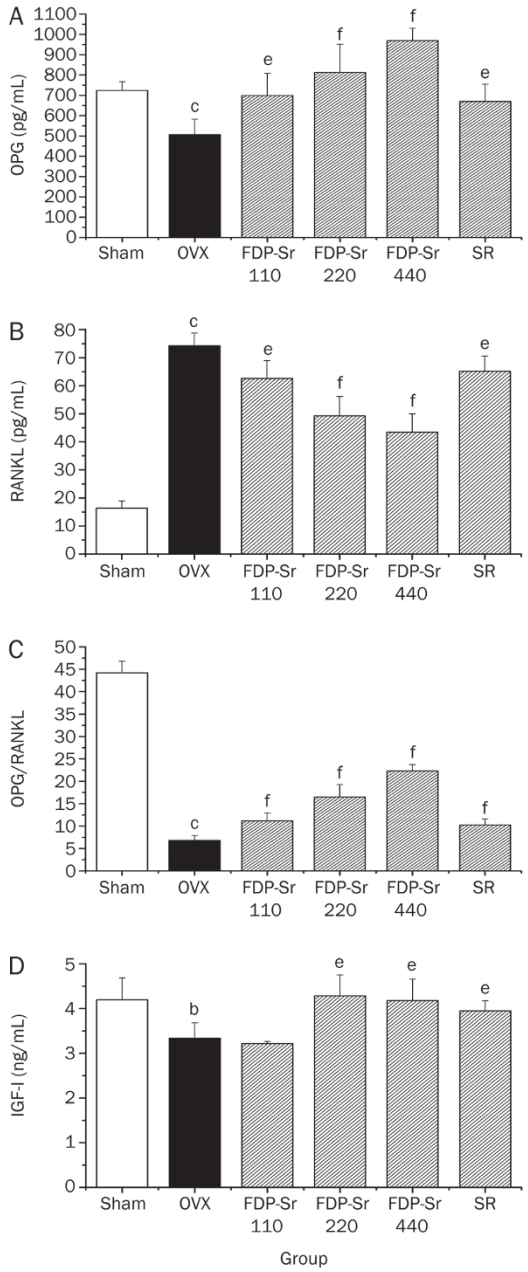

Figure 5. Effect of FDP-Sr on OPG (A), RANKL (B), OPG/RANKL (C) and IGF-I (D) in serum. Each column and bar represents the mean±SD. n=10. Values with a superscript are significantly different from those of the sham-operated group (^{b}P<0.05, ^{c}P<0.01) or OVX group (^{e}P<0.05, ^{f}P<0.01).

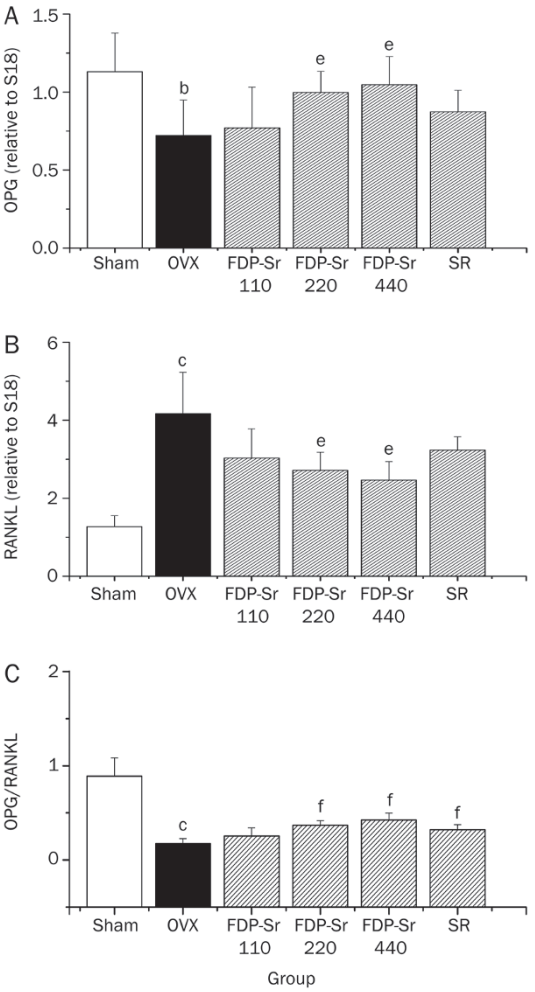

Figure 6. Effect of FDP-Sr on OPG (A), RANKL (B), and OPG mRNA/RANKL mRNA (C) expression in the metaphyseal portion of the tibia. Each column and bar represents the mean±SD. n=10. Values with a superscript are significantly different from those of the sham-operated group (^{b}P<0.05, ^{c}P<0.01) or OVX group (^{e}P<0.05, ^{f}P<0.01).

and biomechanical properties. In this study, Tb.Th and Tb.N decreased and Tb.Sp increased in the OVX control group compared to the sham-operated group. The results indicate that trabecular bone was readily lost as a result of estrogen deficiency, which is typical of cancellous bone loss and has been reported previously. However, this deterioration was ameliorated by FDP-Sr treatment. A gradual increase in Tb.Th and Tb.N and a decrease in Tb.Sp were observed after 3 months of treatment. These findings suggest that FDP-Sr can significantly improve these structural indices, repair lost trabecular connectivity, and prevent the progression of trabecular architecture deterioration. Moreover, these results indicate that FDP-Sr contributes to the restoration of deteriorated trabecular architecture and lumbar morphology.

The loss of bone mass and the deterioration of bone microstructure have been linked to an imbalance between bone formation and bone resorption. Biochemical markers of bone turnover have been widely used to measure the effects of various drugs on bone remodeling[31]. The dual effect of Sr in the treatment of osteoporosis was successfully duplicated *in vivo* in this study. B-ALP activity, an indicator of bone formation, was found throughout the body, and its activity in plasma increased sharply after the ovariectomy surgery. FDP-Sr treatment increased B-ALP by 5.7%, 12.6%, and 15.4% in OVX rats. TRACP 5b is one isoform of TRACP 5 and serum TRACP 5b is derived only from osteoclasts. Thus, TRACP 5b activity can be used as a marker to monitor osteoclastic activ-

ity and bone resorption rates[32]. As expected, the TRACP 5b activity in serum increased sharply after the ovariectomy surgery. FDP-Sr potently inhibited the increase in TRACP 5b levels in plasma and thereby favors bone metabolism. Meanwhile, serum NTx, which also represents a bone resorption biomarker derived from N-telopeptide of type I collagen, rapidly declined after oral administration of FDP-Sr. Overall, these results indicated a positive effect on bone formation and a negative effect on bone resorption and confirmed the dual effect of FDP-Sr *in vivo*.

Physiologically, the balance of bone formation and resorption plays a key role in bone metabolism in healthy individuals. When resorption increases after menopause, bone formation is not able to compensate, leading to the degradation of bone biomechanical properties. Ideally, to reverse this process, treatments should be able rebalance bone remodeling. Fortunately, strontium, a naturally occurring anti-osteoporosis drug, has a dual effect on bone remodeling unlike other drugs. However, the optimal usage of strontium treatment to rebalance bone remodeling and repair bone mass remains unclear. Strontium can directly interact with the calcium-sensing receptor (CaSR), a G protein-coupled receptor first cloned from the parathyroid gland[33–35], as recent work by Naibedya *et al* found that Sr^{2+} is a CaSR agonist, even though strontium's affinity for the receptor is lower than that of calcium[36]. Other studies have demonstrated that the PKC/PKD and p38 pathways could be involved as potential mediators of osteoblast cell replication[37]. In addition, IGF-I can play a role as a key cytokine in bone formation stimulated by strontium[38, 39]. Previous studies have indicated that strontium can inhibit osteoclast proliferation, differentiation and activity[40]; however, the mechanism remains controversial. Strontium was thought to cause osteoclast apoptosis either directly[41] or indirectly through CaSR[42]. Hofbauer *et al* suggest that although multiple hormones and cytokines regulate various aspects of osteoclast formation, the final effectors are in the OPG/RANK/RANKL system, which was regarded as a key factor in inhibiting bone proliferation and directly participates in the process of bone formation and bone resorption[43]. This hypothesis explains the process of bone formation coupled with bone resorption. Our research indicated that FDP-Sr up-regulates OPG and down-regulates RANKL secreted from osteoblasts, thereby inhibiting osteoclastogenesis and promoting bone formation.

Alternatively, oxidative stress is an indirect effect of estrogen deficiency and can be associated with one of several mechanisms related to bone loss[44]. A study by Sridhar *et al* confirmed that estrogen deficiency increases hydrogen peroxide and lipid peroxide in the femur bone and impairs bone antioxidant systems, thereby contributing to accelerated bone loss[45]. Therefore, concentrations of several markers of oxidative stress were determined in plasma and bone marrow six months after surgery. Increased H_2O_2 and MDA in the plasma and bone marrow were found in the rats with estrogen deficiency, and as previously reported, hydrogen peroxide served as the reactive oxygen species responsible for bone loss and the inhibition of osteoblast proliferation[46]. Both decreased H_2O_2 and MDA increased CAT in plasma and bone marrow were observed following FDP-Sr treatment, indicating that FDP-Sr may be a substantial contributor to counteract oxidative stress caused by estrogen deficiency in our study. According to previous reports[47, 48], FDP preserved antioxidant capacity, including CAT activity and GSH content, by indirect radical scavenging. Thus, FDP acting as an anti-oxidant may provide an additional benefit to reduce the risk of bone loss.

Osteoporosis is a chronic bone remodeling disease with increased risk of fracture, which can significantly affect quality of life and life expectancy, and patients suffering from it require long-term medication. Therefore, it is important to carefully evaluate the safety and tolerance of anti-osteoporotic agents. Toxic symptoms from appropriate doses of Sr have not been reported in humans. The only stable Sr-containing acid radical considered to be harmful to humans in small amounts is strontium chromate. In this compound, the toxicity is caused by the chromium, which is a genotoxic carcinogen[49]. Strontium ranelate was selected from among 20 different salts (*eg*, lactate, carbonate or gluconate salts) to treat osteoporosis in the clinical setting. Considerations were based on Sr bioavailability, the gastric tolerance, and the high ratio between Sr and different chemical acid radicals[50]. The biologic activity of ranelic acid as a carrier is unclear. The choice of eligible acid radicals plays a vital role in evaluating the therapeutic effectiveness and safety of a strontium compound. FDP, an endogenous intermediate molecule, has low toxicity[51] and can provide energy[52] and anti-oxidant capabilities[48]. FDP combined with strontium has potential as an anti-osteoporosis drug.

Conclusion

In summary, estrogen deficiency was observed in OVX-rats, similar to that found in postmenopausal women. Three months of FDP-Sr treatment resulted in significant protection against bone loss in both cortical and cancellous bone, as well as maintenance of bone microarchitecture and prevention of deterioration in mechanical parameters. These results suggest that FDP-Sr can prevent vertebral and hip fractures through its protective effects on both trabecular and cortical bone and thereby has potential as an alternative treatment for osteoporosis. In addition, the combination of Sr with fructose 1,6-diphosphate was well tolerated by the rats and did not adversely affect bone quality. Further research is needed to identify the mechanisms of action of FDP-Sr and to explore the potential synergistic functions of FDP in the treatment of osteoporosis.

Acknowledgements

The study was supported by a grant from the National Natural Science Foundation of China (Grant No 30873112) and the Six Talent Peek Project of Jiangsu Province (No 07-C-018).

Author contribution

Bo MA and Qi ZHANG have been involved in all phases

of this project; Qi ZHANG and Han-jie YING designed the research; Yong-lu WANG and Ying-ying HU performed the ELISA and real-time PCR; Yan-ping CHENG, Zhen-dong YANG and Ya-ya ZHENG performed the animal experiments for the project; Di WU wrote and revised the manuscript.

References

1 An overview of osteoporosis. Lancet 1982; 2: 423–4.
2 Yasothan U, Kar S. Osteoporosis: overview and pipeline. Nat Rev Drug Discov 2008; 7: 725–6.
3 Cooper C, Campion G, Melton LJ 3rd. Hip fractures in the elderly: a world-wide projection. Osteoporos Int 1992; 2: 285–9.
4 Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature 2003; 423: 337–42.
5 Petrovic O. Estrogen and postmenopausal osteoporosis. Ann Intern Med 1993; 118: 155–6.
6 Rizzoli R, Burlet N, Cahall D, Delmas PD, Eriksen EF, Felsenberg D, *et al*. Osteonecrosis of the jaw and bisphosphonate treatment for osteoporosis. Bone 2008; 42: 841–7.
7 Hong JW, Nam W, Cha IH, Chung SW, Choi HS, Kim KM, *et al*. Oral bisphosphonate-related osteonecrosis of the jaw: the first report in Asia. Osteoporos Int 2010; 21: 847–53.
8 Bush TL, Helzlsouer K. Estrogen replacement therapy and risk of breast cancer. JAMA 1991; 266: 1357–8.
9 Porch JV, Lee IM, Cook NR, Rexrode KM, Burin JE. Estrogen-progestin replacement therapy and breast cancer risk: the Women's Health Study (United States). Cancer Causes Control 2002; 13: 847–54.
10 Wassertheil-Smoller S, Hendrix SL, Limacher M, Heiss G, Kooperberg C, Baird A, *et al*. Effect of estrogen plus progestin on stroke in postmenopausal women: the Women's Health Initiative: a randomized trial. JAMA 2003; 289: 2673–84.
11 Hendrix SL, Wassertheil-Smoller S, Johnson KC, Howard BV, Kooperberg C, Rossouw JE, *et al*. Effects of conjugated equine estrogen on stroke in the Women's Health Initiative. Circulation 2006; 113: 2425–34.
12 Cabrera WE, Schrooten I, De Broe ME, D'Haese PC. Strontium and bone. J Bone Miner Res 1999; 14: 661–8.
13 Meunier PJ, Roux C, Seeman E, Ortolani S, Badurski JE, Spector TD, *et al*. The effects of strontium ranelate on the risk of vertebral fracture in women with postmenopausal osteoporosis. N Engl J Med 2004; 350: 459–68.
14 Reginster JY, Bruyere O, Sawicki A, Roces-Varela A, Fardellone P, Roberts A, *et al*. Long-term treatment of postmenopausal osteoporosis with strontium ranelate: results at 8 years. Bone 2009; 45: 1059–64.
15 Collette J, Bruyere O, Kaufman JM, Lorenc R, Felsenberg D, Spector TD, *et al*. Vertebral anti-fracture efficacy of strontium ranelate according to pre–treatment bone turnover. Osteoporos Int 2010; 21: 233–41.
16 Liu JM, Wai–Chee KA, Pheng CS, Zhu HM, Zhang ZL, Wu YY, *et al*. Efficacy and safety of 2 g/day of strontium ranelate in Asian women with postmenopausal osteoporosis. Bone 2009; 45: 460–5.
17 Lee HY, Lie D, Lim KS, Thirumoorthy T, Pang SM. Strontium ranelate-induced toxic epidermal necrolysis in a patient with post-menopausal osteoporosis. Osteoporos Int 2009; 20: 161–2.
18 Yin H, Jin XB, Gong Q, Yang H, Hu LY, Gong FL, *et al*. Fructose-1,6-diphosphate attenuates acute lung injury induced by lipopolysaccharide in mice. Int Immunopharmacol 2008; 8: 1842–7.
19 Marangos PJ, Fox AW, Riedel BJ, Royston D, Dziewanowska ZE. Potential therapeutic applications of fructose-1,6-diphosphate. Expert Opin Investig Drugs 1998; 7: 615–23.
20 Chien S, Zhang F, Niu W, Ehringer W, Chiang B, Shi X, *et al*. Fructose-1,6-diphosphate and a glucose-free solution enhances functional recovery in hypothermic heart preservation. J Heart Lung Transplant 2000; 19: 277–85.
21 Tenenbaum HC, Palangio K. Phosphoethanolamine- and fructose 1,6-diphosphate-induced calcium uptake in bone formed *in vitro*. Bone Miner 1987; 2: 201–10.
22 Ma B, Zhang Q, Wang G, Wu Z, Shaw JP, Hu Y, *et al*. Synthesis and pharmacokinetics of strontium fructose 1,6-diphosphate (Sr-FDP) as a potential anti-osteoporosis agent in intact and ovariectomized rats. J Inorg Biochem 2011; 1054: 563–8.
23 Kamel HK. Postmenopausal osteoporosis: etiology, current diagnostic strategies, and nonprescription interventions. J Manag Care Pharm 2006; 12: S4–9; quiz S26–8.
24 Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest 2005; 115: 3318–25.
25 Yamazaki I, Yamaguchi H. Characteristics of an ovariectomized osteopenic rat model. J Bone Miner Res 1989; 4: 13–22.
26 Omi N, Ezawa I. The effect of ovariectomy on bone metabolism in rats. Bone 1995; 17: 163S–168S.
27 Rogers NH, Perfield JW 2nd, Strissel KJ, Obin MS, Greenberg AS. Reduced energy expenditure and increased inflammation are early events in the development of ovariectomy-induced obesity. Endocrinology 2009; 150: 2161–8.
28 Jakubowitz E, Seeger JB, Kretzer JP, Heisel C, Kleinhans JA, Thomsen M. The influence of age, bone quality and body mass index on periprosthetic femoral fractures: a biomechanical laboratory study. Med Sci Monit 2009; 15: BR307–12.
29 Vasikaran SD. Utility of biochemical markers of bone turnover and bone mineral density in management of osteoporosis. Crit Rev Clin Lab Sci 2008; 45: 221–58.
30 Turner CH. Biomechanics of bone: determinants of skeletal fragility and bone quality. Osteoporos Int 2002; 13: 97–104.
31 Bhattacharyya S, Siegel ER, Achenbach SJ, Khosla S, Suva LJ. Serum biomarker profile associated with high bone turnover and BMD in postmenopausal women. J Bone Miner Res 2008; 23: 1106–17.
32 Nenonen A, Cheng S, Ivaska KK, Alatalo SL, Lehtimaki T, Schmidt–Gayk H, *et al*. Serum TRACP 5b is a useful marker for monitoring alendronate treatment: comparison with other markers of bone turnover. J Bone Miner Res 2005; 20: 1804–12.
33 Coulombe J, Faure H, Robin B, Ruat M. *In vitro* effects of strontium ranelate on the extracellular calcium-sensing receptor. Biochem Biophys Res Commun 2004; 323: 1184–90.
34 Pi M, Quarles LD. A novel cation-sensing mechanism in osteoblasts is a molecular target for strontium. J Bone Miner Res 2004; 19: 862–9.
35 Pi M, Faber P, Ekema G, Jackson PD, Ting A, Wang N, *et al*. Identification of a novel extracellular cation-sensing G-protein-coupled receptor. J Biol Chem 2005; 280: 40201–9.
36 Chattopadhyay N, Quinn SJ, Kifor O, Ye C, Brown EM. The calcium-sensing receptor (CaR) is involved in strontium ranelate-induced osteoblast proliferation. Biochem Pharmacol 2007; 74: 438–47.
37 Caverzasio J. Strontium ranelate promotes osteoblastic cell replication through at least two different mechanisms. Bone 2008; 42: 1131–6.
38 Henrotin Y, Labasse A, Zheng SX, Galais P, Tsouderos Y, Crielaard JM, *et al*. Strontium ranelate increases cartilage matrix formation. J Bone Miner Res 2001; 16: 299–308.
39 Gulhan I, Bilgili S, Gunaydin R, Gulhan S, Posaci C. The effect of strontium ranelate on serum insulin like growth factor-1 and leptin levels in osteoporotic post-menopausal women: a prospective study.

Arch Gynecol Obstet 2008; 278: 437–41.
40 Takahashi N, Sasaki T, Tsouderos Y, Suda T. S 12911-2 inhibits osteoclastic bone resorption *in vitro*. J Bone Miner Res 2003; 18: 1082–7.
41 Bonnelye E, Chabadel A, Saltel F, Jurdic P. Dual effect of strontium ranelate: stimulation of osteoblast differentiation and inhibition of osteoclast formation and resorption *in vitro*. Bone 2008; 42: 129–38.
42 Hurtel-Lemaire AS, Mentaverri R, Caudrillier A, Cournarie F, Wattel A, Kamel S, *et al*. The calcium-sensing receptor is involved in strontium ranelate-induced osteoclast apoptosis. New insights into the associated signaling pathways. J Biol Chem 2009; 284: 575–84.
43 Hofbauer LC, Schoppet M. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. JAMA 2004; 292: 490–5.
44 Wauquier F, Leotoing L, Coxam V, Guicheux J, Wittrant Y. Oxidative stress in bone remodelling and disease. Trends Mol Med 2009; 15: 468–77.
45 Muthusami S, Ramachandran I, Muthusamy B, Vasudevan G, Prabhu V, Subramaniam V, *et al*. Ovariectomy induces oxidative stress and impairs bone antioxidant system in adult rats. Clin Chim Acta 2005; 360: 81–6.
46 Lean JM, Jagger CJ, Kirstein B, Fuller K, Chambers TJ. Hydrogen peroxide is essential for estrogen-deficiency bone loss and osteoclast formation. Endocrinology 2005; 146: 728–35.
47 Tang XY, Zhang Q, Dai DZ, Ying HJ, Wang QJ, Dai Y. Effects of strontium fructose 1,6-diphosphate on expression of apoptosis–related genes and oxidative stress in testes of diabetic rats. Int J Urol 2008; 15: 251–6.
48 Ahn SM, Hwang JS, Lee SH. Fructose 1,6-diphosphate alleviates UV-induced oxidative skin damage in hairless mice. Biol Pharm Bull 2007; 30: 692–7.
49 Pors NS. The biological role of strontium. Bone 2004; 353: 583–8.
50 Reginster JY. Strontium ranelate in osteoporosis. Curr Pharm Des 2002; 8: 1907–16.
51 Wang M, Yao J, Chen JY, Bai WX, Mei SY, Pan WN, *et al*. A subchronic intravenous toxicity study of magnesium fructose-1,6-diphosphate in beagle dogs. Basic Clin Pharmacol Toxicol 2009; 104: 93–100.
52 Hua D, Zhuang X, Ye J, Wilson D, Chiang B, Chien S. Using fructose-1,6-diphosphate during hypothermic rabbit-heart preservation: a high-energy phosphate study. J Heart Lung Transplant 2003; 22: 574–82.