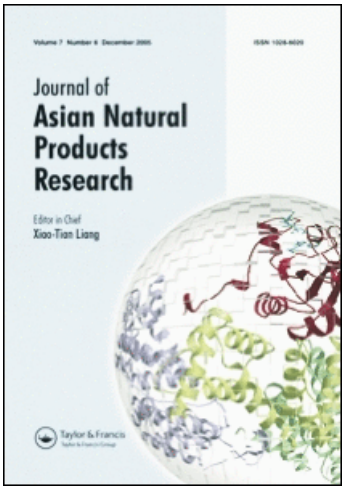

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Y. -S. Gong[a]; J. Chen[a]; Q. -Z. Zhang[a]; J. -T. Zhang[a]
[a] Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

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Effect of 17β-oestradiol and ginsenoside on osteoporosis in ovariectomised rats

Y.-S. GONG, J. CHEN, Q.-Z. ZHANG and J.-T. ZHANG*

Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China

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To study the anti-osteoporosis effects and mechanism of action of oestradiol (E_2) and ginsenoside (tR), we measured the bone mineral densities (BMD) of lumbar vertebra and tibia and analysed the tibia histological morphological data, as well observed the activity and the number of osteoblasts and the activity of alkaline phosphatase (ALP) and the concentration of cAMP. Results showed that E_2 ($400\,\mu g\,kg^{-1}$ $week^{-1}$) and tR (10, 20, $30\,mg\,kg^{-1}\,day^{-1}$) were able to countervail the decreasing in BMDs of lumbar vertebra and tibia induced by OVX in rats ($P < 0.05$); E_2 ($0.1\,\mu mol\,l^{-1}$) and ginsenoside Rg_1 ($1\,\mu mol\,l^{-1}$ and $10\,\mu mol\,l^{-1}$) were able to increase the number of osteoblasts, the activity of ALP and the concentration of intercellular cAMP in cultured osteoblast cells. The present findings suggest that E_2 and tR have an anti-osteoporosis effect in ovariectomised rats.

Keywords: Osteoporosis; Ovariectomy; 17β-Oestradiol; Ginsenoside; Histomorphometry; Osteoblast

1. Introduction

Osteoporosis is a systemic skeletal disease characterised by loss of bone mass and microarchitectural deterioration of bone tissue. Postmenopausal osteoporosis is a major public health problem in bone fragility and susceptibility to fracture. Oestrogen deficiency in postmenopausal women results in a number of detrimental effects on bone, including suppression of osteocyte survival as well as impairment of osteoblast response to mechanical stimuli and repair of ageing bone.

Administration of oestrogen at or after the menopause preserves bone mass and reduces fracture rate. Its effects are believed to be mediated predominantly by inhibition of osteoclastic bone resorption [1] and suppression of bone turnover [2]. Oestrogen might also increase bone mass, not only by decreasing the bone absorption but also by promoting the activity of osteoblasts [1], mainly by stimulating oestrogen receptors and osteoprogesterin expression in osteoblasts [3]. It was also found that long-term administration of high doses of oestrogen produces anabolic effects in cancellous bone through an increase in osteoblast activity [4,5].

*Corresponding author. E-mail: zhangjt@imm.ac.cn

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Osteoblast lineage in bone formation synthesises and secretes molecules that in turn initiate and control osteoblast differentiation in bone resorption. Osteoblasts have a critical role in bone formation and normal bone density. The cellular events involved in bone formation include the proliferation and differentiation of osteoblast precursors [6]. In these processes, oestrogen plays an important role in maintaining normal bone metabolism via the direct or indirect regulation of bone cells.

However, oestrogen therapy must be long-term, possibly lifelong, to have any lasting impact on bone health. The safety of long-term treatment with oestrogen is much worried about because oestrogen may increase the incidence of uterine and breast cancer [2]. In addition, oestrogen replacement has many non-fatal side effects [3–5].

To find an appropriate drug for treatment of postmenopausal osteoporosis one should take into account the long-term effects in preventing bone calcium loss and stimulating osteoblast activity, as well as potentially beneficial effects on other tissues and safety concerns. Many bioactive compounds have been discovered, such as flavonoids, phenolic acids and phytoestrogens, which may act as oestrogen agonists/antagonists with beneficial health effects, for example reducing the risk of osteoporosis, cancer and cardiovascular disease [7].

Ginseng has been used as a traditional Chinese medicine for more than two thousand years in Asia. Ginseng and its active ingredients show wide beneficial health effects in animals and humans. This study aimed at finding a new non-steroid bioactivity compound for osteoporosis derived from ginseng, such as ginsenoside (tR) and its ingredients.

Ovariectomised (OVX) rats showed the symptoms of osteoporosis similar to the pathological changes apparent in postmenopausal women, such as decreased bone calcium, increased bone reabsorption and sizeable decrease in the number and activity of osteoblasts [6]. In this experiment, the OVX rats were used as an osteoporosis model to observe the effect of oestradiol (E_2) and tR on bone calcification and histological morphology, and the cultured osteoblasts were used to detect the effect of E_2, Rb_1 and Rg_1 (two main ingredients of tR) on the activities of osteoblasts.

2. Result and discussion

2.1 Effect of E_2 and tR on the equilibrium of bone calcium and histological morphology

Ovariectomy (OVX) caused a significant decrease in the secretion of oestrogen. Six months after OVX, the uterus in rats of the sham-operated control group still existed, but the uterus in rats of the ovariectomised group clearly atrophied and became just like the small vessels. Oestrogen thus prevented the uterus from atrophy. In this respect, the effect on the ginsenoside group was not obvious.

Bone density measurement showed that bone density in lumbar vertebrae and tibia in the ginsenoside group increased significantly compared with that in OVX animals. Oestrogen treatment prevented the loss of bone density at these two sites, but its effect was lower than ginsenoside. The BMDs of lumbar vertebrae and tibia were significantly lower in the OVX group than in the control group ($0.15 \pm 0.01\ g\,cm^{-2}$ and $0.205 \pm 0.019\ g\,cm^{-2}$ versus $0.217 \pm 0.018\ g\,cm^{-2}$ and $0.265 \pm 0.021\ g\,cm^{-2}$, respectively, $P < 0.05$). As shown in table 1, E_2 and tR were able to elevate the BMDs in these bones in OVX rats ($P < 0.05$). The values of bone density in lumbar vertebrae and tibia are shown in table 1.

Table 1. Effect of E_2, tR on the bone mineral density (BMD) in OVX rats.

	BMD ($g\,cm^{-2}$)	
	Lumbar vertebrae	*Tibia*
E_2		
$400\,\mu g\,kg^{-1}\,week^{-1}$	0.203 ± 0.017^{bd} (35.4%)	0.239 ± 0.018^{bd} (16.6%)
TR		
$10\,mg\,kg^{-1}$	0.172 ± 0.020^{ae} (16.7%)	0.223 ± 0.018^{ae} (8.8%)
$20\,mg\,kg^{-1}$	0.198 ± 0.018^{bd} (32.0%)	0.243 ± 0.007^{bd} (18.5%)
$40\,mg\,kg^{-1}$	0.205 ± 0.020^{bd} (36.7%)	0.246 ± 0.018^{bd} (20.0%)
OVX rat	0.150 ± 0.011^{f}	0.205 ± 0.019^{e}
Control	0.217 ± 0.018 (44.7%)	0.265 ± 0.021 (29.3%)

Mean $\pm$ SD, $n = 8$. (%): variational percent vs. OVX rat.
$^{a}P > 0.05$, $^{b}P < 0.05$, $^{c}P < 0.01$ vs. OVX rats; $^{d}P > 0.05$, $^{e}P < 0.05$, $^{f}P < 0.01$ vs. control.

The results of cortical bone histomorphometry showed that, compared with the normal group, OVX resulted in trabecula rarefaction, bone lacuna increase, ossein in bone plate, and immature and incomplete calcification. Ginsenoside and oestrogen treatment prevented deleterious changes by OVX. The effect of a high dose of ginsenoside was stronger than that of the oestrogen treatment in these respects (figures 1 and 2; table 2).

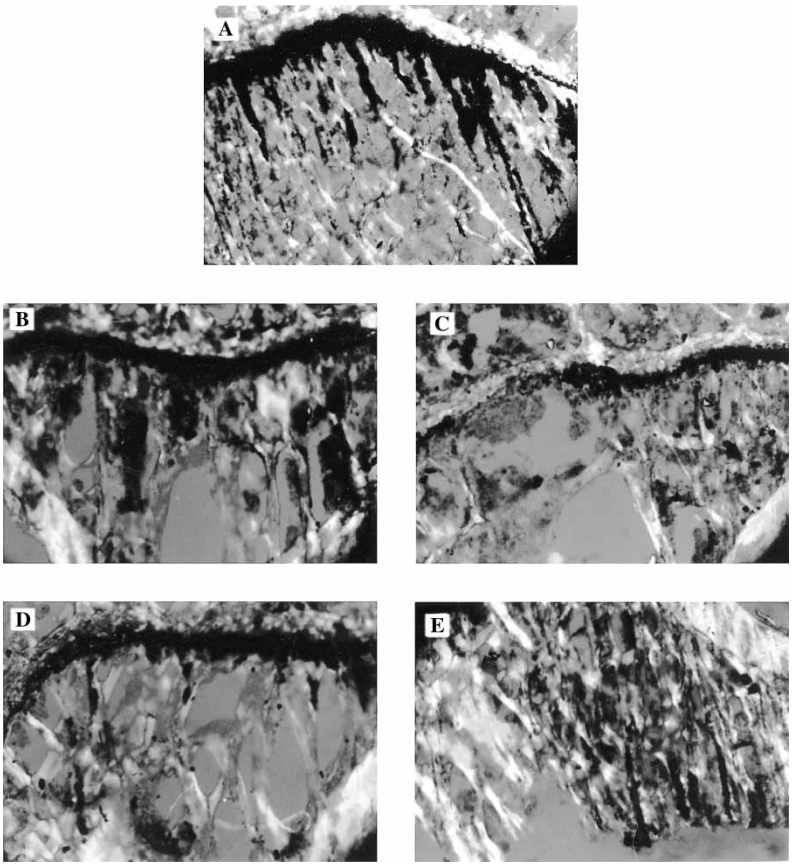

Figure 1. Effect of 17β-oestradiol and ginsenoside on the bone trabecula and lacuna of tibia in OVX rats. (A) Control, (B) OVX rats, (C) 17β-oestradiol, (D) ginsenoside $20\,mg\,l^{-1}$, (E) ginsenoside $40\,mg\,l^{-1}$. 17β-Oestradiol or ginsenoside were able to increase the density of bone trabecula and improve bone lacuna of tibia in OVX rats.

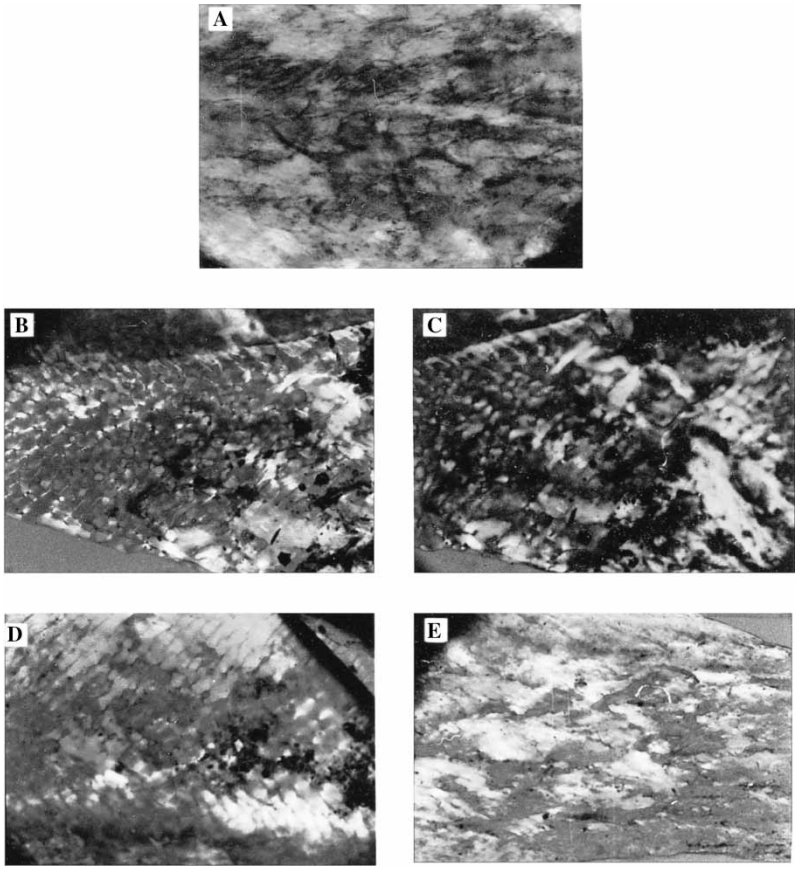

Figure 2. Effect of 17β-oestradiol and ginsenoside on the bone collagen and the bone calcification in lamina bone of tibia in OVX rats. (A) Control, (B) OVX rats, (C) 17β-oestradiol, (D) ginsenoside 20 mg·l^{-1}, (E) ginsenoside 40 mg l^{-1}. 17β-Oestradiol or ginsenoside were able to improve the distribution of collagen and increase the calcification of tibia in OVX rats.

Previous studies have shown that oestrogen deficiency is closely associated with osteoporosis [7]. Oestrogen is available as a treatment option against postmenopausal bone loss, but long-term treatment with oestrogen may have side effects on the reproductive system, whereas some ingredients from ginseng, such as tR or Rg_1 and Rb_1, have an anti-osteoporosis effect without adverse effects. We found that tR had a strong effect on preventing bone loss and markedly increasing bone density of the tibia.

Table 2. Effect of E_2, tR on the area of bone lacuna.

	Average area (μm^2)
E_2	
400 μg kg^{-1} week^{-1}	79.84 ± 53.49* (24.59%)
tR	
10 mg kg^{-1}	90.97 ± 69.54 (14.08%)
40 mg kg^{-1}	33.51 ± 26.88** (68.35%)
OVX rat	105.88 ± 80.75$^{\#\#}$
Control	14.99 ± 7.54 (85.84%)

Mean ± SD, $n = 8$. (%): variational percent vs. OVX rat.
*$P < 0.05$, **$P < 0.01$ vs. OVX rats; $^{\#\#}P < 0.01$ vs. control.

2.2 Effect of E_2, Rg_1 and Rb_1 on osteoblast from calvaria of newborn rats

After osteoblasts had been cultured for 4 days, the cell amount increased markedly. Then E_2, Rg_1 and Rb_1 were added and the osteoblasts continued to be cultured for 5 days, but they were still unable to reach contact inhibition. However, we found that $0.1\,\mu mol\,l^{-1}$ E_2 increased cell division and Rg_1 at $10\,\mu mol\,l^{-1}$ also was able to increase proliferation of osteoblasts (table 3). Cultured cells were digested with 0.025% pancreatin. It was found that E_2 and Rg_1 were able to increase the ability of osteoblast division, but Rb_1 was not.

Alkaline phosphatase (ALP) is a symbol of mature osteoblasts. The higher the activity of ALP in blood, the more active metabolism of osteoblasts was observed. After osteoblasts were cultured for 10 days following serum removal, E_2, Rg_1 and Rb_1 were added to osteoblast culture dishes. It was found that the activity of ALP in the E_2 and Rg_1 groups increased markedly, but the Rb_1 group did not (table 4).

cAMP is the second messenger of cell growth and metabolism. The increase of cAMP in cells promotes proliferation of cells. After osteocytes were cultured for 4 days and E_2, Rg_1 and Rb_1 were respectively added, E_2 and Rg_1 increased the content of cAMP in osteoblasts, but Rb_1 was unable to do so (table 5).

Calcium supplementation, because of its safety, high toleration and low price, is suitable for to prevention of osteoporosis the elderly whose bone density is still normal, but it is not ideal for osteoporosis patients [8]. The current treatment for osteoporosis is not limited to inhibiting activity of osteoclasts or simple calcium supplementation. More importantly, as well as supplying enough calcium for an osteoporosis patient, the doctor should try to promote the patient's osteoblast activity and increase the synthesis of bone stromatin, and make more sites of calcification available. It was found that osteogenesis could induce the division of osteoblasts [9]. Oestrogen could markedly increase the secretion of bone morphogenetic protein-6 from osteoblasts and increase the activity of osteoblasts [10], and could effectively prevent osteocytes from apoptosis and maintain the vitality of osteocytes [11]. *In vitro*, oestrogen could enhance osteoblast proliferation, promote anabolism of osteoblasts and promote synthesis of collagen. On the genetic level, it could not only affect the vitality of osteoblasts, but also adjust the number of osteoclasts [12]. We found that tR had a strong effect on preventing bone loss and increasing bone density of the tibia markedly, while Rg_1 increased division capability of osteoblasts.

Our findings may help in the understanding of the pathophysiology of postmenopausal osteoporosis and in trying to find more plant-derived compounds to prevent osteoporosis.

Table 3. Effect of E_2, Rg_1 and Rb_1 on the number of osteoblasts.

	Percent of cell number
Control	$(2.43 \pm 0.23) \times 10^4$ (100%)
E_2	
0.1 μmol	$(3.14 \pm 0.44) \times 10^4$ ** (129%)
Rg_1	
1 μmol	$(3.01 \pm 0.46) \times 10^4$* (124%)
10 μmol	$(3.09 \pm 0.58) \times 10^4$** (128%)
Rb_1	
1 μmol	$(2.60 \pm 0.25) \times 10^4$ (107%)
10 μmol	$(2.63 \pm 0.41) \times 10^4$ (109%)

Mean ± SD, $n = 8$. (%): variational percent vs. control.
*$P < 0.05$, **$P < 0.01$ vs. control.

Table 4. Effect of E_2, Rg_1 and Rb_1 on the ALP activity.

	ALP activity (APA μmol 30 min^{-1} μg protein^{-1})
Control	112.3 ± 10.2 (100%)
E_2	
0.1 μmol	234.7 ± 19.1** (209%)
Rg_1	
1 μmol	142.7 ± 14.5** (127%)
10 μmol	157.2 ± 18.2** (140%)
Rb_1	
1 μmol	121.2 ± 11.2 (108%)
10 μmol	127.9 ± 12.6* (114%)

Mean ± SD, $n = 8$. (%): variational percent vs. control.
*$P < 0.05$, **$P < 0.01$ vs. control.

3. Experimental

3.1 Material and methods

3.1.1 Rats. (1) Female Wistar rats ($n = 8$, 200–240 g, Grade II, from the Centre of Experimental Animals, Chinese Academy of Medical Sciences) were fed laboratory chow and water *ad libitum* and maintained in a thermoregulated environment (19–23°C) during a 12-h light/dark cycle.

(2) 1–2-day-old Wistar rats were obtained from the Centre of Experimental Animals, Chinese Academy of Medical Sciences.

3.1.2 Drugs and drug delivery. 17β-Oestradiol, provided by the Third Pharmacy of Beijing, was dissolved with peanut oil; ginsenoside, provided by the Pharmacy in Dan Dong, was dissolved with deoxidised distilled water; Rg_1 and Rb_1 was provided by the Chinese Assay Institute of Biological Products. Other reagents were DMEM, phenol red-free embryo bovine (Gibco, USA), collagenase II (Sigma, USA), HEPES, determining reagent of alkaline phosphatase.

3.1.3 Ovariectomy and drug delivery. Female Wistar rats was either sham-operated (sham-op) or ovariectomised (OVX) at 90 days after birth. Commencing from the eighth day after surgery, OVX rats received vehicle, 17β-oestradiol and ginsenoside, respectively, for 6 months

Table 5. Effect of E_2, Rg_1 and Rb_1 on the cAMP of osteoblasts.

	Concentration of cAMP (μmol × 10)
Control	0.619 ± 0.025 (100%)
E_2	
0.1 μmol	1.431 ± 0.12** (231%)
Rg_1	
1 μmol	0.924 ± 0.056** (149%)
10 μmol	1.206 ± 0.11** (195%)
Rb_1	
1 μmol	0.686 ± 0.072* (111%)
10 μmol	0.746 ± 0.081** (121%)

Mean ± SD, $n = 8$. (%): variational percent vs. control.
*$P < 0.05$, **$P < 0.01$ vs. control.

(180 days). One group of OVX rats received 400 $\mu g\,kg^{-1}$ 17β-oestradiol by OP/iP in turn. Three additional groups were administered ginsenoside 10, 20 and 40 $mg\,kg^{-1}$,respectively, by gavage.

3.2 Measurement of bone density

After E_2 and tR were given for OVX rats, the rats were anaesthetised with ketamine hydrochloride and their bone density was measured by dual X-ray absorptiometry (Lunar, USA).

3.2.1 Analysis of bone tissue. After the bone density was measured, the rats were anaesthetised with ketamine hydrochloride. The right tibiae were removed and stripped of musculature. A longitudinal cut was made in the centre from the proximal tibiae; one half was used for longitudinal sections, the other for transverse sections. Longitudinal and transverse sections 150–200 μm thick were ground. Under the micropolariscope, the changes as follows were observed: (1) calcification index; (2) the degree of osteoporosis. Bone mineral density is defined as the mean values of the marrow cavity of trabecula by proximal tibia.

3.3 Cell culture of osteoblasts

Calvaria of newborn rats were sequentially digested and put in a PBS buffer solution containing 4 $mmol\,l^{-1}$ EDTA and cut up in 10 min, and then cut calvaria were digested with collagenase II (200 $\mu g\,ml^{-1}$) in PBS at 37°C. The released cells in initial two runs (20 min) were given up, and the released cells in the latter three runs (15 min each) were mixed and seeded at 10^3 cells per culture dish (six-well culture plate).

After 4 days of cell culture, 10 $\mu mol\,l^{-1}$ E_2, 1 $\mu mol\,l^{-1}$, and 10 $\mu mol\,l^{-1}$ Rg_1 and Rb_1 were added. Cell growth and morphological changes were observed and pictures taken under a phase contrast microscope.

3.4 Measurement of alkaline phosphatase

After serum was removed on the 8th day, E_2, Rg_1 and Rb_1 were added and osteoblasts continued to be cultured for 3 days. For measurement of ALP, osteoblasts in a 2 cm^2 culture area were treated by 0.5% Triton X-100 and crushed by supersonic wave. After 30 min the extraction hydrolysate phospho-*m*-nitrophenol released *m*-nitrophenol. Measured by UV absorption at 410 nm, the enzymatic activity was determined by the quantitation of *m*-nitrophenol per microgram extracted per minute.

3.5 Measure of cAMP

3.5.1 Sample treatment. After treatment with E_2, Rg_1 and Rb_1, culture dishes were added to 1 ml of 1 $mol\,l^{-1}$ perchloric acid, the cells were scraped into the medium and collected. After they were homogenised by supersonic wave and centrifugation, part of the supernatant was neutralised by 20% KOH and centrifuged and sequestered during deposition, then all parts of

the supernatant were evaporated to dryness. The dried remainder was solved into TE buffer solution for measuring cAMP.

3.5.2 Sample solution. cAMP standard solution was made up and the cAMP standard curve described; the sample solution was measured according to the manufacturer's instructions (Chinese Atomic Energy Institute of Chinese Scientific Academy).

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