Puerarin combined with estradiol for treatment of osteoporosis in ovariectomized rats**

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Abstract

BACKGROUND: Estradiol produces great adverse effects in treatment of postmenopausal osteoporosis.
OBJECTIVE: To investigate the effects of small dose of estradiol combined with estradiol on bone tissue of ovariectomized rats.
METHODS: A total of 120 healthy female rats were randomly and evenly divided into five groups: sham-operated (only resection of a small block of para-ovarian fat pad), ovariectomized (without drugs), puerarin, estradiol, and puerarin + estradiol. One week later, rats from the puerarin, estradiol and puerarin + estradiol groups were subcutaneously injected with puerarin (50 mg/kg, once a day), estradiol (200 μg/kg, twice a week), estradiol puerarin (25 mg/kg, once a day) + (100 μg/kg, twice a week), respectively.
RESULTS AND CONCLUSION: In the ovariectomized group, rat bone tissue was sparse, bone calcium and phosphorus levels as well as bone mineral density were significantly lower compared with sham-operated group (P < 0.01). After estradiol and/or puerain treatment, rat bone tissue morphology was obviously improved, and bone calcium and phosphorus levels and bone mineral density were significantly increased. The above-mentioned indices were similar after small dose of puerarin combined with estradiol treatment and high dose of puerarin combined with estradiol treatment. These findings suggest that small dose of estradiol combined with estradiol treatment yields satisfactory curative effects in treatment of osteoporosis in ovariectomized rats.

INTRODUCTION

Estrogen replacement therapy for treatment of type Ⅰ osteoporosis shows obvious curative effects, but produces great adverse effects and many complications; while simple traditional Chinese medicine does not show ideal curative effects all the time, traditional Chinese combined with western medicine can reduce hormone use and thereby reduce adverse effects and complications[1-5]. There have been many reports describing simple use of western medicine or traditional Chinese medicine for treatment of postmenopausal osteoporosis in animal models[6-10], but animal experimental studies regarding traditional Chinese combined with western medicine for treatment of postmenopausal osteoporosis are rarely reported. This study was to investigate the effects of small dose of puerarin combined with estradiol on bone tissue and bone mineral density in ovariectomized (OVX) rats, so as to provide experimental evidence for treatment of postmenopausal osteoporosis using traditional Chinese combined with western medicine.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

This study was performed at the Department of Histology and Embryology, Central Laboratory of Morphology, and Central Laboratory of Science, Youjiang Medical College for Nationalities in China between May 2010 and January 2011.

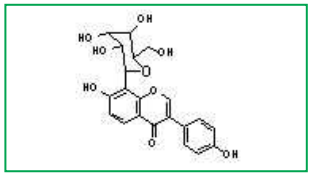
Materials

Animals

A total of 120 healthy female Sprague-Dawley rats of specific pathogen free grade, aged 5 months old weighing 280±20 g, were provided by Laboratory Animal Center of Guangxi Medical University (certification No. SCXK (gui) 2009-002).

Drugs

The main component of puerarin injection was puerarin, with the chemical structure of



Puerarin injection, with a specification of 100 mg/ampoule, was purchased from Chengdu Tiantai Pharmaceutical Factory, China (Certification No. 0905053).
Estradiol benzoate was purchased from Zhejiang Xianju Pharmaceutical Factory, China (Certification No. 0908072).

Main reagents and instruments

Reagent and instrument	Source
Phosphorus agent kit	Nanjing Jiancheng Bioengineering Co.,Ltd., China
Optical microscope	Olympus, Japan
XR-600 dual-energy X-ray absorptiometer	Norleand, USA
Analytical balance	Shanghai Fangrui Instrument Co.,Ltd., China
WFX-IE2 atomic absorption spectrophotometer	Beijing Ruili Analytical Apparatus Company, China
722 visible spectrophotometer	Shanghai Optical Instrument Factory, China

Methods

A total of 120 Sprague-Dawley rats were divided into five groups according to body mass (n=24):

sham-operated,ovariectomized (OVX), puerarin, estradiol, and puerarin + estradiol. All rats were raised in separate cages with standard chow and free access to water. The raising environment was the same (illumination, ventilation, temperature, and humidity) for all groups of rats.

Establishment of OVX rat models

Rats from the OVX, puerarin, estradiol, and puerarin+estradiol groups were intraperitoneally anesthesized using 10% 3.0 mg/kg sodium pentobarbital. Under sterile condition, through the incision made on each back, bilateral ovaries were resected and then incisions were sutured layer by layer. The resected ovaries were histologically identified. After surgery, rats were injected with gentamycin for 3 days to prevent wound infection. One week later, rats from the puerarin, estradiol, and puerarin + estradiol groups were given corresponding drugs. In the sham-operated group, a small block of para-ovarian fat pad was resected.

Drug intervention

Rats from the puerarin, estradiol and puerarin + estradiol groups were subcutaneously injected with puerarin (50 mg/kg, once a day), estradiol (200 μg/kg, twice a week), estradiol puerarin (25 mg/kg, once a day) + (100 μg/kg, twice a week), respectively.

Observation of morphological structure of bone tissue

After drug intervention for 4, 8, 12 and 20 weeks, six rats of each group were intraperitoneally anesthesized with 10% 3.0 mg/kg sodium pentobarbital and then left femur was harvested, fixed, decalcified, dehydrated, embedded with paraffin, sliced into 3 μm sections, stained with hematoxylin-eosin, and finally observed under 200-fold optical microscope.

Measurement of femoral bone mineral density

After drug intervention for 4, 8, 12 and 20 weeks, the right femur of six rats was harvested from each group and dried, and then bone mineral density was measured through the use of dual-energy X-ray absorptiometer, with a scanning width of 1.5 cm, scanning length of 4.3 cm and scanning speed of 4.0 s/m.

Measurement of femoral calcium and phosphorus levels

After measurement of bone mineral density, the harvested right femur was dried again at 105 ℃ for 48 hours till constant weight, then transferred into crucible for charring till no fume, cooled to 600 ℃ and maintained this temperature for 60 minutes, and dissolved with 20% hydrochloric acid. Deionized water was added till the containing mark, bone calcium level was measured through the use of atomic absorption spectrophotometer. Using the same method for measurement of bone calcium level, bone phosphorus level was measured as follows: femoral tissue was dried till constant weight and then transferred into a 10 mL ampoule, which was enveloped after 20% hydrochloric acid was added. Subsequently, the ampoule was placed in a baker at 110 ℃ for 24 hours to sufficiently dissolve the femoral tissue. Finally, following addition of deionized water till the containing mark, bone phosphorus level was measured through the use of visible spectrophotometer.

Main outcome measures

Morphological structure of bone tissue, bone calcium and phosphorus levels, and bone mineral density.

Statistical analysis

All experimental data were expressed as mean±SD and statistically processed using SSTP software (version 2.0; Shanghai Science and Technology Publishing House, China. Analysis of variance and test of the significance of difference were performed. A level of $P < 0.05$ was considered statistically significant.

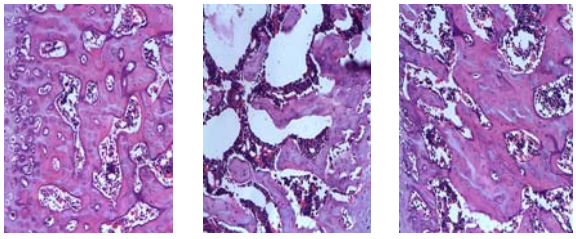
RESULTS

Quantitative analysis of experimental animals

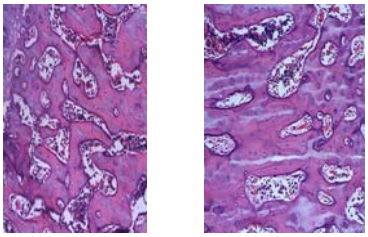
A total of 120 rats were initially included and divided into five groups. All rats were included in the final analysis.

Morphological structure of bone tissue

In the sham-operated group, compact bone was dense, osteocytes were orderly arranged, trabecular bone was sparse and slender, fractured in large fragments, and poorly arranged, bone marrow cavity was enlarged, and deposited calcium salt was reduced. After estradiol and/or puerarin treatment, pathological changes of osteoporosis were obviously improved, compact bone was thickened, tracebular bone became thick and compact. Under the optical microscope, there was no obvious difference in morphological structure of bone tissue among the puerarin, estradiol and puerarin + estradiol groups (Figure 1).



a: Sham-operated group b: Ovariectomized group c: Puerarin group



d: Estradiol group e: Puerarin + estradiol group

Figure 1 Morphological structure of bone tissue after intervention for 20 wk in each group (hematoxylin-eosin staining, ×200)

Rat femoral bone mineral density in each group

Rat femoral bone mineral density was significantly lower in the OVX group than in the sham-operated group ($P < 0.01$). After estradiol and/or puerarin treatment, rat bone mineral density was significantly increased ($P < 0.01$) (Table 1).

Table 1 Bone mineral density at different time points after estradiol and/or puerarin treatment in each group ($\bar{x}$±s, n=6, g/cm^2)

Group	Time after treatment (wk)			
	4	8	12	20
Sham-operated	0.31±0.02	0.30±0.01	0.29±0.02	0.32±0.01
Ovatiectomized	0.25±0.01[a]	0.23±0.01[a]	0.24±0.02[a]	0.26±0.01[a]
Puerarin	0.33±0.01[b]	0.28±0.03[b]	0.30±0.01[b]	0.32±0.02[b]
Estradiol	0.29±0.03[b]	0.27±0.02[b]	0.28±0.03[b]	0.30±0.01[b]
Puerarin+ estradiol	0.30±0.01[b]	0.29±0.01[b]	0.27±0.01[b]	0.31±0.03[b]

[a]P < 0.01, *vs.* sham-operated group; [b]P < 0.01, *vs.* ovariectomized group

Rat bone calcium levels in each group

Rat bone calcium level was significantly lower in the OVX group than in the sham-operated group (P < 0.01). After estradiol and/or puerarin treatment, rat bone calcium level was significantly increased (P < 0.01) (Table 2).

Table 2 Bone calcium level at different time points after estradiol and/or puerarin treatment in each group ($\bar{x}$±s, n=6, mg/g)

Group	Time after treatment (wk)			
	4	8	12	20
Sham-operated	170.34±8.47	168.67±17.23	173.13±14.25	172.53±11.30
Ovatiectomized	151.24±11.30[a]	148.61±9.71[a]	153.57±12.25[a]	148.63±10.81[a]
Puerarin	165.26±10.52	161.34±11.14	164.72±15.40	167.81±15.57
Estradiol	163.50±13.08	167.18±20.32	164.34±13.21	170.93±16.15
Puerarin+ estradiol	159.46±16.40	160.55±13.48	162.06±10.85	164.15±14.23

[a]P < 0.01, *vs.* sham-operated group

Rat bone phosphorus levels in each group

Rat bone phosphorus level was significantly lower in the OVX group than in the sham-operated group (P < 0.01). After estradiol and/or puerarin treatment, rat bone phosphorus level was significantly increased (P < 0.01) (Table 3).

Table 3 Bone phosphorus level at different time points after estradiol and/or puerarin treatment in each group ($\bar{x}$±s, n=6, mg/g)

Group	Time after treatment (wk)			
	4	8	12	20
Sham-operated	103.43±15.1	98.72±10.28	94.67±11.15	97.86±11.21
Ovatiectomized	87.5±13.26[a]	79.87±12.5[a]	84.46±9.65[a]	82.4±10.42[a]
Puerarin	98.75±9.86	93.45±14.16	92.53±8.94	90.76±15.12
Estradiol	101.24±12.37	96.31±14.07	98.25±10.15	94.13±12.11
Puerarin+ estradiol	97.26±11.01	91.32±13.23	90.15±12.08	92.85±15.38

[a]P < 0.01, *vs.* sham-operated group

DISCUSSION

Murine bone tissue structure is similar to human bone tissue structure. Murine ovaries are easily recognized and removed without damage to other organs during the surgery because their location and morphological structure are relatively clear. Removal of adult rat ovary can decrease estrogen level in vivo and effectively simulate postmenopausal decreased ovarian function and reduced estrogen secretion-caused postmenopausal osteoporosis in middle-aged and elderly women. OVX rats have been considered the preferred animal models of postmenopausal osteoporosis[11-13]. Results from this study showed that after ovariectomy, bone calcium and phosphorus levels and bone mineral density were significantly decreased and pathological changes of osteoporosis were observed, indicating that OVX rats are a good animal model of postmenopausal osteoporosis. Osteoblasts and osteoclasts or other cells of bone tissue have estrogen receptor[14-17]. Estrogen combined with osteoblast receptor promotes secretion of osteoprotegerin, formation of osteoid, and deposition of calcium salt, and thereby forming bone tissue. If estrogen level decreases, osteoprotegerin secretion would decrease and interleukin 1, 6 secretion would increase, which cause decreased osteogenic function of osteoblasts and increased osteolytic function of osteoblasts, leading to osteolysis stronger than osteogenesis and finally sclerotin loss[18-21]. In the OVX group, estrogen level was decreased, and bone calcium and phosphorus levels as well as bone mineral density were decreased with sclerotin loss, leading to osteoporosis. After estradiol and/or puerarin treatment, estrogen level was increased, osteogenic function of osteoblasts was enhanced, and osteolytic function of osteoclasts was decreased. Thus, increased bone calcium and phosphorus levels as well as bone mineral density were increased, and pathological changes of osteoporosis were obviously improved. Bone mineral density and bone calcium level were increased while bone phosphrous level was decreased with the prolongation of treatment time, but there was no significant difference, and the underlying mechanisms need to be studied. Radix Puerariae isoflavone, the main component of puerarin, exhibits estrogen-like pharmacological action, can inhibit the generation and activation of osteoclasts, without adverse effects of estrogen. Strong evidence exists that puerarin shows good curative effects on osteoporosis of OVX rats[22-23]. Bone tissue morphology, which can directly reflect bone morphology and structure, is the most objective diagnostic index of osteoporosis. Bone mineral density is a golden standard of clinical diagnosis of osteoporosis. The present study investigated the curative effects of puerarin, estradiol, and puerarin combined with estradiol treatment on osteoporosis in OVX rats by comparing changes in bone tissue morphology, bone calcium and phosphrous levels, and bone mineral density. Results from this study demonstrated that low dose of puerarin combined with estradiol increased compact bone and promoted the recovery of bone calcium and phosphrous levels as well as bone mineral density. This therapeutic protocol can reduce estradiol use and thereby reduce estradiol-caused adverse effects, providing experimental evidence for treatment of postmenopausal osteoporosis. In this study, only one group of puerarin combined with estradiol was designed, which is not enough to conclude that this combination is the optimal, so future studies are needed to search the best combination through different dose matches.

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葛根素联合雌二醇对去卵巢大鼠骨质疏松症的治疗作用**

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摘要

背景：雌激素治疗绝经后骨质疏松症产生的不良反应较大。

目的：观察小剂量葛根素联合雌二醇对去卵巢大鼠骨组织的影响。

方法：120只健康雌性大白鼠等分成只切除卵巢旁的一小块脂肪垫的假手术组和切除双侧卵巢的去卵巢模型组、葛根素组、雌二醇组及葛根素+雌二醇组。1周后葛根素组、雌二醇组和雌二醇+葛根素组分别皮下注射葛根素(50 mg/kg，1次/d)、雌二醇(200 μg/kg，2次/周)和雌二醇(100 μg/kg，2次/周)+葛根素(25 mg/kg，1次/d)。

结果与结论：去卵巢模型组大鼠骨组织稀疏，骨钙、磷水平和骨密度明显低于假手术组($P < 0.01$)，而经雌二醇和/或葛根素治疗后大鼠骨组织形态明显改善、骨钙、磷水平和骨密度明显升高。其中小剂量的葛根素联合雌二醇治疗与较大剂量的葛根素或雌二醇治疗效果接近。提示较小剂量的雌二醇与葛根素联合治疗也可对去卵巢大鼠骨质疏松症产生良好的治疗效果。

关键词：葛根素；雌二醇；去卵巢；大鼠；骨质疏松症；组织工程

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