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Preventive effects of ginsenosides on osteopenia of rats induced by ovariectomy

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KEY WORDS *ginseng*; 17α-*ethynylestradiol*; ovariectomy; bone and bones; osteoclasts; developmental bone diseases; histocytochemistry

ABSTRACT

AIM: To determine the effect of ginsenosides (GSL) on *ovariectomized rats by analysis of cancellous bone histomorphometry*. **METHODS**: Forty Sprague-Dawley female rats at age of 3 months were sham-operated (Sham, $n=8$) and treated orally with vehicle, or ovariectomized (OVX, $n=32$ which were divided into three group with $n=8$ per group) and treated orally with either vehicle, 17α-ethynylestradiol (EE, 100 $\mu g \cdot kg^{-1} \cdot d^{-1}$), or ginsenosides (GSL) at 100 or 300 $mg \cdot kg^{-1} \cdot d^{-1}$ for 10 weeks. Double *in vivo* fluorochrome labeling was administrated. The undecalcified longitudinal proximal tibial metaphyseal sections were cut and stained with Goldner's Trichrome (4-μm thickness) or unstained (8-μm thickness) for the bone histomorphometric analysis. **RESULTS**: After 10 weeks post OVX the cancellous bone mass was lost markedly and showed high bone turnover indices (increased bone resorption and formation). EE decreased the resorptive surface and bone formation rate related to bone turnover and prevented bone loss. GSL at the two doses (100 and 300 $mg \cdot kg^{-1} \cdot d^{-1}$) reduced the resorptive surfaces as did EE, but did not depress the mineral bone formation. High dose of GSL greatly increased bone mass and had a tendency to decrease bone turnover when compared with OVX group. **CONCLUSION**: GSL partially prevented OVX-induced cancellous bone loss by inhibiting osteoclast bone resorption and by a mild depression of bone turnover.

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INTRODUCTION

Ginsenosides (GSL) are total saponins extracted from the herbal medicine of stem and leaves of *Panax ginseng* CA Mey. It is reported to have sex hormone-like effects[1,2], enhance protein and DNA synthesis[3-5], have anti-aging effect[6,7], and influences cardiovascular and immunological systems[8]. Our previous studies have also shown ginsenosides can inhibit lipid peroxidation[9,10]. Since primary osteoporosis is not only associated with the disorder of sex hormones (include estrogen and androgen), but is also a pathologic procedure observed with increasing age. The stratagems for prevention and treatment of osteoporosis also include the use of anabolic agents besides the anti-resorptive agents. GSL has established anabolic pharmacological actions and sex hormones like effects, but whether GSL also has effects on bone metabolism and osteoporosis is now under active research. In this study we first report the effects of GSL on an animal model of ovariectomy-induced osteoporosis by analysis of bone histomorphometry.

MATERIALS AND METHODS

Drugs and reagents Ginsenosides from stem and leaves of *Panax ginseng* CA Mey, purity 94 %, light yellow powder, were purchased from Yanji Pharmaceutical Factory; 17α-ethynylestradiol (EE), Goldner's staining reagents (Iron hematoxylin, Anhydrous Ferric, Beibrich Scarlet, Acid Fuchsin, and Fast green) and calcein were purchased from Sigma Chemical Co; Tetracycline hydrochloride (Shanghai Xinya Pharmaceutical Factory), methyl methacrylate (Beijing Chemical Factory 940117), dibutyl phthalate (Guangdong Shantou Chemical Co 940122) and benzoyl peroxide (Hubei University Chemical Factory 940302) were purchased as indicated.

Animals and experimental protocol Forty 3-months-old Sprague-Dawley female rats weighing 267 g ± 20 g (Nanhai Animal Center, Grade II, Guangdong)

were acclimated to local vivarium conditions (temperature 24 ℃ - 26 ℃, humidity 67 %) for two weeks. The rats were allowed free access to water and pelleted diets containing 1.33 % calcium and 0.76 % phosphorus. Eight rats were sham-operated and treated with vehicle as the aging control group (Sham + Veh). The remaining rats were bilaterally ovariectomized and randomly divided into four groups with 8 rats per group, they were treated with either vehicle (OVX + Veh), 17α-ethynylestradiol at dose of 100 $\mu g \cdot kg^{-1} \cdot d^{-1}$ (*po*) (OVX + EE), or ginsenoside at dose of 100 or 300 $mg \cdot kg^{-1} \cdot d^{-1}$ (*po*) (OVX + GSL) for 10 weeks. Rats received treatment from the second day of OVX. All rats were given sc injections of tetracyclin 25 $mg \cdot kg^{-1}$ (first fluorochrome bone marker) on 14 and d 13, and calcein 10 $mg \cdot kg^{-1}$ (second fluorochrome bone marker) on 4 and d 3 before sacrifice.

Bone sections preparation At autopsy, the rats were killed by cardiac puncture under pentobarbital sodium 40 $mg \cdot kg^{-1}$ anesthesia. The soft tissue (lungs, heart, thymus, liver, spleen, kidneys, adrenal glands, uterine, gastrocnemius, and soleus muscles) were removed and weighed separately.

The right tibiae were removed and dissected free of muscles, the proximal tibiae were opened to expose the marrow cavity using an isomet low speed saw (Buechler LTD, USA) and fixed in 10 % phosphate buffered formalin. They were then dehydrated in ethanol, defatted in xylene and embeded undecalcified in methyl methacrylate[11]. The frontal sections were cut at 4-μm and 10-μm thickness with microtome (Leica RM2155, Germany). The 4-μm section was stained with Goldner's Trichrome staining for bone static and cells parameters measurements, the unstained 10-μm sections were used for dynamic histomorphometric analysis of fluorochrome labeling.

Cancellous bone histomorphometric analyses A digitizing image analysis system (DIAS; KSS Image, Magna, UT, USA) was used for quantitative bone histomorphometric measurements. This system consisted of a light and epifluorescent microscope coupled to an Apple Macintosh computer. The measurement site on the bone section was the proximal tibial metaphyses between 1 and 4 mm distal to the growth plate-epiphyseal junctions. The 1 mm metaphyseal region was omitted to restrict measurements to the secondary spongiosa[12]. The measured parameters and their nomenclature was followed according to Parfitt *et al*[13] and Jee *et al*[14]:

Measurements and calculations relating to trabecular bone volume and structures:

1) Total tissue (metaphyseal) area (TV, mm^2): metaphyseal area between 1 and 4 mm distal to the growth plate-epiphyseal junction.

2) Trabecular bone area (BV, mm^2): total area of trabeculae within TV.

3) Trabecular surface (BS, mm): the length of total perimeter of trabeculae.

4) Trabecular bone volume (BV/TV, %): BV/TV × 100

5) Trabecular number (Tb.N, mm^{-1}): (1.199/2) × BS/TV

6) Trabecular thickness (Tb. Th, μm): (2000/1.122) × BV/BS

7) Trabecular separation (Tb. Sp, μm): (2000 × 1.199) × (TV-BV)/BS

Measurements and calculations relating to bone resorption:

1) Osteoclasts perimeter (Oc. Pm, mm): total length of osteoclasts occurred on trabecular surface.

2) Osteoclasts number (Oc. No): total number of osteoclasts occurring on trabecular surface.

3) Osteoclasts number divided by bone volume (Oc. No/BV, mm^{-2}): Oc. No/BV.

4) Percent osteoclast surface (Oc. Pm/BS, %): Oc. Pm/BS × 100

Measurements and calculations relating to bone formation and turnover

1) Single labeled perimeter (sL. Pm, mm): total length of trabecular surface labeled with one calcein or/and one tetracyclin label deposited.

2) Double labeled perimeter (dL. Pm, mm): total length of trabecular surface labeled with tetracyclin and calcein simultaneously deposited.

3) Inter-labeled width (IrL. Wi, μm): average distance between tetracyclin and calcein labels.

4) Percent mineralizing surface (MS/BS, %): (sL. Pm/2 + dL. Pm)/BS × 100

5) Percent active mineralizing surface (double label perimeter, dL. Pm/BS, %): dL. Pm/BS × 100.

6) Mineral apposition rate (MAR, μm/day): IrL. Wi/label interval.

7) Bone formation rate per bone surface (BFR/BS, μm /d × 100): (sL. Pm/2 + dL. Pm) × (MAR/BV) × 365 × 100

Statistical analysis Data are expressed as $\bar{x} \pm s$. The percent changes between two comparative groups were calculated. Statistical differences among aging control and treated group were evaluated using ANOVA with

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Fisher's PLSD test. Probability (P) less than 0.05 was considered significant.

RESULTS

Body weight and uterine weights At the end of the study, body weight was increased by 42 % in OVX group *vs* only 9 % increase in sham-operated group over the 10 weeks of study. This increase was not found in EE treated group (the same as sham group). GSL at the two doses did not have effect on body weight when compared to OVX control.

Uterine weight in OVX control dropped by 70 % as compared to Sham control. In EE (100 $\mu g \cdot kg^{-1} \cdot d^{-1}$) treated group uterine weight markedly increased by 7 folds as compared to sham and by 16 folds as compared to OVX. GSL at two doses did not have effect on uterine weight when compared to OVX control (Tab 1).

Cancellous bone histomorphometry

Effect of ovariectomy Compared to sham control, in OVX treated with vehicle group the trabecular volume decreased by 76 % (Fig 1A, B) and showed high bone turnover indices: markedly increased osteoclasts number and surface (228 % and 322 %, Tab 2), greatly increased bone formation: MAR by 27 %, BFR/BS by 240 % and bone turnover of BFR/BV by 263 % (Tab 3).

Effect of EE and GSL treatments Compared to the OVX control, EE (100 $\mu g \cdot kg^{-1} \cdot d^{-1}$) greatly increased trabecular volume (162 %) and trabecular number (164 %), decreased trabecular separation (64 %), and effectively inhibited OVX-induced osteoclast bone resorption and high bone turnover indices (Tab 2, Tab 3, Fig 1C). GSL 100 $mg \cdot kg^{-1} \cdot d^{-1}$ decreased osteoclast parameters (Oc.N/BV by 56 %, Oc. Pm/BS by 70 %), but tended to increase bone formation rate when compared to OVX control (Tab 3). BV tended to increase but the difference was not significant. GSL 300 $mg \cdot kg^{-1} \cdot d^{-1}$ decreased Oc. N/BV by 58 % and Oc. Pm/BS by 68 %, however, trabecular volume and Tb.N were greatly increased (56 % and 51 %) accompanied by decrease of MAR (17 %) and bone turnover (BFR/BV, 33 % Tab 3, Fig 1D).

Comparison between EE and GSL treatments Both the low and high dose of GSL had similar effect as EE on bone resorption (Tab 2). Low dose of GSL in contrast, tended to increase bone formation rate as compared with either OVX or EE group. High dose of GSL did not differ from EE in osteoclast bone resorption, bone formation rate, and bone turnover, but the bone volume was lower in high dose of GSL than in EE treatment, double label perimeter (usually an index of osteoblast perimeter) was higher in high dose of GSL than in EE treatment (Tab 3).

DISCUSSION

Our data demonstrated that 10 weeks after ovariectomy, the rats had been induced with marked bone loss and high bone turnover as indicated by histomorphometry. 17α-ethynylestradiol completely inhibited OVX-induced osteoclast bone resorption and high bone turnover therefore prevented bone loss, but the bone volume still did not reach to the level of the value in sham group in this study. GSL 100 $mg \cdot kg^{-1} \cdot d^{-1}$ greatly inhibited osteoclast surface, but it tended to increase bone formation and turnover, although bone mass tended to increase but the difference was not significant. GSL at a dose of 300 $mg \cdot kg^{-1} \cdot d^{-1}$ retained its ability of inhibiting osteoclast resorption, however it decreased mineral apposition rate and bone turnover mildly leading to a marked increase of bone volume when compared with OVX group. It indicated that high dose of GSL partially prevented OVX-induced cancellous bone loss by inhibiting osteoclast activity and a mild depression of bone turnover. The effect of GSL on high bone turnover-induced bone loss (OVX) was weaker than that of estrogen treatment.

Tab 1. Effects of GSL on whole body weight (BW) and uterine weight. $n = 8$ rats. $\bar{x} \pm s$. $^cP < 0.01$ *vs* Sham. $^fP < 0.01$ *vs* OVX + Veh.

Parameters	BW/g Beginning	BW/g Endpoint	± BW/%	Uterine weight/mg
Sham + Veh	267 ± 20	291 ± 25	9	700 ± 115
OVX + Veh	277 ± 18	394 ± 47^c	42^c	110 ± 20^c
OVX + EE 100 $\mu g \cdot kg^{-1}$	266 ± 11	291 ± 19^f	10^f	1620 ± 110^{cf}
OVX + GSL 100 $mg \cdot kg^{-1}$	278 ± 14	384 ± 32^c	38^c	101 ± 20^f
OVX + GSL 300 $mg \cdot kg^{-1}$	276 ± 11	398 ± 35^c	42^c	110 ± 20^f

± BW/%: Percent changes from beginning to endpoint.

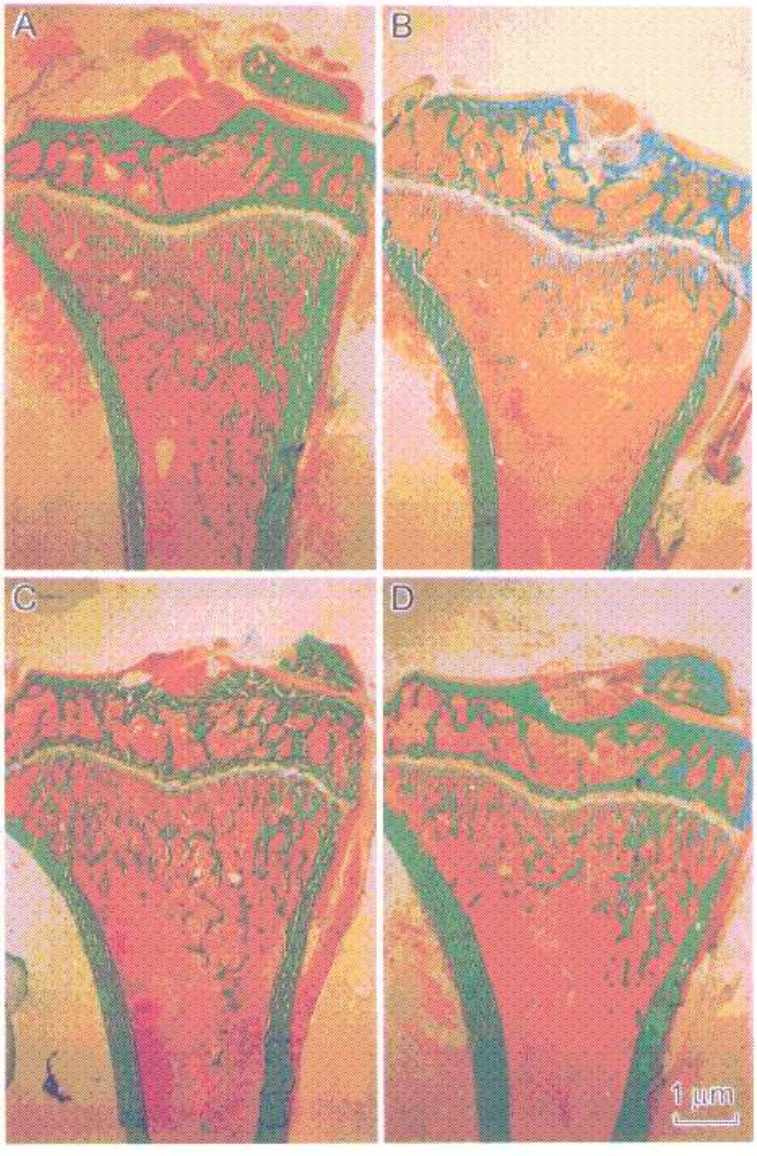

Fig 1. Micrographs of proximal tibiae from vehicle treated sham control (A: Sham + Veh), OVX control (B: OVX + Veh), and OVX rats treated with 17α-ethynylestradiol (C: OVX + EE 100 $\mu g \cdot kg^{-1} \cdot d^{-1}$) and GSL (D: OVX + GSL 300 $mg \cdot kg^{-1} \cdot d^{-1}$) for 10 weeks. Goldner's Trichrome stain. × 13.5.

It is well known that estrogen deficiency induce bone loss is associated with an increase in bone resorption, accompanied by an increase in bone formation that is insufficient to compensate for increase in resorption thereby leading to a net bone loss[15]. Inhibition of bone resorption is a critical stratagem to prevent bone loss in this animal model. Estrogen replacement mainly suppresses resorption to prevent further bone loss[16]. GSL was seen to decrease bone resorption, however the mechanism of GSL on bone resorption is unclear. Kuang reported that GSL increased serum estradiol levels in aged rats[1], thus its affect on the osteoclast activity may be related to its estrogen-like effect. On the other hand, our previous study showed that GSL reduced myocardial ischemia reperfusion injury and arrhythmia in a high cholesterol state through anti-lipidperoxidation action and by inducing

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Tab 2. Effects of GSL on static parameters of proximal tibial cancellous bone histomorphometry. $n = 8$ rats. $\bar{x} \pm s$. $^cP < 0.01$ *vs* **OVX + Veh.**

Parameters	Trabecular volume (%)	Trabecular thickness (μm)	Trabecular number (mm^{-1})	Trabecular separation (μm)	Osteoclast number/BV (mm^{-2})	Osteoclast perimeter/BS (%)
Sham + Veh	23 ± 6	55 ± 4	4.2 ± 1.4	215 ± 115	11 ± 4	0.7 ± 0.3
OVX + Veh	5.5 ± 1.5	52 ± 5	1.0 ± 0.3	960 ± 238	35 ± 13	2.8 ± 1.2
± % *vs* Sham	−76	−5	−75	346	228	322
OVX + EE 100 $\mu g \cdot kg^{-1}$	14 ± 5	52 ± 5	2.8 ± 0.9	342 ± 118	11 ± 5	0.6 ± 0.3
± % *vs* OVX	162^c	−1	164^c	−64^c	−69^c	−77^c
OVX + GSL 100 $mg \cdot kg^{-1}$	7 ± 3	58 ± 8	1.2 ± 0.5	1082 ± 915	15 ± 8	0.9 ± 0.5
± % vs OVX	23	11	14	13	−56^c	−70^c
OVX + GSL 300 $mg \cdot kg^{-1}$	8.5 ± 1.5	55 ± 6	1.6 ± 0.3	604 ± 135	15 ± 6	0.9 ± 0.4
± % *vs* OVX	56^c	4	51^c	−37^c	−58^c	−68^c
± % *vs* GSL-100 $mg \cdot kg^{-1}$	26	−6	32	−44	−5	5
± % *vs* EE	−41	5	−43	76	38	41

± %: Percent changes from the group with which to be compared.
BV: trabecular bone area. BS: trabecular surface.

Tab 3. Effects of GSL on dynamic parameters of proximal tibial cancellous bone histomorphometry. $n = 8$ rats. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* **OVX + Veh.**

Parameters	Parameters perimeter (%)	Double label perimeter (%)	Mineral apposition rate (μm/d)	Bone formation rate/BS μm/d × 100	Bone formation rate/BV %/year	Bone formation rate/TV %/year
Sham + Veh	5.8 ± 2.4	2.2 ± 1.5	1.33 ± 0.16	8.0 ± 3.7	87 ± 38	18 ± 6
OVX + Veh	16 ± 7	11 ± 6	1.69 ± 0.12	27 ± 13	315 ± 155	17 ± 9
± % *vs* Sham	172	385	27	240	263	−5
OVX + EE 100 $\mu g \cdot kg^{-1}$	10 ± 3	5.6 ± 2.2	1.42 ± 0.15	15 ± 6	175 ± 60	24 ± 8
± % *vs* OVX	35	−47	−16^c	−45^b	−45^b	39
OVX + GSL 100 $mg \cdot kg^{-1}$	22 ± 12	16 ± 9	1.60 ± 0.25	36 ± 22	363 ± 169	22 ± 8
± % *vs* OVX	37	46	−5	32	15	26
OVX + GSL 300 $mg \cdot kg^{-1}$	14 ± 3	8.7 ± 2.7	1.40 ± 0.17	19 ± 6	210 ± 51	18 ± 6
± % *vs* OVX	−15	−18	−17^c	−30	−33	6
± % *vs* GSL-100	−38	−44	−13	−47	−42	−17
± % *vs* EE	30	55	−1	27	20	−24

± % *vs*: Percent changes from the group with which to be compared.
BS: trabecular surface. BV: trabecular bone area. TV: total tissue (metaphyseal) area.

the production of nitric oxide (NO)[10]. NO has been implicated to play an important role in bone metablism. The inhibition of NO production leads to bone loss; treatment of rats with aminoguandine, which inhibits nitric oxide synthase (NOS), reduces bone mass in growing rats[17] and increases bone loss in ovariectomized adult rats, presumably by increasing bone resorption[18]. Treatment with the NO donor protected rats from ovariectomy-induced bone loss[19]. Thus the GSL mediated inhibition of osteoclast surface might be attributed to its influence on NO.

In many studies including the current one, besides inhibiting bone resorption, estrogen was also observed to inhibit mineral bone formation that resulted in decrease of bone turnover which in turn was mediated by increase in resorption[15]. However, suppression of mineral bone formation by anti-resorptive agents like estrogen that is of less benefit to the quality of bone structure[16]. Estrogen deficiency causes resorption over formation, and the insufficient bone formation is not compensated by anti-resorptive agents. Recently anabolic agents were approached for the aspect of increasing bone formation. Parathyroid hormone (PTH) and prostaglandin E_2 (PGE_2), as potent anabolic agents, have been confirmed to increase bone volume and bone mineral density in either intact animals, osteoporosis models, or senile osteo-

porosis by stimulating osteoblast bone formation over bone resorption[20,21]. Androgens are another kind of anabolic agents, hence treatment with dihydrotestosterone increased bone mass in ovariectomized rats[22]. From this point of view GSL, which has been report to enhance protein and DNA synthesis and possess androgen-like effect, may be assumed to have similar effect on bone formation. Our data also showed that GSL increased active bone mineral surface especially in double fluorescent labels (as index of osteoblast surface)[16] even though compared to OVX control (Tab 3). This indicated that GSL does not weaken osteoblast function and does not harm bone mineral formation as other anti-resorptive agents do, and it may possess similar effects as of anabolic agents. Hence the effect of GSL on bone formation seems somehow different from the estrogen.

As indicated in data and Fig 1C and D, the amounts of bone gain from OVX rats is smaller in GSL than in EE treatment. This may be due to the following reasons: 1) we used a high bone turnover model which induced a rapid bone loss, GSL only mildly depresses bone turnover index; 2) Although GSL increased bone mineral formation, its effect is not as potent as anabolic agents such as PTH and PGE_2, which increase both resorption and formation in favor of formation thus leading to a net bone gain; 3) Estrogen deficiency causes production of interleukin-6 (IL-6) which plays an important role in bone resorption[23], and GSL was reported to induce IL-6 secretion by fibroblasts[24], the fibroblasts probably are the osteoblast precursor in bone marrow[25]. If this is established, GSL will be a double-modulating agent on bone resorption and formation. The results imply that GSL can be combined with other agents (such as anti-resorptive agents) for added advantage in prevention and treatment of osteoporosis. The potential bone metabolic effects of GSL to treat osteoporosis need to be further studied in senile osteoporosis, male osteoporosis, or drug related osteoporosis.

In conclusion, GSL partially prevented OVX-induced cancellous bone loss in rat by inhibiting osteoclast bone resorption and a mild depression of bone turnover, GSL did not inhibit active mineral bone formation hence differing from the estrogen treatment.

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人参皂甙对去卵巢大鼠骨质丢失的防治作用[1]

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关键词 人参; 17α-乙炔基雌二醇; 卵巢切除术; 骨和骨组织; 破骨细胞; 发育性骨疾病; 组织细胞化学

目的: 研究人参皂甙(GSL)对去卵巢大鼠所致骨丢失的预防作用. **方法**: 采用双侧卵巢摘除术, 体内双荧光标记法, 胫骨上段硬组织包埋切片, 全自动图象分析及松质骨形态计量学软件处理, 观察药物对骨形态计量参数的影响. **结果**: 大鼠去卵巢 10 周后骨量丢失(−75 %, $P<0.01$)伴随破骨细胞活性、骨形成率及骨转换率明显增加. GSL 100 和 300 mg·kg^{-1}·d^{-1}均抑制破骨细胞骨吸收(−68 %, −70 %, $P<0.01$), 低剂量组略增加骨形成和骨转换率(+32 %, +15%, $P>0.05$ *vs* OVX). 高剂量组则略抑制骨形成及骨转换率(−30 %, −33 % *vs* OVX, $P>0.05$), 并增加骨量(+56 %, $P<0.01$ *vs* OVX), 部份预防骨丢失. 17α-乙炔基雌二醇 100 μg·kg^{-1}·d^{-1}抑制骨吸收和骨转换率(−45 %, −45 % *vs* OVX, $P<0.05$), 增加骨量(+162 % *vs* OVX, $P<0.01$), 预防骨丢失. **结论**: 人参皂甙对去卵巢所致高转换型骨丢失有预防作用, 与其抑制骨吸收和轻度抑制骨转换率有关. 其低剂量增加骨形成. 人参皂甙抑制骨转换率和预防骨丢失作用比 17α-乙炔基雌二醇弱.

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