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원 저

실험적으로 유발된 골손실에 미치는 骨碎補의 예방 효과

이구형, 정지천
동국대학교 한의과대학 내과학교실

Preventive Effects of *Drynariae Rhizoma* on Experimental Bone Disorder in Rats

Gu-Hyong Lee, Ji-Cheon Jeong
Dept. of Internal Medicine, College of Oriental Medicine, Dongguk University

흰쥐의 난소 절제로 야기된 골손실에 미치는 骨碎補의 영향을 관찰하였다. 난소 절제 후 흰쥐의 체중은 증가하고 자궁의 무게는 감소하였는데, 骨碎補는 특별한 영향을 주지 않았다. 그러나, 난소 절제 후 흰쥐의 tibia에서 측정한 골밀도는 대조군에 비하여 22% 감소되었으나, 骨碎補와 17beta-estradiol를 투여한 경우는 골밀도의 감소가 현저히 억제되었다. 난소를 절제한 실험군에 骨碎補와 17beta-estradiol를 투여하고 전자 현미경으로 관찰한 결과, trabecular bone의 미세분자 표면이 보호되는 것으로 관찰되었다. 이러한 결과들을 볼 때, 骨碎補가 17beta-estradiol 만큼 난소 절제술 후 진행되는 골손실을 예방하는데 효과적임을 강하게 나타내고 있다고 사료된다.*(J Korean Oriental Med 2001;22(4):22-28)*

Key Words: *Drynariae Rhizoma*, bone loss, bone mineral density, osteoporosis, ovariectomy

Introduction

Since a large decrease in bone mass occurs in the postmenopause state, women are vulnerable to the osteoporosis known as postmenopausal osteoporosis[1]. The primary cause of postmenopausal osteoporosis is an estrogen deficiency resulting in the decrease in bone mass. An ovariectomized rat model, which artificially produces the depleted state of estrogen, has been used for the study of postmenopausal osteoporosis: Both aged rats and mature rats have been used as animal models to study experimentally-induced osteoporosis and the mature rat model has characteristics that are comparable with those of early postmenopausal trabecular bone loss[2].

Several medications have been reported to be effective for curing osteoporosis based upon the results obtained using these animal models. Estrogen[1,2], bisphosphonates[2,3], calcitonin[2], calcium products[2,4], ipriflavone[5] and anabolic steroids[6] are clinically employed as effective medications.

Oriental medicines have been reevaluated by clinicians[7], because these medicines have fewer side

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· 교신저자 : 정지천, 서울시 강남구 논현1동 37-21 동국대학교 강남한방병원 내과
(Tel. 02-3416-9731, E-mail: jjcjh@hitel.net)

effects and because they are more suitable for long-term use as compared to chemically synthesized medicines. About forty kinds of oriental medicines are claimed to be effective for gynecological diseases such as climacteric psychosis, feeling of cold, menstrual disorders, dysmenorrhea, and low back pain. It has been suggested that the effectiveness of oriental medicines on low back pain seems to correspond to their efficacy in curing osteoporosis[8].

From ancient times in China, Korea and Japan, women who have had low back pain in climacteric and senescent periods have been treated with oriental medicines. For example, some formula have been used in treating ovary function failure, used in treating low back pain during the climacteric period, and also used after oophorectomies because of malignant tumors[8,9]. However, no data are available as to the recovery of bone mass by any of these oriental medicines.

Drynariae Rhizoma (DR) is effective for the treatment of deficient kidneys manifested as lower back pain, weakness of the legs, tinnitus or toothache by function of tonifing the kidney, and invigorating blood and stop bleeding according to the Chinese medicinal literature[10]. To treat the osteoporosis, a herbal formula containing DR is used in oriental medicine[11]. According to the previous study, DR significantly decreased the level of urine deoxypyridinoline to creatinine ratio inadministered group[12]. Thus, in order to evaluate the effectiveness of oriental medicines on osteoporosis, author examined whether DR could prevent the progression of bone loss induced by ovariectomy in rats.

Materials and Methods

1. Oriental Medicines and ChemicalsDried extract powders of traditional medicines, DR was supplied by Oriental Medical Hospital, Dongguk University (Kyungju, Korea). A 17beta-estradiol was purchased from Sigma Chemicals (St. Louis, MO). All other reagents were purchased from Wako Chemicals (Osaka, Japan).

2. Ovariectomy and Administration of DR medicines45 female Sprague-Dawley rats, aged 6 weeks, were purchased from KRIBB (Taejon, Korea). Twenty-seven days later (at 90-days-old; a mature rat model[3]) was used), five rats (as baseline control) were killed by exanguination under chloroform anesthesia for the evaluation of bone mineral density (BMD) as described in the text that follows. Eight rats were given a sham operation (control rats; first group), and 16 rats were ovariectomized under nembutal (Pentobarbital sodium; 50 mg/kg body weight: Abott Laboratories, IL) anesthesia. They were divided into fourth groups, those being the second group (4 rats per group) after the first group of Sham rats. The first group received distilled water as drinking water. The second group received 0.054% (w/w) DR. The concentration of DR was calculated from the daily fluid consumption and the body weight of the rats (Table 1). It was assumed that the daily doses of DR in humans was 5.0 g.

Each ovariectomized rat in the third group was injected subcutaneously with 17beta-estradiol (in 20% polyethylene glycol 3,000) 5 days/week at a dose of 10 μg/kg body weight. Rats in the fourth group were injected with 20% polyethylene glycol 3,000 in the same manner as were rats in the seventh group.

To develop bone loss in ovariectomized rats, all the animals were maintained for seven weeks under regulated 12 hour/12 hour light-dark illumination cycles at constant temperature (24 $\pm$ 0.5 ℃) and humidity (45%-50%). Food (MF pellets, Oriental Yeast Company, Ltd., Tokyo, Japan) and drinking water were supplied ad libitum.

Daily fluid consumption, daily food consumption, and body weight were measured at a specified time

during the course of the experiment. After terminating the animal by exanguination under chloroform anesthesia, the right leg was dissected from each animal and was stored in a 7.5% formalin-neutral buffer solution (pH 7.2). Longitudinal sections of the tibiae from the right legs were made as described in the following text. The uterus of each rat was dissected out and was weighed.

3. Mesurement of Bone Mineral Density (BMD) -1

After dissecting the adhering soft tissues, longitudinal sections of tibiae were made by manual grinding with whetstones (#600). The right tibiae were dehydrated by a step-wise application of 70 and 99.9% (v/v) ethanol solutions. These samples were used for both the measurement of BMD and scanning electron microscopic observation. The BMD was quantitatively determined by the computed X-ray densitometry (CXD) method (Bonalyzer: Teijin Co., Tokyo, Japan)[14].

As an index of BMD and bone mineral content (BMC), ΣGS/D and ΣGS (mm Al) were used, respectively; ΣGS is a value obtained by computer integrating the pattern area, which is obtained opticodensitometrically and is converted into the number of steps on the aluminum step-wedge. For determining the area, one line was placed on the boundary between the epiphyseal cartilage and the epiphysis and another line was made at 1/6 of the length from the tibial mesial end.

4. Determination of BMD-2

Each fixed femur was dissected free from adhering soft tissues, and microradiographed (Softex, Softex Co., Tokyo, Japan) (at 90 kV, 1 mA for 60-90 s) together with a standardized step-wedge made of syntheti hydroxyapatite (HA; Mitsubishi Kasei Co. Ltd, Tokyo, Japan). Since there is a linear relationship between the lorarithms of HA density ($\mu g/mm^2$) of the step-wedge and gray levels (256 steps) of the microradiographic image of the step-wedge (3), the BMD (μg HA/mm^2) was determined by analyzing the grey levels in the micrograph with an image analyzer (Winroof, Mitani Corp., Fukui, Japan). Then, then BMD of the whole femur was determined by analyzing the total content of HA in the femur (μg HA/femur) and the femoral volume, and was expressed as μg HA/mm^3.

5. Statistics

Data were obtained from 3-5 measurements and were expressed as the means ± standard deviations. Statistical comparisons were made by ANOVA and Scheffe' s tests using a statistic software program. The difference was considered significant when $P < 0.05$.

Results

1. Body Weight

Increases in the body weight in ovariectomized (OVX) rats were significantly higher than those in sham control (Sham) rats (Fig. 1). After seven weeks, the mean body weights ± SD in Sham and OVX rats were 262±2.4 (n =5) and 351.2±2.1 (n = 4), respectively. Increase in the body weight of the groups, which were given DR (OVX + DR) was almost the same as those in OVX rats.

With the administration of 17beta-estradiol to OVX rats (OVX+Es), the increase was almost the same as those in Sham rats. The administration of the vehicle alone had no effect (data not shown).

2. Consumption of Fluid and Food

Since both fluid and food consumptions in the first one week were smaller than those in the following weeks, two groups (0-1 week and 2-6 weeks) were compared. As shown in Table 1, fluid consumption of OVX+DR rats in week 0-1 was higher than that in

OVX rats. Both consumptions of Sham and OVX + DR rats in weeks 2-6 were higher than those in others. Food consumption of Sham rats in week 0-1 was higher than that in others. Food consumption of OVX rats in weeks 2-6 was higher than that of Sham rats by 26%. Food consumption of OVX + Es rats in weeks 2-6 was comparable to that of Sham rats.

3. Uterine weight

The uterine weights of Sham (491.3±43 mg) and OVX rats (112.5±7 mg) differed significantly ($P<0.01$). Those of the groups given DR were 126.5±7 mg. Those of the groups that were injected 17 beta-estradiol and its vehicle were 473.4±34 and 116.4±12 mg, respectively. The difference between OVX rats and OVX + DR was not statistically significant (Table 2).

4. Bone Mineral Density (BMD)

As shown in Table 3, bone mineral density (BMD) of Sham and OVX rats were 1.0 ±0.01 and 0.80 ± 0.01, respectively. This indicated that the ovariectomy decreased in the BMD by 21%. The administration of DR to the ovariectomized rats clearly restored the BMD to the level of Sham rats. The injection of 17beta-estradiol to OVX rats also restored it to the level of Sham rats, whereas the vehicle had no effect (Table 3).

The BMD (ΣGS/D) of normal rats (baseline control) was 0.94±0.01. The value of OVX rats after seven weeks (0.81±0.01) was lower than the baseline BMD ($P<0.05$).

5. SEM Analyses

The scanning electron micrograph of the proximal tibiae taken at seven weeks after the ovariectomy is shown in Fig. 2. Compared to that of Sham rats, the connectivity of cancellous bone in the epiphysis and that of trabecular bone in the metaphysis exhibited greater loss than those in the Sham rats. At increased magnification (×20,000), the surface of trabecular bones of Sham rats appeared to be composed of fine particles (Fig. 2A), but that of trabecular bones in OVX rats had a porous or an erosive appearance (Fig. 2B). However, the appearance of the surface of the trabecular bones in OVX rats was restored to that of Sham rats by the administration of DR, although the surface of DR bones had some porous appearance (Fig. 2C). 17beta-estradiol similarly preserved the surface appearance (Fig. 2D), whereas the vehicle had no effect (data not shown).

Three trabecular bones were randomly chosen from one tibia and their surface state was observed. The frequency of the occurrence of three types of the surface states, namely fine particle, porous or erosive, and intermediate between these two types, is shown semiquantitatively in Table 4. Both the Sham and 17beta-estradiol rats were 100% fine particle type. The OVX rats were 96.6% porous or erosive and 4.4% intermediate type. The frequency of the occurrence of the fine particle type was restored to 80-93% by the administration of the three DR medicines.

Discussion

Ovariectomy caused an increase in body weight (Fig. 1). This is one of the prominent features that has been postulated to provide a partial protection against the development of osteoporosis in long bones[15]. DR delayed the body weight increasing only in the first week. Subcutaneous injection of 17beta-estradiol characteristically inhibited the increase of body weight (Fig. 1). It is not known whether DR has a weak estrogen-like effect.

Ovariectomy caused a decrease in fluid consumption but an increase in food consumption in the rats in 2-6 weeks (Table 1). However, DR noticeably increased fluid consumption through the entire experimental

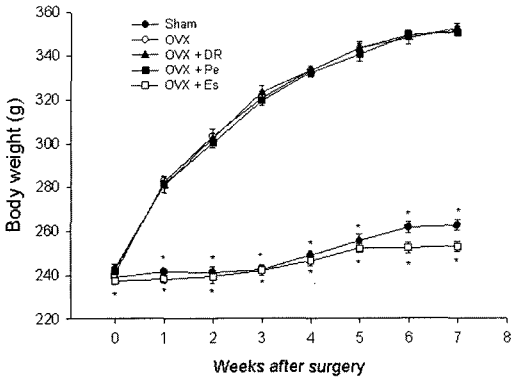

Fig. 1. Changes in body weight in rats. Rats (n = 4) were sham-operated (Sham), ovariectomized (OVX), OVX given DR (OVX+DR), OVX given polyethylene glycol (OVX+Pe), and OVX given 17beta-estradiol (OVX+Es), respectively. The changes of OVX(OVX+Pe), which were given a vehivle, were the same as those of ovariectomized. Each point represents the mean value ±SD (n=4). * Significant difference (P < 0.05) from the ovariectomized group at corresponding times

Table 1. Daily Fluid and Food Consumption in Rats

	Fluid (ml) per rat		Food (g) per rat	
	0-1 week	2-6 weeks	0-1 week	2-6 weeks
Sham	26.4±2.1	44.8±2.3	12.3±1.2	16.3±1.3
OVX	28.4±2.2	37.3±3.2a	8.4±0.2a	21.4±1.2a
OVX + DR	34.1±2.2b	46.4±2.2b	7.3±0.3a	20.3±1.5a
OVX + Pe	26.8±1.2	35.4±1.6a	7.6±0.2a	23.4±1.0a
OVX + Es	30.2±2.3	34.5±2.3a	6.7±0.3a	17.0±1.4

Rats (n = 4) were sham-operated (Sham), ovariectomized (OVX), OVX given DR (OVX + DR), OVX given polyethylene glycol (OVX + Pe), and OVX given 17beta-estradiol (OVX + Es), respectively. a : Significant difference (P < 0.05), when compared to Sham rats, b : Significant difference (P < 0.05), when compared to OVX rats.

Table 2. Changes in Uterine Weight in Rats

	Uterine weight (mg)
Sham	491.4±43
OVX	112.5±7a
OVX + DR	126.5±7
OVX + Pe	116.4±12
OVX + Es	473.4±34b

Rats (n = 4) were sham-operated (Sham), ovariectomized (OVX), OVX given DR (OVX + DR), OVX given polyethylene glycol (OVX + Pe), and OVX given 17beta-estradiol (OVX + Es), respectively. a : Significant difference (P < 0.01) when compared to Sham rats, b : Significant difference (P < 0.001) when compared to OVX rats.

Table 3. Bone Mineral Density (BMD) in Sham-Operated and in Ovariectomized Rats Treated with DR

	Bone mineral density (ΣGS/D)
Sham	1.00±0.01
OVX	0.80±0.01a
OVX + DR	0.95±0.02
OVX + Pe	0.82±0.02a
OVX + Es	0.97±0.01

Rats (n = 4) were sham-operated (Sham), ovariectomized (OVX), OVX given DR (OVX + DR), OVX given polyethylene glycol (OVX + Pe), and OVX given 17beta-estradiol (OVX + Es), respectively. a : Significant difference (P < 0.05), when compared to Sham rats

Table 4. Frequency of Occurrence of Three Types of Surface of Trabecular Bones in the Metaphysis

	Fine particles	(%)	Intermediate	(%)	Porous or erosive	(%)
Sham	4/4	100	0/4	0	0/4	0
OVX	0/4	0	1/4	25	3/4	75
OVX + DR	2/4	50	1/4	25	1/5	20
OVX + Pe	0/4	0	1/5	20	4/5	80
OVX + Es	4/4	100	0/4	0	0/4	0

Rats (n=4 or 5) were sham-operated (Sham), ovariectomized (OVX), OVX given DR (OVX + DR), OVX given polyethylene glycol (OVX + Pe), and OVX given 17beta-estradiol (OVX + Es), respectively. Using 4 or 5 rats, one trabecular bones in the metaphysis per one tibia were analyzed on their surface by SEM at increased magnification.

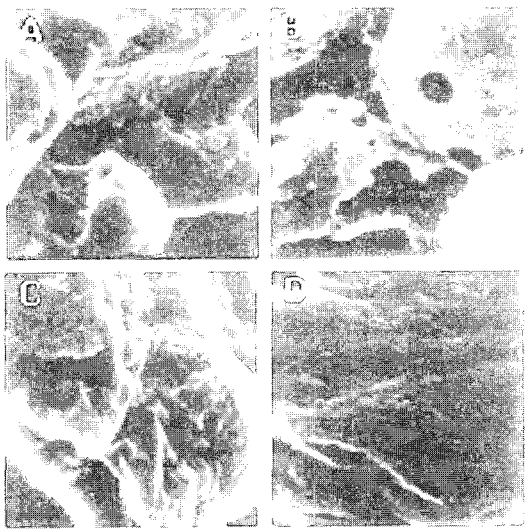

Fig. 2. The scanning electron micrograph (×20,000) of the proximal tibiae at seven weeks after the ovariectomy. A : sham-operated (Sham), B : ovariectomized (OVX), C : OVX given DR (OVX + DR), D : OVX given 17beta-estradiol (OVX + Es) rat.

period. This may be related to the diuretic action of DR in the rat. The increase in the food consumption in ovariectomized rats (24 increase in OVX over Sham rats in 2-6 weeks) may correlate with the increase of body weight15). The decreased food consumption of OVX rats in 0-1 week may be caused by surgical insult. The food consumption in week 0-1 of OVX+DR rats was the same as that of Sham rats. The administration of 17beta-estradiol preserved the food consumption (Table 1). Because the orental medicines did not cause a decrease in food consumption in ovariectomized rats (during weeks 2-6), their effect may be different from that of estrogen.

Ovariectomy resulted in the dramatic decrease in uterine weights (Sham vs. OVX; $P<0.01$). Since none of the DR medicine increased the uterine weight, it is suggested that these agents do not function as estrogen agonists. The BMD of OVX rats after seven weeks of the experiment was lower than that of the beginning of the study (baseline control). Our results indicate that true bone loss occurs in the ovariectomized rats[2]. It has been previously documented that the bone density of Sprague-Dawley rats continues to increase until approximately six months of age13). Therefore, 20% bone loss over a seven-week period (Table 2) may precisely express the 20% arrest of bone density increase.

The administration of the DR and the injection of 17beta-estradiol to OVX rats restored the BMD to the level of Sham rats (Table 3). This strongly suggests that those gynecological medicines are as effective as 17beta-estradiol in preventing bone loss, although it is not clear whether the same mechanism as estrogen would be functioning. It has been reported that both bone resorption and bone formation are promoted by ovariectomies and the prominent increase of the bone resorption is termed high-turnover osteoporosis[16]. It is possible that the porous or erosive appearance of the tibiae surface in OVX rats resulted from this high-turnover osteoporosis. DR may be effective in inhibiting the elution of bone calcium.

Oriental medicines, which have been developed over some 3000 years[7] and are known to have low toxicity, may offer advantages over the longer term over synthetic agent medication. Although the preventive mechanism of these agents remains to be explained, this initial study does show that oriental medicines that have traditionally been effective for the gynecological diseases[8,9] may also be administered for the prevention of osteoporosis.

In conclusion, the DR as well as 17 beta-estradiol, could prevent the development of bone loss induced by ovariectomy in rats. This result strongly suggests that these oriental medicines are useful for preventing postmenopausal osteoporosis and osteoporosis associated with both the ovary function failure.

Conclusion

Preventive effects by oriental medicine, Drynariae Rhizoma (DR), on the progress of bone loss induced by ovariectomy in rats were investigated for a period of 6 weeks. The bone mineral density (BMD) of tibia in ovariectomized (OVX) rats decreased by 22% from those in sham-operated (Sham) rats, with the decrease completely inhibited by the administration of the DR or 17beta-estradiol. The administration of the DR and 17beta-estradiol to OVX rats preserved the fine particle surface of the trabecular bone. These results strongly suggest that any of the DR as effective as 17beta-estradiol in preventing the development of bone loss induced by ovariectomy in rats.

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