

Dioscorea improves the morphometric and mechanical properties of bone in ovariectomised rats

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Abstract

BACKGROUND: The aim of this work was to determine the effects of oral administration of dioscorea on the morphometric and mechanical properties of the femur in ovariectomised (OVX) rats. Female Wistar rats that had undergone surgery for ovariectomy were used as a model of menopause and osteoporosis. Four weeks after surgery the animals were given oral dioscorea (0, 250, 750 or 1500 mg kg⁻¹ day⁻¹) for 27 days, then the porosity, mineral fraction, stiffness and toughness of the femur and the ultimate force needed to break the femur were measured.

RESULTS: Ovariectomy resulted in an increase in the total volume, dry weight and porosity but a decrease in the mineral fraction of femora. Subsequent chronic administration of dioscorea reversed the effect on porosity and increased the ultimate force of the femur in OVX rats but did not affect the bone properties of sham-operated rats.

CONCLUSION: These results suggest that chronic administration of dioscorea may enhance bone strength and provide insight into the role of dioscorea in bone remodeling and osteoporosis during the menopause. However, the benefit is not clear for the reproductive female.

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Keywords: dioscorea; bone properties; ovariectomy; osteoporosis

INTRODUCTION

Osteoporosis is a highly prevalent disease in postmenopausal women. Bone loss in postmenopausal women is a result of oestrogen deficiency, which results in an increase in osteoclast activation rate and the risk of fracture.¹ Fifty percent of western females and 33% of males are reported to suffer from osteoporosis.² Decreased blood levels of sex hormones are thought to be involved in osteoporosis, as postmenopausal syndrome is significantly improved by hormone replacement therapy, especially a combined oestrogen/progesterone regimen.³ Thus administration of foods containing hormone or hormone precursor might be effective in preventing osteoporosis.

Dioscorea (wild yam) has long been used as a Chinese medicine for improving gastrointestinal, sensory, memory and sexual-related functions as well as hot flush and frequency of urination in postmenopausal women. Animal studies have been used to evaluate the effect of dioscorea

on osteoporosis,⁴ diabetes⁵ and hyperlipidemia,⁶ disorders which are very common in postmenopausal women. Furthermore, effects of dioscorea on bone function at the cellular and genomic levels have been reported.^{4,7} Diosgenin, the main steroidal saponin in dioscorea,^{8,9} is used to manufacture steroidal hormones such as progesterone, oestrogen, testosterone and cortisone^{10,11} by *in vitro* chemical modification.¹² Because diosgenin stimulates the growth of the mammary epithelium¹³ and inhibits weight gain following ovariectomy,¹⁴ it has been suggested to be responsible for the effects of dioscorea.

The mechanical properties of bone are determined by its morphometric properties, e.g. its mineral content^{15–17} and porosity.¹⁸ Hernandez *et al.*¹⁹ found that the volume fraction of bone (one minus porosity) and the mineral fraction (bone mineral content divided by bone dry weight) are not correlated, suggesting that they are two independent parameters. The bone mineral fraction and bone volume fraction may

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be affected differently during bone remodeling. In addition, the bone mineral fraction is considered as a component of 'bone quality', while the bone volume fraction is used as an index of 'bone quantity'. Analysing the relationship between the morphometric and mechanical properties of the bone of ovariectomised (OVX) animals can provide insights into the pathophysiological role of osteoporosis in menopausal animals.

OVX rats are used as a menopausal animal model because the changes in biochemical and physiological functions seen in these animals are similar to those in menopausal women,²⁰ i.e. decreased levels of progesterone and oestrogen,²¹ an increased risk of cardiovascular disease²² and an enhanced rate of bone loss,^{7,23} as well as increased anxiety levels.²⁴ There are no published studies on the effect of dioscorea on the morphometric and mechanical properties of bone in OVX rats. Therefore the aim of this study was to use dual-energy X-ray absorption (DEXA) scans and a three-point bending test to evaluate the effects of chronic oral administration of dioscorea on the morphometric and mechanical properties of the femur in OVX rats that had undergone ovariectomy 4 weeks previously.

MATERIALS AND METHODS

Animals

Adult female Wistar rats (3 months old, 258.8 ± 3.94 g, $n = 90$; National Laboratory Animal Center, Taiwan) were used in the experiment. They were housed in groups of five in acrylic cages (35 cm \times 56 cm \times 19 cm) in an animal room with a 12/12 h light/dark cycle (lights on at 07:00) and provided with food and water *ad libitum*. Each animal was handled for 15 min per day on two consecutive days prior to the experiment. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of Chung Shan Medical University (Reference No. 179).

Surgery and general procedures

The rats were divided into two groups which either underwent surgery for ovariectomy (OVX group, $n = 60$) or were sham-operated ($n = 30$). Since ovariectomy may have higher risk of causing death compared with sham operation, a larger number of rats were prepared for this group. The sampling rate for the OVX group was 1.5 times higher than that for the sham-operated group. However, in this study the surgery did not cause any loss of rats. The surgery was the same as described in our previous report.²⁵ Briefly, the rats were anaesthetised using ketamine (100 mg kg⁻¹, intramuscular injection (IM)), then a 2 cm incision was made in the skin through the musculature and peritoneum and the ovaries were retracted and removed. Immediately after surgery, each rat was injected with penicillin-G procaine

(0.2 mL, 20 000 IU, IM) to reduce the chance of post-operative infection. The sham-operated group underwent the same surgical procedure except for the removal of the ovaries. After surgery the rats were housed individually in plastic cages (25 cm \times 41 cm \times 19 cm) for about 10 days for recovery, then regrouped in their home cages. Four weeks after surgery the rats were subjected to dioscorea or vehicle (distilled water) treatment. Because equal sample size for each group was not intended, the allocation was based on a convenient random manner.

Administration of dioscorea

Dioscorea (*Dioscorea alata* L. var. *purpurea* (Roxb.) M. Pouch; Tainung No. 1 Shan-Yao) was purchased from Ming-Jean Town, Nan Tao County, Taiwan. The yam tubers were cleaned, peeled, sliced into 1 cm thick slices and boiled for 30 min to inhibit the browning reaction. The cooked sample was then placed in a grinder set at a moisture level of around 10% and was milled to a flour that passed through a 60-mesh sieve, which was then stored at -25°C until use. The dose was freshly prepared before use, by adding 1 mL of doubly distilled water to 1000 mg of dioscorea flour and mixing. The dosage expressed in this study is the dry weight of dioscorea flour. From day 28 after ovariectomy or sham operation the animals were given dioscorea (250, 750 or 1500 mg kg⁻¹ day⁻¹) or distilled water by oral gavage for 27 days. Similar conditions of 4–6 weeks after ovariectomy^{14,26,27} and a duration of treatment for osteoporosis of 4–6 weeks^{27,28} have been employed by other authors using the rat menopausal model. The dosage used in the present study was based on the results of our previous study, in which treatment with 250–1500 mg kg⁻¹ day⁻¹ of dioscorea for 27 days caused pronounced changes in behaviour and biochemistry,²⁵ and our wish that the amount of dioscorea administered per day be less than 10% of the average food intake (around 10 g) to avoid an effect on calorie intake; in the highest-dose rats the dose was about 450 mg, i.e. 4.5%. The rats were then sacrificed using CO₂ and their femora were immediately removed.

Preparation of femora and determination of morphometric and mechanical properties

During removal of the muscle and fibrous periosteum the femora were kept wet in distilled water. After defatting in chloroform²⁹ and drying, the right femur was used to sequentially measure the morphometric parameters of wet weight, total volume, dry weight and mineral content to calculate the porosity and bone mineral fraction. The left femur was kept at -20°C until mechanical testing. A previous report has shown that drying, rewetting and freezing have no effect on the mechanical properties of bone.³⁰

Measurement of wet weight

The right femur was placed in an unstoppered glass vial containing distilled water and the vial was placed in

a vacuum desiccator for 90 min to remove air diffusing out of the bone.³¹ After gently wiping off the water on the surface of the specimen, the femur was weighed on an analytical balance (AE240-S, Mettler, Zurich, Switzerland) to give the wet weight W_W .

Measurement of total volume

According to the theory of porous media,³² the porous structure of bone consists of the 'solid skeleton' (bone frame volume V_B) and the 'interstitial fluid' (void volume V_P).³³ Instead of using a conventional caliper or mathematical equations, we have, by combining Newton's third law and Archimedes' principle, developed a novel and accurate method to directly measure the total volume V_T ($V_T = V_B + V_P$, cm^3) of the femur.^{18,34} Briefly, each right femur was suspended by a thin silk yarn and fully immersed in water in a beaker on the analytical balance, then the buoyancy force \bar{B} (g) was read directly from the balance display. The total volume of the femur was calculated as $V_T = \bar{B}/0.9971$, where 0.9971 is the density (g cm^{-3}) of distilled water at 25 °C and 1 atm.³⁵

Measurement of dry weight

After measuring the total volume, each right femur was placed in an incubator at 50 °C for 72 h to remove the interstitial fluid until a constant weight (<0.05% change) was obtained in weighings at 1 h intervals. The dry weight W_D was immediately measured on the analytical balance.

Measurement of bone mineral content

To measure the bone mineral content W_M ,³⁶ each specimen was immersed in 10 cm of distilled water,³⁷ which has been demonstrated to simulate the surrounding soft tissue in the *in vivo* situation,³⁸ and underwent DEXA scans in the EXPERT-XL system (Lunar, Madison, WI, USA). The DEXA scan is a common technique for assessing bone mineral density.³⁹ Our previous study showed that the measurement of the bone mineral content is constant irrespective of the scan direction.¹⁸ Each specimen underwent three scan trials, with an inter-trial interval of 1 week, which were performed by a senior radiologist with more than 5 years' experience. The code of the femur was reassigned before each trial so as to perform a single-blind test.

Calculation of morphometric parameters

The values of two morphometric parameters, bone porosity and bone mineral fraction, were calculated as follows. In the saturated right femur the void space is filled with W_f g of distilled water. The void volume V_P was calculated using the equations $V_P = W_f/0.9971$ and $W_f = W_W - W_D$. The porosity P was calculated using the equation $P = V_P/V_T$. The bone mineral fraction was calculated by dividing the bone mineral content W_M by the bone dry weight W_D .

Evaluation of mechanical properties

Before mechanical testing, the left femur was soaked in saline at room temperature for 12 h. A material testing system, INSTRON 4464-Standard (Instron, Massachusetts, USA), was used to perform the 'three-point bending test', where the span width was 15.7 mm. The rate of compression was 0.5 mm min^{-1} and the relationship between load (force, N) and displacement (mm) was recorded at a sampling rate of 10 Hz. The slope of the linear section of the load–displacement curve gives the stiffness S (N mm^{-1}). The force required to fracture the bone is the ultimate force F_{ult} (N), which causes the ultimate displacement D_{ult} (mm). The work (energy) needed to fracture the bone, or the toughness (N mm), was calculated as the area under the curve from zero displacement to ultimate displacement. Since the central portion of a femur is similar to a cylinder and the length of the span used in the bending test was consistent, the present model was performed. However, it is noteworthy that, when the shape and length of bone used are different, Young's modulus and the stress–strain curve are useful for calculating certain measures.⁴⁰

Statistical analysis

To analyse the effects of surgery and dioscorea treatment, two-way analysis of variance (ANOVA) was carried out. When interactions existed, the independent t test was used to analyse the effects of ovariectomy. To evaluate the effects of dioscorea treatment, one-way ANOVA was performed, followed by the least significant difference (LSD) *post hoc* test. The test of homogeneity of variances of the parameters measured showed no significance (values of Levene statistic >0.598), suggesting that differences in sample size between the groups may not affect the statistical results. All results are expressed as mean \pm standard error of mean (SEM). The level of significance was defined as $P < 0.05$.

RESULTS

At 4 weeks after ovariectomy the percentage change in body weight was significantly higher in OVX rats than in sham-operated rats (degrees of freedom (DF) = 88, $t = -2.377$, $P = 0.02$; Fig. 1(A)). A subsequent 4 weeks of dioscorea treatment did not affect the change in body weight over this 4 week period in either OVX or sham-operated rats (Fig. 1(B)).

As shown in Table 1, neither ovariectomy nor dioscorea treatment affected bone mineral content. Two-way ANOVA showed main effects of surgery on total volume ($F(1, 83) = 38.447$, $P < 0.001$) and dry weight ($F(1, 83) = 22.287$, $P < 0.001$). The LSD *post hoc* test indicated that total volume and dry weight were significantly higher in OVX rat groups than in sham-operated rats receiving 0 mg kg^{-1} of dioscorea treatment. However, bone mineral content, total volume and dry weight in both sham-operated and OVX rats were not affected by dioscorea

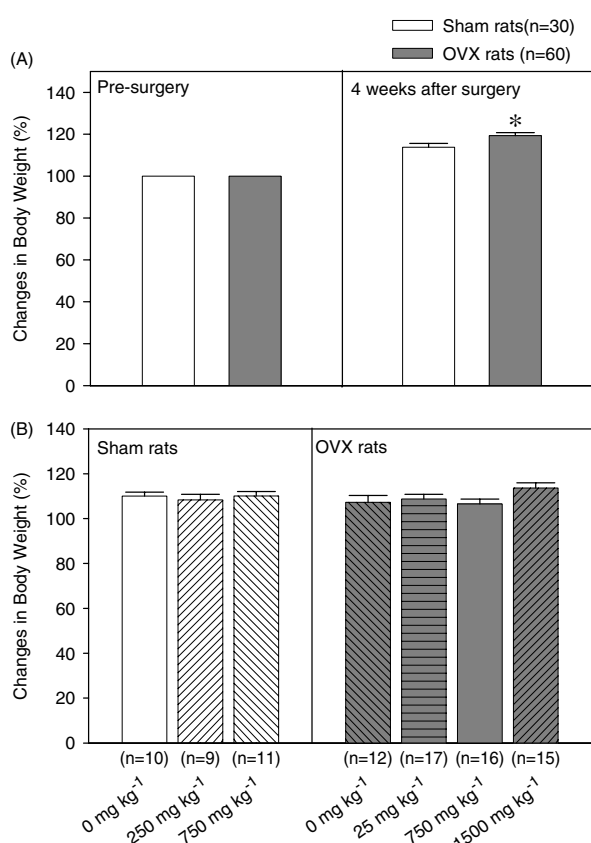


Figure 1. Effects of ovariectomy and subsequent dioscorea treatment on changes in body weight. (A) Change in body weight in the different groups at 4 weeks after ovariectomy or sham operation expressed as a percentage of the pre-surgery value. The body weight before surgery was 231.7 ± 7.1 and 272.3 ± 3.5 g in the sham-operated and ovariectomised (OVX) rats respectively. (B) Change in body weight in the different groups over the 4 week period of dioscorea treatment expressed as a percentage of the weight at the start of dioscorea treatment of the sham-operated or OVX rats, as appropriate. * $P < 0.05$ compared with sham-operated rats. Data are shown as mean \pm SEM.

treatment. Moreover, these three bone parameters were significantly correlated with body weight (values of Pearson correlation >0.515 , P values <0.001).

Furthermore, in addition to the main effects of surgery on stiffness ($F(1, 83) = 13.848$, $P < 0.001$), ultimate force ($F(1, 81) = 27.359$, $P < 0.001$) and toughness ($F(1, 83) = 21.174$, $P < 0.001$), there were interactions between surgery and dose for porosity ($F(2, 83) = 3.676$, $P = 0.030$) and ultimate force ($F(2, 81) = 3.306$, $P = 0.042$). The independent t test revealed that at 8 weeks after OVX the porosity of the femora was higher (DF = 20, $t = -2.395$, $P = 0.027$) but the bone mineral fraction (DF = 20, $t = 2.629$, $P = 0.016$) lower in OVX rats than in sham-operated rats. ANOVA followed by the LSD test showed significant differences between the OVX rat groups in porosity ($F(3, 56) = 3.264$, $P = 0.028$) and ultimate force ($F(3, 54) = 3.158$, $P = 0.032$) of the femora. That is, in OVX rats, oral administration of dioscorea at the dosage of 750 or 1500 mg kg⁻¹ for 27 days returned the porosity of the femora to control levels (both P values <0.05); furthermore, all three dosages of dioscorea increased the ultimate force (all P values <0.05). In contrast, dioscorea treatment did not affect the morphometric and mechanical properties of the femur in sham-operated rats (Table 1).

DISCUSSION

Four weeks after ovariectomy the body weight increase in OVX rats was significantly higher than that in sham-operated rats, consistent with previous reports for menopausal animal models.^{14,26,27} Ovariectomy resulted in an increase in the total volume, dry weight and porosity but a decrease in the mineral fraction of femora. Subsequent chronic administration of dioscorea reversed the effect on porosity and increased the ultimate force of the femora in OVX rats but did not affect the bone properties of sham-operated rats.

OVX rats are used as a menopausal animal model, since the changes in biochemical and physiological functions are comparable to those seen in menopausal women.^{20–22} The OVX-induced increase in bone loss causes osteopaenia in rats⁷ and is similar to that seen in postmenopausal women.²³ Dioscorea has

Table 1. Effects of dioscorea on bone properties in sham-operated and OVX rats

| Property | Sham-operated rats | | | OVX rats | | | |
|---------------------------------|-----------------------------------|------------------------------------|-------------------------------------|-----------------------------------|-------------------------------------|-------------------------------------|--------------------------------------|
| | 0 mg kg ⁻¹ (n = 10) | 250 mg kg ⁻¹ (n = 9) | 750 mg kg ⁻¹ (n = 11) | 0 mg kg ⁻¹ (n = 12) | 250 mg kg ⁻¹ (n = 17) | 750 mg kg ⁻¹ (n = 16) | 1500 mg kg ⁻¹ (n = 15) |
| Bone mineral content (g) | 0.37 \pm 0.01 | 0.37 \pm 0.02 | 0.34 \pm 0.01 | 0.39 \pm 0.01 | 0.39 \pm 0.01 | 0.39 \pm 0.01 | 0.39 \pm 0.01 |
| Total volume (cm ³) | 0.48 \pm 0.01 | 0.48 \pm 0.01 | 0.48 \pm 0.01 | 0.53 \pm 0.01 ^{##} | 0.54 \pm 0.01 ^{##} | 0.53 \pm 0.01 ^{##} | 0.53 \pm 0.01 ^{##} |
| Dry weight (g) | 0.52 \pm 0.02 | 0.52 \pm 0.02 | 0.51 \pm 0.01 | 0.56 \pm 0.01 [#] | 0.56 \pm 0.01 [#] | 0.56 \pm 0.01 [#] | 0.56 \pm 0.01 [#] |
| Porosity (%) | 12.7 \pm 0.4 | 12.9 \pm 0.7 | 13.8 \pm 0.5 | 14.0 \pm 0.3 [#] | 13.3 \pm 0.4 | 12.7 \pm 0.3* | 12.3 \pm 0.3** |
| Bone mineral fraction (%) | 71.3 \pm 1.2 | 71.0 \pm 1.2 | 68.3 \pm 0.5 | 67.8 \pm 0.7 [#] | 69.4 \pm 1.1 | 70.6 \pm 1.1 | 71.6 \pm 1.8 |
| Stiffness (N mm ⁻¹) | 283.9 \pm 8.3 | 283.5 \pm 13.2 | 277.4 \pm 9.4 | 299.0 \pm 10.5 | 316.4 \pm 8.8 | 314.0 \pm 6.7 | 307.4 \pm 6.8 |
| Ultimate force (N) | 117.4 \pm 4.2 | 113.4 \pm 5.3 | 113.0 \pm 4.2 | 122.9 \pm 4.0 | 135.0 \pm 4.2* | 139.5 \pm 3.2** | 136.3 \pm 3.2* |
| Toughness (N mm) | 38.4 \pm 2.1 | 36.7 \pm 2.4 | 37.8 \pm 2.2 | 43.5 \pm 2.0 | 46.5 \pm 1.8 | 48.4 \pm 2.0 | 50.1 \pm 2.4 |

[#] $P < 0.05$ and ^{##} $P < 0.01$ compared with sham-operated group receiving 0 mg kg⁻¹ of dioscorea. * $P < 0.05$ and ** $P < 0.01$ compared with OVX rats receiving 0 mg kg⁻¹ of dioscorea. Data are mean \pm SEM for number of rats indicated in parentheses.

long been used as a Chinese medicine for improving the symptoms seen in the menopause.^{5,6} No toxic effect was seen when rats were given 50% (w/w) of uncooked dioscorea in the diet for 3 weeks⁴¹ or a dosage of dioscorea of 500–2000 mg day⁻¹ for 28 days.⁴² Water extracts of dioscorea inhibit the bone resorption induced by parathyroid hormone in a bone culture system.⁴ Oral administration of methanol and ethanol extracts of dioscorea causes antiosteoporotic activity in OVX rats, enhancing osteoblast differentiation and matrix mineralisation⁴³ as well as decreasing parathyroid hormone-induced bone resorption.⁴⁴ Furthermore, a histomorphometric study using X-ray analysis of the femur in OVX Sprague–Dawley rats showed that 33 days of treatment with a sustained release capsule of diosgenin, the major steroidal saponin in dioscorea, reverses the changes in the endosteal perimeter and cortical area to control levels.⁷ The present study showed that oral administration of dioscorea also had antiosteoporotic-like activity, promoting the morphometric and mechanical properties of femora in OVX rats. These results demonstrate that dioscorea and/or its ingredients have beneficial effects on the bone in OVX rats.

Although the bone mineral content, total volume and dry weight were significantly correlated with body weight, these data *per se* do not provide much functional information. To show the physical properties, these data were used to calculate morphometric and mechanical parameters. Porosity reflects the proportion of bone frame that is made of organic and inorganic components. Mineral fraction is the content of inorganic phase in the bone frame and resists the force acting on the bone. All the above contribute to the mechanical properties, e.g. ultimate force, stiffness and toughness, of bone⁴⁵ that represent the ability of the bone to bear external loads. Although the stiffness, ultimate force and toughness of the femora were not affected by ovariectomy, the increase in porosity and decrease in mineral fraction, indicating a decrease in the quality and quantity of bone,^{7,23,46,47} may increase the risk of fracture. Chronic dioscorea treatment significantly increased the ultimate force of the femora in OVX rats, suggesting that the mechanical strength was increased. Our previous studies involving mechanical tests on the bovine femur demonstrated relationships between the mechanical properties, porosity and mineral fraction and showed that the contribution of porosity to the ultimate force was significantly higher than that of the mineral fraction.^{18,34} This may explain the observation in the current study that the increase in the ultimate force of the femur in dioscorea-treated OVX rats was accompanied by a decrease in porosity but no change in the mineral fraction. In addition, the relationship between porosity and mineral fraction depends on the experimental subjects and possibly also on the pathophysiological conditions. The literature shows that porosity and mineral fraction in human bone are independent parameters,¹⁹ but there

is a positive correlation between bone mineral density and volume fraction in bovine cortical bone.³⁴ The current data showing that dioscorea at 750 mg kg⁻¹ suppressed the porosity but did not affect the bone mineral fraction are consistent with the findings in bisphosphonate treatment of osteoporosis in dogs, where the changes in porosity and mineral density were not parallel, suggesting that time-dependent factors may have been involved.⁴⁸ Furthermore, the porosity and bone mineral fraction in OVX rats receiving dioscorea treatment were similar to the levels in sham-operated controls. However, the mechanical ultimate force was better in the OVX rats than in the controls. This suggests that the treatment with dioscorea may affect not only the inorganic but also the organic components in the bone. Therefore the effect of dioscorea on bone remodeling, especially the organic phase, e.g. collagen,⁴⁹ during the postmenopause deserves further study.

The sex hormone system may be involved in these effects of dioscorea. Decreased blood levels of sex hormone are thought to be involved in the disorders seen in the postmenopause,⁵⁰ as postmenopausal syndrome is significantly improved by hormone replacement therapy.³ Diosgenin, the main steroidal saponin in dioscorea,^{8,9} is used to manufacture steroidal hormones such as progesterone, oestrogen, testosterone and cortisone^{10,11} by *in vitro* chemical modification.¹² There are no reports on the exact mechanisms by which diosgenin is converted to other hormones *in vivo*, but a previous study showed that hypertrophy of the adrenal cortex in OVX animals was reversed towards control values after continuous supplementation with diosgenin.⁵¹ Furthermore, the consumption of wild Mexican yam products containing diosgenin increases progesterone activity in the saliva,⁵² suggesting that the steroidal hormone system is affected. However, it should be noted that a recent study on menopausal animals indicated that sex hormone levels might not be affected by diosgenin treatment.⁵¹ Moreover, dietary supplementation with dioscorea does not affect dehydroepiandrosterone levels in the blood.⁵³ Thus dioscorea and/or diosgenin may not serve as a precursor of sex hormones *in vivo* but may affect menopausal symptoms by another mechanism, e.g. an anti-inflammatory action, as dioscorea modulates the production of cytokines *in vivo*^{25,54} and *in vitro*.⁵⁵ Furthermore, the effects of dioscorea observed in this work may be mediated through its antioxidant activity or by modulating lipid metabolism,⁵³ as the antioxidative capacity of foods has positive effects on bone.²⁸ In addition, dioscorea treatment improves nutritional status and the synthesis of proteins and related hormones,⁴² in which ingredients of dioscorea (diosgenin and related steroidal saponins) are reported to be involved.⁵⁶

The conditions used in testing, e.g. dryness, humidity and age of the bone, the manner in which the force is applied and whether the bone is kept

whole, affect the mechanical characteristics of bones. Thus the conditions applied here were consistent. Because the main goal of this work was not to focus on the characteristics of compact or cancellous bone but to study the effects of bending force on the whole bone, the whole femur was used for mechanical testing to mimic the situation in which the bone is hit laterally or in a fall. The changes in porosity and bone mineral fraction and their relationship to the mechanical properties seen in this study should not be compared with those for compact or cancellous bones, as the performance of anisotropic bones under axial compression and axial tension differs from that of a long bone under a bending force.

CONCLUSIONS

The present data show that ovariectomy causes an increase in porosity and a decrease in bone mineral fraction in the femora of rats. Subsequent chronic administration of dioscorea reverses the effect on porosity and increases the ultimate force. These data provide an insight into the effect of dioscorea on bone remodeling and osteoporosis in the menopause.

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