Improving Bone Microarchitecture in Aging with Diosgenin Treatment: A Study in Senescence-Accelerated OXYS Rats

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Abstract

Osteoporosis is a major disease associated with aging. We have previously demonstrated that diosgenin prevents osteoporosis in both menopause and D-galactose-induced aging rats. OXYS rats reveal an accelerated senescence and are used as a suitable model of osteoporosis. The aim of the present study was to analyze microarchitecture and morphological changes in femur of OXYS rats using morphological tests and microcomputed tomography scanning, and to evaluate the effects of oral administration of diosgenin at 10 and 50 mg/kg/day on femur in OXYS rats. The result showed that, compared with age-matched Wistar rats, the femur of OXYS rats revealed lower bone length, bone weight, bone volume, frame volume, frame density, void volume, porosity, external and internal diameters, cortical bone area, BV/TV, Tb.N, and Tb.Th, but higher Tb.Sp. Eight weeks of diosgenin treatment decreased porosity and Tb.Sp, but increased BV/TV, cortical bone area, Tb.N and bone mineral density, compared with OXYS rats treated with vehicle. These data reveal that microarchitecture and morphological
changes in femur of OXYS rats showed osteoporotic aging features and suggest that diosgenin may have beneficial effects on aging-induced osteoporosis.

Key Words: aging, bone loss, diosgenin, microcomputed tomography, osteoporosis, OXYS rats

Introduction

Osteoporosis is one of the most prevalent and serious diseases in the elderly. Aging-induced osteoporosis is associated with a reduction in mineralization and increase in porosity in the cortical and trabecular bones, which results in bone loss and increased risk of fracture. Oxidative stress has been proposed as a major cause of aging (46, 56) and age-related bone loss (62).

Laboratory rodents are the most convenient animal models for osteoporosis research (36), but there are only a few examples of rodent genetic models of osteoporosis. Over the last few years, a large amount of experimental data has demonstrated that accelerated senescent OXYS rats are a suitable model of osteoporosis. OXYS rats were produced in the Institute of Cytology and Genetics of the SB Russian Academy of Sciences (Novosibirsk, Russia) by selective breeding of Wistar rats that were highly sensitive to the cataractogenic effect of D-galactose (51). These rats develop early spontaneous cataracts and degenerative features that are regarded as syndromes of accelerated senescence (33). OXYS rats have a shortened lifespan and show early development of age-related pathological phenotypes similar to geriatric disorders observed in humans, including senile osteoporosis (41), cataracts, retinopathy (42, 64), and signs of accelerated aging (31, 44). These features make it possible to use OXYS rats to evaluate the efficacy of treatments for osteoporosis. Thus, detailed analysis of microarchitecture and morphological changes in the bone of OXYS rats is needed. Furthermore, OXYS rats have low glutathione levels, high malondialdehyde (MDA) levels, and high superoxide dismutase (SOD) activities (50). Recently, some aging-related features of OXYS rats were found to be corrected or improved by supplementation with anti-oxidants (4, 32, 45, 53). Therefore, OXYS rats are useful for detecting effects of antioxidant drugs on osteoporosis.

Antioxidants scavenge free radicals and protect cells and organs from oxidative damage. Among the natural antioxidants, Dioscorea (wild yam) is noteworthy. Dioscorea, a common food and Chinese medicine (40), contains phytosteroids, such as diosgenin and steroidal saponins (19), and has long been used to treat menopausal syndrome and has anti-osteoporotic activities (10, 61). Dioscorea also decreases inflammatory cytokine levels in the brain of menopausal rats (22). Diosgenin, the main steroidal saponin in Dioscorea, has a chemical structure similar to steroid hormones, and is used as a precursor in the manufacture of estrogen, progesterone, testosterone and cortisol (17, 49). In addition, it has antioxidant and free radical scavenging activities in rats (12, 52), shows anti-aging effects in menopausal animals (21), improves epidermal functions in aging mice (54). Diosgenin also improves learning and memory in a D-galactose-induced aging model in mice (12).

Materials and Methods

Animals and Drug Administration

Twelve-week-old male OXYS rats (weighing 268.0 ± 4.9 g, n = 29), obtained from the Institute of Cytology and Genetics, Russian Academy of Sciences, Russia, and the same age male Wistar rats (weighing 416.2 ± 9.8, n = 8, used as health control) were housed in groups of four or five in acrylic cages (35 × 56 × 19 cm) in an animal room with a 12 h light-dark cycle (lights on at 07:00 h) with food and water available ad libitum. The OXYS rats were divided into three experimental groups: aging control rats (n = 10), or rats treated with diosgenin at a dose of 10 mg/kg/day (n = 10) or 50 mg/kg/day (n = 9). 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 (IACUC approval No.: 1018). All
efforts were made to minimize the number of animals used and their suffering.

**General Procedure:** Starting at 12 weeks of age (day 0), the OXYS rats underwent diosgenin treatment (0, 10 or 50 mg/kg/day, p.o.) for 8 weeks. The Wistar control rats received the vehicle. All the rats were sacrificed on day 57 by exposure to CO₂, and the femur bones dissected out, frozen and stored at -70°C until use.

**Drugs and Drug Administration:** Diosgenin was purchased from Sigma-Aldrich (St. Louis, MO, USA). An appropriate amount (see below) of the drug was mixed with flour and water to produce 0.8 g pellets. One pellet was given per day to each rat at 12:00-13:00 h for 56 days; the control rats received pellets without the drug. The body weight of each rat was measured daily, then the pellet mixture for that day was prepared individually for each rat based on its body weight.

**Preparation of Femora and Determination of the Morphometric Properties**

During the removal of the muscle and the fibrous periosteum, the femora were kept wet using distilled water. After defatting in chloroform and drying, the right femur was used to sequentially measure the morphometric parameters of wet weight, total volume, dry weight, and frame volume, which were then used to calculate the void volume and porosity.

**Measurement of the Wet Weight:** The femur was placed in an unstoppered glass vial containing distilled water. The vial was then placed in a vacuum desiccator for 90 min to remove air diffusing out of the water. After gently wiping off the water on the surface of the specimen, the femur was weighed using an analytical balance to obtain the wet weight (WW).

**Measurement of the Dry Weight:** The femur was analyzed by μCT (Skyscan 1176, SKYSCAN, Belgium) without further sample preparation. Sequential transaxial images through the distal half of the femoral shaft were obtained using an isotropic voxel size of 9 μm, current of 500 μA, exposure time of 1,000 ms, peak tube voltage of 50 kV, and a 0.5 mm aluminum filter. The scanning angular rotation was 180 degrees in angular increments of 0.5 degrees. Data sets were reconstructed using the software provided with the equipment (Skyscan™ NRecon software, version 1.6.9.3) and segmented into binary images (16-bit BMP images). To analyze the microarchitectural properties of the trabecular bones, volumes of interest (VOIs) in the femur were evaluated. For analyzing the trabecular bone, a VOI was selected starting 0.45 mm (50 image slices) from the growth plate (GP) and extending a longitudinal distance of 3.60 mm in the proximal direction (400 image slices analyzed, cortical bone excluded), while, for an image slice, taken at the middle of the bone, was used for analyzing the cortical bone (Fig. 1).

Regions of interest in the trabecular and cortical bone were obtained by manual drawing on the scanned image and analyzed using the software provided with the equipment (Skyscan™ CT-analyzer software, version 1.6.0). Morphometric indices of the trabecular bone region were determined from the microtomographic data sets (integrated over a VOI) using direct 3D morphometry. The total volume of the VOI (tissue volume TV, mm³) and the trabecular bone volume (BV; mm³) were calculated based on the hexahedral marching cubes volume model of the VOI. The BV/TV (%) was then calculated. The Tb.Th (μm), Tb.Sp (μm), Tb.N (mm⁻¹) and bone mineral density (BMD) were obtained by image analysis.

Tomographic scans were performed ex vivo on the excised femur as described above. Each pixel of the reconstructed 8-bit BMP image had a color or grey value between 0 and 255. A grey value of 255 was assumed to be white (void space), whereas a value of 0 was taken as black or the densest part of the image.
Effects of Diosgenin on Bone Loss in Aging

Statistical Analysis

One-way ANOVA, followed by the least-significant difference (LSD) post hoc test, was used to evaluate differences between the groups. A test of homogeneity of the variances of the parameters measured showed no significance (values of Levene statistic > 0.598), indicating that the differences in sample size between the groups did not affect the statistical results. All results are expressed as the mean ± SEM. The level of significance was defined as $P < 0.05$.

Results

The OXYS rats showed lower body weight than the control Wistar rats before and after the experiment ($F(3,36) > 69.28$, $P < 0.001$). Paired-samples $t$ test revealed that all the rats showed higher body weight at the end of the experiment, compared with that before the experiment (all $t$-values $> 7.05$, all $P$-values $< 0.001$). Diosgenin treatment, at the dose of 50 mg/kg/day, significantly decreased the body weight, compared with the OXYS rats treated with vehicle ($P < 0.05$) (Fig. 2). However, the percentage of body weight changes, which ranged from $21.4 \pm 2.6\%$ to $31.7 \pm 5.3\%$, did not show between-group differences ($F(3,36) = 1.18$, $P = 0.33$). The ANOVA followed by LSD post hoc test revealed differences of morphological properties of femur between the groups, where bone length ($F(3,36) = 71.49$, $P < 0.001$), dry weight ($F(3,36) = 100.58$, $P < 0.001$), wet weight ($F(3,36) = 115.46$, $P < 0.001$), bone volume ($F(3,36) = 46.17$, $P < 0.001$), frame volume ($F(3,36) = 15.22$, $P < 0.001$), and frame density ($F(3,36) = 13.09$, $P < 0.001$) of OXYS rats were significantly lower than those in the Wistar rats.
Chronic administration of diosgenin at the dosage of 50 mg/kg/day, but not 10 mg/kg/day, resulted in a significant decrease in dry weight, wet weight and bone volume (all \(P\)-values < 0.05), compared with the OXYS rats treated with vehicle (Table 1).

The void volume (\(F(3,36) = 79.24, P < 0.001\)) and porosity (\(F(3,36) = 19.15, P < 0.001\)) of the OXYS rats treated with vehicle were also significantly increased compared to the Wistar control. **\(P < 0.01\) and *** \(P < 0.001\) compared to the Wistar control. # \(P < 0.08\), ## \(P < 0.01\), ### \(P < 0.001\) compared to the OXYS rats treated with vehicle. Data are expressed as the mean ± SEM.
Effects of Diosgenin on Bone Loss in Aging

327

rats were significantly lower than that in the Wistar rats. Diosgenin treatment, at the dosage of 10 and 50 mg/kg/day, decreased the void volume and porosity (both $P$-values < 0.05) (Fig. 3).

Using the images obtained by μCT, the microarchitecture of the cortical bone and trabecular bone of the femur was analyzed. The images used for analyzing BV, TV and trabecular parameters are shown in Fig. 4A. The TV, BV ($F(3,36) = 12.329$, $P < 0.001$) (data not shown), BV/TV (Fig. 4B), Tb.N ($F(3,36) = 11.57$, $P < 0.001$, partial Eta squared = 0.51) (Fig. 4C), and Tb.Th ($F(3,36) = 86.62$, $P < 0.001$, partial Eta squared = 0.89) (Fig. 4D) of the OXYS rats were significantly lower than that in the Wistar rats. The OXYS rats showed higher Tb.Sp ($F(3,36) = 13.74$, $P < 0.001$, partial Eta squared = 0.56) (Fig. 4E) and BMD ($F(3,36) = 98.79$, $P < 0.001$, partial Eta squared = 0.90) (Fig. 4F), compared to that in the Wistar rats. Diosgenin did not affect the TV, BV (data not shown), and Tb.Th. However, diosgenin at 50 mg/kg/day slightly increased the BV/TV ($P = 0.089$) (Fig. 4B). Diosgenin, at both 10 and 50 mg/kg/day, significantly increased the Tb.N (both $P$-values < 0.05) (Fig. 4C) and BMD (both $P$-values < 0.01) (Fig. 4F) but reduced the Tb.Sp (both $P$-values < 0.001) in a dose-dependent manner (Fig. 4E).

The images used for analyzing bone diameter and cortical bone area are shown in Fig. 5A. OXYS rats revealed lower external diameter ($F(3,36) = 51.92$, $P < 0.001$) (Fig. 5B), internal diameter ($F(3,36) = 23.73$, $P < 0.001$) (Fig. 5C) and percentage of cortical area ($F(3,36) = 8.10$, $P < 0.001$) (Fig. 5D), compared with the Wistar control. Diosgenin treatment, at the dosage of 50 mg/kg/day, decreased the external diameter and internal diameter (both $P$-values < 0.01), but increased the percentage of cortical area ($P < 0.001$), compared with the OXYS rats treated with vehicle (Fig. 5). Femur microarchitecture of the animals is shown in Fig. 6.

![Figure 5](image_url)

Fig. 5. Effects of diosgenin on the cortical bone of femur in OXYS rats analyzed by μCT. (A) The images, taken at the middle of the bone, were used for analyzing the cortical bone; bar, 1 mm. (B) external diameter, (C) internal diameter, (D) percentage of cortical bone area. *$P < 0.05$ and ***$P < 0.001$ compared to the Wistar control. ###$P < 0.001$ compared to the OXYS rats treated with vehicle. Data are expressed as the mean ± SEM.
Discussion

Morphological and μCT image analysis of the femur revealed that OXYS rats show lower bone length, bone weight, bone volume, frame volume, frame density, void volume, porosity, external and internal diameters, percentage of cortical bone area, BV/TV, Tb.N and Tb.Th, but higher Tb.Sp, compared with the Wistar control. Eight weeks of diosgenin treatment decreased porosity and Tb.Sp, but increased BV/TV, cortical bone area and Tb.N, compared with the OXYS rats treated with vehicle. We also found that the BV/TV was positively correlated with the Tb.N (r = 0.817) and negatively correlated with the Tb.Sp (r = -0.555) (data not shown). This is the first study thoroughly examining microarchitecture and morphological changes in the femur of OXYS rats. The smaller physical size of OXYS femur is parallel to their smaller body size and weight. The bone features observed in OXYS rats are similar with that in menopausal rats (24) and in human (43). Treatment with diosgenin improved trabecular and cortical structure of the femur, suggesting that diosgenin has potential for the treatment of osteoporosis during aging.

An antioxidative action may be one of the mechanisms by which diosgenin decreases bone loss in OXYS rats. Accelerated senescence OXYS rats show aging features, including osteoporosis, cognitive deficit, reproductive dysfunction and neurodegeneration as early as 3 months of age (2, 34), and have high levels of free radicals (32) and oxidative damage to DNA and proteins in liver mitochondria and cytosol (26, 27, 30). We, therefore, propose that oxidative damage may play a role in the bone loss seen in the OXYS aging rat model. Diosgenin, one of the important bioactive ingredients in dioscorea, has antiaging and antioxidant activities (39). A previous study reported that administration of diosgenin to D-galactose-induced senescent mice increases SOD and glutathione peroxidase activities and decreases MDA levels, suggesting that diosgenin has beneficial effects on aging and oxidative stress-related disorders (12). Similarly, our previous study (10) has demonstrated that diosgenin improves morphometric and mechanical properties of bone in menopausal animals. Our recent report (55) also showed an increase in sperm motility in aging rats after diosgenin treatment at the dosages of 10 and 50 mg/kg/day. Decreasing male motility of spermatozoa with aging is usually associated with oxidative stress due to accumulated seminiferous tubule damage (11).

Decreased blood levels of some sex hormones are also involved in osteoporosis. Postmenopausal women have low estrogen levels and exhibit osteoclast activation and increased risk of fracture and osteoporosis (35), and bone loss in these women can be prevented using estrogen (9). Lower serum androgen levels have been reported in idiopathic osteoporosis in males, and androgen has anabolic effects on bone and increases its mineral density (18). In addition, decreased levels of testosterone or dehydroepiandrosterone sulfate are seen in D-galactose-induced aging models in rats (63). Diosgenin has a similar chemical structure to sex hormones and has long been used as a precursor in the manufacture of steroid hormones, such as estrogen, progesterone, testosterone and cortisol (17, 49). Although nothing is known about the pathways by which diosgenin is converted into other hormones in vivo, chronic administration of diosgenin to rats has been found to increase progesterone levels (8) and reverse menopause-induced hypertrophy of the adrenal gland (5). Recovery of sex hormone levels have also been observed in postmenopausal women (60) and ovariectomized rats (8) treated with diosgenin. In addition, diosgenin can enhance bone formation by stimulating the synthesis and secretion of bone marker proteins, which increase the formation of Ca²⁺ deposits in the extracellular matrix, thereby increasing bone formation (3). Interestingly, it is known that diosgenin enhances formation of osteoprogenitor cells in vitro in the bone marrow (47). These

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Fig. 6. Effects of diosgenin on the trabecular bone of the femur in OXYS rats. The figure shows a representative 3D architecture measured using μCT for (A) a Wistar control rat, (B) an OXYS rat treated with vehicle, (C) and (D) OXYS rats treated with diosgenin at 10 or 50 mg/kg/day, respectively.
data lead us to suggest that the effects of diosgenin on bone loss in OXYS rats may, at least partially, attribute to its effects on hormonal systems and protein synthesis. Further studies are needed to address this issue.

Bone quality is related to its mechanical and morphological properties, which determine fracture risk. The lowered Tb.N and higher Tb.Sp in OXYS rats is similar to the trabecular bone loss in the osteoporotic rat model (24). In the present study, 2 months of diosgenin administration resulted in significant increases in BV/TV, cortical bone area and Tb.N and significant decreases in void volume, porosity, and Tb.Sp. Higher bone porosity has been correlated with increased risk of fracture (14, 15). Further, an improvement of cortical component was seen after effective anti-osteoporotic therapy (24). Thus, the above diosgenin-induced bone changes may increase bone quality.

The trabecular microarchitecture also contributes to bone strength. 3D μCT and high resolution peripheral quantitative computer tomography analysis of the same bone biopsy has shown that trabecular architecture contributes to bone strength (13). Thus, assessment of trabecular architecture using μCT can predict fracture. A correlation has been found between morphological parameters of the femur, such as Tb.Sp, Tb.N and BV/TV, and mechanical properties, such as failure load and stiffness (37). Other studies have suggested that the Tb.Sp is an important determinant of bone strength. A μCT study of bone reported that women with documented vertebral fractures showed a 49% increase in the Tb.Sp compared to women without vertebral fractures (28). Similarly, in a study comparing 2D histomorphometry and 3D μCT parameters in Japanese women with or without spinal fractures (23), the Tb.N was significantly lower and the Tb.Sp significantly higher in those with spinal fractures. Furthermore, thinning of trabeculae has been documented in steroid-induced osteoporosis, which increases the risk of fracture (1). The present study did not find any change in the Tb.Th but revealed an increase in Tb.N after diosgenin treatment. In addition, decreases in the Tb.Sp, void volume and porosity were observed after diosgenin treatment, showing that diosgenin increases trabecular number and density, but not trabecular thickness. These changes may, therefore, increase the mechanical strength. There is evidence that when bone loss occurs in osteoporosis, trabecular bone is affected at an earlier date and more severely than cortical bone (24, 37). Interestingly, our data showed that diosgenin enhanced not only Tb.N but also cortical bone area. Further investigations are needed on the effect of diosgenin on mechanical properties.

In conclusion, the present study showed microarchitecture and morphological changes in the femur of OXYS rats, and indicated that the bone of OXYS rats reveals osteoporotic features. Our data demonstrate that extended administration of diosgenin to OXYS rats increases the BV/TV, cortical bone area, Tb.N and BMD, and decreases Tb.Sp, void volume and porosity. This study suggests that diosgenin may have potential in the treatment of aging-induced osteoporosis. By using the body surface area normalization method (48), the effective dose, 50 mg/kg/day, of diosgenin in improving bone quality in rats can be translated to the human equivalent dose of 8.1 mg/kg/day. Further studies are needed to determine the optimal and safe dose for elderly.

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**Conflicts of Interest**

The authors declare no conflicts of interest for the material in the manuscript.

**References**


Tikhonova, Ting, Kolosova, Hsu, Chen, Huang, Tseng, Hung, Kao, Amstislavskaya and Ho


Effects of Diosgenin on Bone Loss in Aging

331


