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Long-term effects of ovariectomy on the properties of bone in goats

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Abstract. Large animal models of osteoporosis are essential for osteoporosis research. However, the time required to establish an accurate osteoporosis model is unknown. Therefore, the aim of the present study was to establish a large animal model of osteoporosis in goats. In total, 14 Chinese goats were divided into an ovariectomized (OVX, n=7) or sham-operated (SHAM, n=7) group. Vertebral bodies were used to measure the bone mineral density (BMD) prior to the ovariectomy and at 24 months after the ovariectomy. In addition, the BMD of the femoral neck, femoral diaphysis and tibial diaphysis were measured 24 months postoperatively. Bone samples from the vertebral body, femoral head and femoral neck were scanned by micro-computed tomography (CT) to visualize the trabecular and cortical microstructure. Furthermore, the vertebral body, femoral head, femoral neck and tibial diaphysis were analyzed for mechanical strength. The BMD of vertebral body of the OVX group decreased significantly (P<0.01) at 24 months after the ovariectomy when compared with the baseline measurements. Micro-CT scans of the vertebral body revealed that the bone volume fraction, trabecular number, trabecular thickness and the degree of anisotropy decreased by 37.1, 36.7, 10.5 and 16.5%, respectively (P<0.01) in the OVX group when compared with the SHAM group. Additionally, the specific bone surface and trabecular spacing significantly increased by 37.7 and 62%, respectively in the OVX group (P<0.001). Cortical bone porosity in the vertebral body and femoral neck was greater in the OVX group when compared with the SHAM group (P<0.05). In addition, mechanical testing revealed a statistically significant difference between the vertebral bodies of the OVX group and the SHAM group. In conclusion, the present study demonstrated that an ovariectomy was able to induce significant osteoporosis and deterioration of mechanical properties in the bones of goats.

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Key words: osteoporosis model, Chinese mountain goats, bone mineral density, biomechanical test, estrogen deficiency

Introduction

Osteoporosis is one of the main geriatric problems worldwide, occurring frequently in postmenopausal females (1), while postmenopausal osteoporosis is a common systemic skeletal system disease occurring in middle-aged females. The functional decline of the ovaries leads to decreased estrogen levels, which triggers osteoporotic changes (2). Osteoporosis becomes a clinical issue when fragility fractures occur in weakened bones. Osteoporotic fractures of the hip and spine can lead to serious complications, including loss of mobility and independence, and even mortality. With the aging of a large portion of the worldwide population, considerable sums of money are spent managing osteoporosis and associated fractures (3,4).

A large portion of osteoporosis research is aimed at prevention, medical treatment of low bone mass and surgical treatment of osteoporotic fractures. Thus, large animal models that accurately portray human osteoporotic changes are required for these studies. In previous studies, rats (5), large osteopenic animal models, such as nonhuman primates and sheep, have been used as models (6,7). However, the time required to establish an accurate osteoporosis model is unknown (8-12). Therefore, the aim of the present study was to use locally available Chinese goats to establish a large osteopenic animal model through application of an ovariectomy (OVX), with a follow-up period of 24 months.

Materials and methods

Ovariectomized goat animal model. In total, 14 skeletally mature female Chinese mountain goats, with a body weight between 27 and 32 kg, were used for the study. The goats were randomly divided into an ovariectomized group (OVX, n=7) or a sham group (SHAM, n=7). The animals were aged 2.5 years, and skeletal maturity was determined by radiographical confirmation of the closure of the distal femoral and proximal tibia growth plates (12). The goats were housed on a farm and cared for by a qualified veterinarian during the entire study. Animal Research Ethics approval was obtained from the Research Ethics

Committee of Shanghai Ninth People's Hospital (Shanghai, China). A bilateral ovariectomy was performed under general anesthesia, using a standard aseptic surgical technique, on the seven goats in the OVX group. The same surgical procedure was performed on the seven goats in the SHAM group, without the ligation of the oviduct and the excision of the ovary. All the goats were housed for 24 months, and no goats were excluded from the study due to disease or any other reasons. The development of osteopenia was confirmed by measuring the changes in bone mineral density (BMD), bone microstructure and alterations in biomechanical properties at several skeletal sites.

Measurement of the serum estrogen concentration. A 20-ml blood sample was collected from the jugular vein of the goats at the beginning of study and prior to being euthanized with pentobarbital sodium (100 mg/kg, intravenously). After leaving the blood samples to stand for 30 min at room temperature, the blood was centrifuged for 10 min at 1,000 x g. The serum estrogen (pg/ml) concentration was measured using a radioimmunoassay (RIA) following manufacturer's instructions. A gamma counter (University of Science and Technology of China, Hefei, China) was employed and RIA kits were obtained from the Institute of Radioactive Medicine at Fudan University (#1031990; Shanghai, China).

BMD measurement by dual-energy X-ray absorptiometry (DXA). DXA (Discovery DXA System; Hologic, Bedford, MA, USA) was used to measure the BMD (g/cm^2) of the first to fourth lumber vertebrae, femoral neck, femoral diaphysis and tibial diaphysis. The BMD of the vertebral body was measured at the baseline and at 24 months after surgery. All measurements were obtained by the same individual.

Microstructure analysis by micro-computed tomography (micro-CT). Vertebral bodies from the goats were isolated by carefully removing the surrounding muscles, ligaments and intervertebral discs. The femoral head and femoral neck bone specimens were subjected to a similar treatment. All the samples were scanned using micro-CT (μCT 80; Scanco Medical AG, Brüttisellen, Switzerland) at 70 kVp, 117 mA and 20-μm slice thickness. After scanning, a constant volume of interest (VOI) centered over the specimen was selected for analysis of all the study samples. Three-dimensional (3-D) images were reconstructed based on the VOI. The bone volume fraction (BV/TV; %), trabecular thickness (Tb.Th; μm), specific bone surface (BS/BV; %), trabecular number (Tb.N; 1/mm), trabecular spacing (Tb.Sp; mm), connectivity density (Conn.D; 1/mm^3) and structure model index (SMI; %) were calculated using the Image Processing Language software, version 4.29d (Scanco Medical AG) provided with the instrument. The SMI is a topological index used to estimate the characteristic form of bone in terms of the plates and rods that compose the 3-D structure. This index assumes integer values of 0 and 3 for ideal plates and rods, respectively; for a mixed structure containing plates and rods, the value lies between 0 and 3 (13). Cortical bone porosity (%) in the vertebral body and femoral neck was also measured.

Mechanical testing. Prior to mechanical testing, samples were defrosted overnight in a 0.15-M NaCl solution at 5˚C.

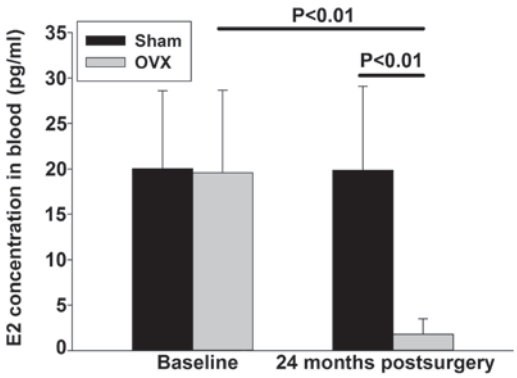

Figure 1. Level of E2 in the SHAM and OVX goats at the baseline and at 24 months after surgery. E2, estradiol; OVX, ovariectomized.

At 3 h prior to mechanical testing, the samples were removed from the refrigerator and allowed to reach room temperature. A previously described mechanical testing method was applied (8), which utilized a testing machine (model 8874; Instron Corporation, Norwood, MA, USA). The samples were kept moist with a saline-soaked gauze throughout the experiment. Prior to the cyclic loading the vertebral body, femoral head and femoral neck were preloaded to 50 N and cycled for 200 cycles between 50 and 450 N at 1 Hz for preconditioning. Immediately after cyclic loading, the sample was compressed under displacement control at a rate of 2 mm/min. During compression, load and displacement data were recorded. Each specimen was compressed in a longitudinal direction between two plates at a rate of 2 mm/min. The test concluded upon the failure of the specimen. The ultimate stress and elastic modulus were obtained from the stress-strain curve.

The frozen tibial samples were thawed prior to the three-point bending assessment. The tibia was placed on a lateral surface on two rounded support bars spaced 2.4 cm apart. A preload was applied at the medial surface of the diaphysis by lowering a third rounded bar. A constant displacement rate of 2 mm/min was applied until failure occurred.

Statistical analysis. All data are expressed as the mean ± standard deviation. The Student's t-test was used to compare the mean values between the OVX and SHAM groups, while a paired t-test was used to compare the baseline data and the data collected at 24 months after surgery. All statistical analyses were performed using a commercial software package (SPSS 16.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Serum estrogen levels in the blood. At 24 months after the ovariectomy, the serum estrogen levels in the OVX group (1.78±1.71 pg/ml) were significantly lower compared with those in the SHAM group (19.86±9.24 pg/ml). When compared with the baseline levels, the 24-month estrogen level of the SHAM group did not change significantly, whereas a statistically significant decrease in the estrogen levels of the OVX group was observed (Fig. 1).

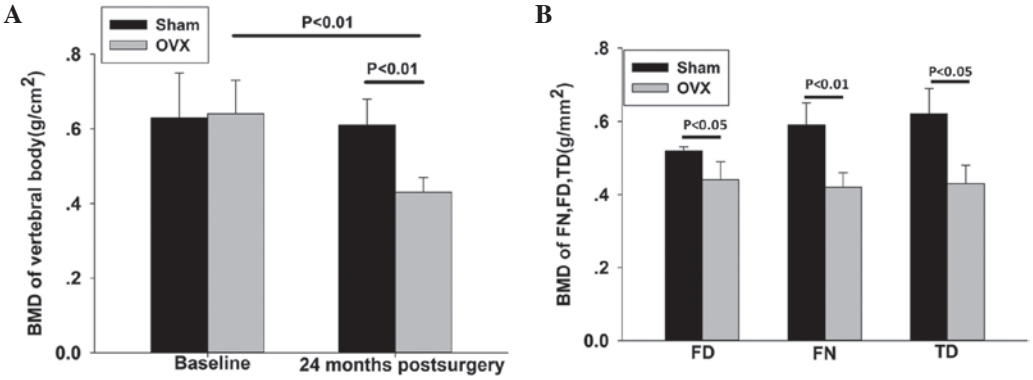

Figure 2. (A) BMD of the vertebral body at the baseline and at 24 months after surgery. (B) BMD of the FN, FD and TD at 24 months after surgery. BMD, bone mineral density; FN, femoral neck; FD, femoral diaphysis; TD, tibial diaphysis; OVX, ovariectomized.

BMD. At 24 months after the ovariectomy, when compared with the SHAM group, the OVX group exhibited a significantly reduced BMD in the lumbar spine, femoral neck, femoral diaphysis and tibial diaphysis [29.5, 28.5 (P<0.01), 23 and 28.8% (P<0.05), respectively] (Fig. 2).

Microstructure analysis by micro-CT. The trabecular microstructure of the vertebral body revealed the following results. Compared with the SHAM group, the OVX group exhibited a significantly decreased BV/TV, Tb.N, Conn.D and DA (37.1, 36.7, 17.3 and 16.5%, respectively; P<0.01), Tb.Th (0.5%; P<0.05) and significantly increased BS/BV and Tb.Sp (37.7 and 62%, respectively; P<0.01). In addition, the SMI was lower in the SHAM group compared with the OVX group; however, the difference was not statistically significant (P>0.05). The BV/TV and DA of the femoral head were significantly lower in the OVX group (15.5 and 12.7%, respectively; P<0.05) when compared with the SHAM group. BS/BV, SMI and Tb.Sp exhibited upward trends, while the remaining parameters exhibited a significant downward trend; however, no statistically significant differences were observed. The Tb.Th and Conn.D of the femoral neck were lower in the OVX group compared with the SHAM group, while the other parameters showed no significant difference (P<0.05; Fig. 3).

The cortical bone porosity of the vertebral body and femoral neck were higher in the OVX group when compared with the SHAM group (P<0.05; Fig. 3).

Mechanical testing. At 24 months after the surgery, the failure load and elastic modulus of the vertebral body were significantly lower in the OVX group compared with the SHAM group (P<0.05), with an overall decrease of ~24%. The failure load of the femoral head and femoral neck were also significantly lower in the OVX group when compared with the SHAM group, decreasing by ~30% (P<0.05) and 17% (P>0.05), respectively (Fig. 4).

Results of the three-point bending test revealed that the maximum bending load of the tibia in the OVX group was less than that in the SHAM group, with a decrease of ~7%; however, this difference was not statistically significant (P>0.05). The ultimate strength and elastic modulus in the OVX group decreased by 4 and 7%; however, the differences were not statistically significant (P>0.05; Fig. 5).

Discussion

Loss of bone mass and damaged bone microstructure significantly weakens the mechanical strength of the bone. Osteoporotic bones are prone to brittle fractures, which seriously threaten the quality of life in elderly female patients (1). The pathogenesis of osteoporosis is very complex, and research into the condition is expensive and time-consuming. Estrogen plays a fundamental role in skeletal growth and bone homeostasis in males and females. In postmenopausal females, longitudinal loss of bone mass increases in association with reduced levels of endogenous estrogen (8,14). Although marked progress has been made in understanding how estrogen deficiency causes bone loss, the mechanisms involved are complex and multifaceted (15). Therefore, selecting and establishing an ideal experimental animal model is essential for further osteoporosis research.

Sheep have been widely used as an animal model in orthopedic research (16), and as osteoporosis models in numerous studies (17-19). However, the time required to establish an accurate osteoporosis model remains inconsistent between studies. A number of previous studies (20-23) have found that bone formation in sheep continues to decline between 10 weeks and 6 months after an ovariectomy, with certain studies reporting that the BMD significantly decreases 6 months after an ovariectomy (24); however, other studies have not observed these results (25).

A number of scholars have proposed that an osteoporosis model can be established within 6 months through the use of various testing methods, such as biomechanical testing, bone histophotometry analysis or DXA (18,26-28); however, there are limitations, including a small number of samples and single specimen testing methods. Recent research has demonstrated that the BMD of the vertebral body in ovariectomized sheep exhibits a significant downward trend after 1 year; however, no statistically significant difference was identified when compared with a sham-control group (18). It has been suggested that a short-term ovariectomized sheep model should be defined as an osteopenia model, rather than an osteoporosis model (29). Lill *et al* (12) hypothesized that >1 year was required to establish an osteoporosis model using an ovariectomy alone. Short-term ovariectomies are unable to guarantee the establishment of an effective osteoporosis

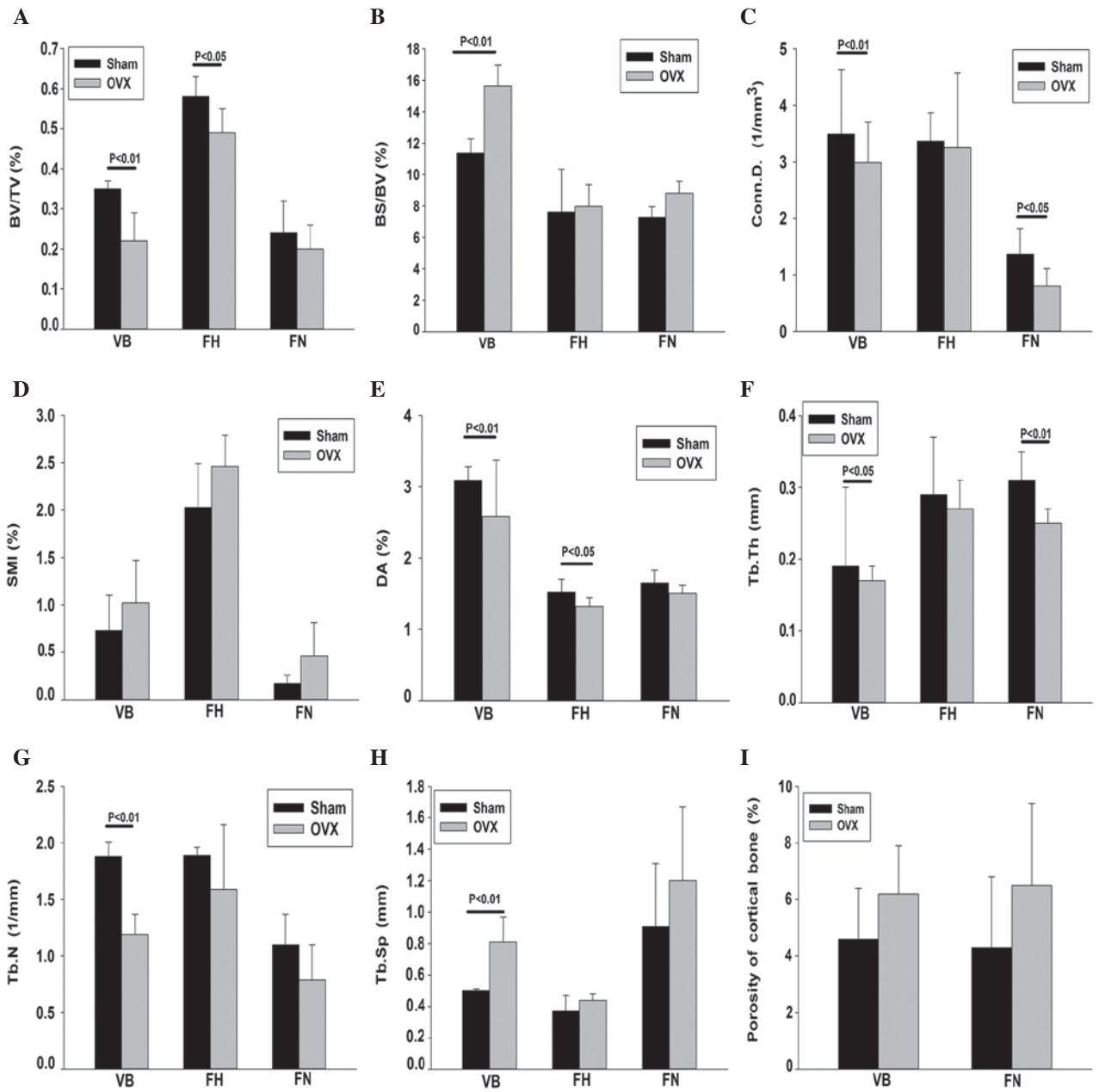

Figure 3. Trabecular microstructure parameters were measured using micro-computed tomography at 24 months after surgery. (A) BV/TV; (B) BS/BV; (C) Conn.D; (D) SMI; (E) DA; (F) Tb.Th; (G) Tb.N; (H) Tb.Sp; and (I) porosity of cortical bone. OVX, ovariectomized; VB, vertebral body; FH, femoral head; FN, femoral neck; BV/TV, bone volume fraction; BS/BV, specific bone surface; Conn.D, connectivity density; SMI, structure model index; DA, degree of anisotropy; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular spacing.

model. Therefore, the time required to establish an osteoporosis model remains controversial. In the present study, the changes in BMD, bone microstructure and biomechanical properties were analyzed in different skeletal locations in ovariectomized goats for 24 months to further clarify the time period required to establish an effective osteoporosis model.

Since sheep are most fertile in the autumn and winter, an increase in hormone levels occurs during these seasons, which can affect the result of an ovariectomy. Furthermore, the BMD of ewes is influenced by seasons and is generally reduced in the winter (30-32). To account for seasonal influences, the present study was initiated in July and finished in the same season 24 months later. Evaluation of the serum estrogen levels is essential for the assessment of the model. Johnson *et al* (25) found that an ovariectomy was unable to completely eliminate 17β-estradiol synthesis (4-6 pg/ml) in sheep. Furthermore, Karch *et al* (26) found that the normal estrogen level in sheep was ~1 pg/ml, and estrogen levels were significantly lower following an ovariectomy. The results of the present study demonstrated that 24 months after an ovariectomy, the serum estrogen levels were significantly decreased in the OVX group (1.78±1.71 pg/ml) when compared with the SHAM group (19.86±9.24 pg/ml; P<0.001).

Geusens *et al* (29) observed that at 6 months after an ovariectomy, bone mass in the femoral neck of sheep decreased by between 3 and 9%; however, no statistically significant difference was observed when compared with a

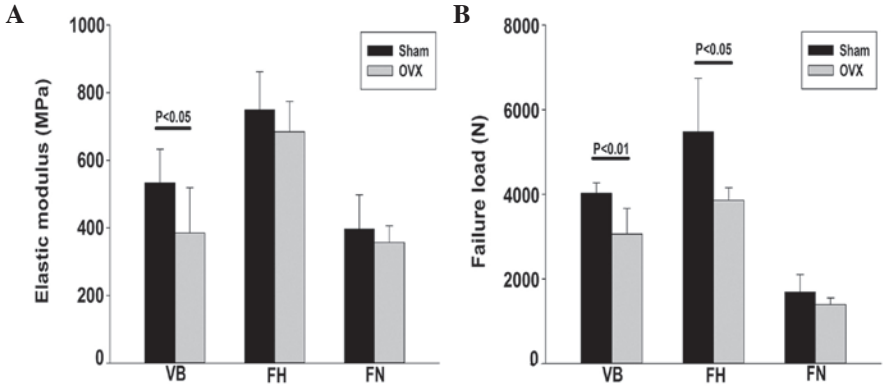

Figure 4. (A) Elastic modulus (MPa) and (B) failure load (N) in compression testing of specimens from the VB, FH and FN. OVX, ovariectomized; VB, vertebral body; FH, femoral head; FN, femoral neck.

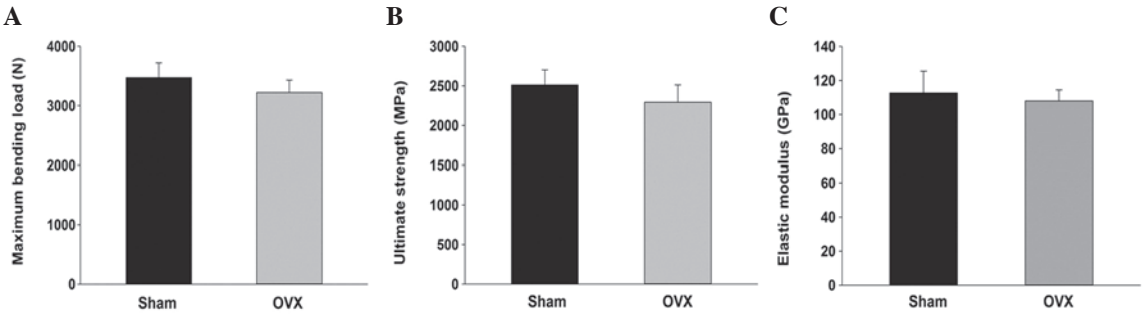

Figure 5. (A) Maximum bending load (N), (B) ultimate strength (MPa) and (C). elastic modulus in the three-point bending analysis of specimens from the tibial diaphysis. OVX, ovariectomized.

control group (33). Lill *et al* (9,12) found that following application of an ovariectomy and restricted calcium intake, the BMD of the radius in sheep decreased by 5.5%. In the study by Turner *et al* (32), the BMD was shown to decrease by 3-8%. These observations indicate that short-term estrogen deficiency can increase bone turnover by up to 10% in an ovariectomized sheep model. However, whether short-term ovariectomized sheep can effectively simulate the long-term human postmenopausal process that leads to osteoporosis is yet to be determined. A previous study demonstrated that limiting calcium and vitamin D intake can enhance the effect of bone mass loss caused by estrogen deficiency (34). Food-induced metabolic acidosis may also enhance the effects of an ovariectomy (35). In the present study, long-term estrogen deficiency (2 years) was investigated. The BMDs of the lumbar spine, femoral neck and femoral head were significantly lower in the OVX group when compared with the SHAM group, decreasing by 28.5, 28.8 and 23% (P<0.05), respectively. The BMDs of the femoral and tibial shafts were also reduced by 15.3 and 30.6%, respectively; however, the decreases were not statistically significant when compared with those in the SHAM group (P>0.05). Changes in the microstructure of the cortical bone play an important role in bone quality. Increased cortical bone porosity can lead to decreased structural integrity (35) and is closely associated with the occurrence of fractures (36). The results of the present study revealed that at 24 months after the ovariectomy, the cortical bone porosity in the vertebral body and femoral neck significantly increased by 6.2±1.7 and 6.5±2.9%, respectively. These changes may have been caused by long-term estrogen deficiency.

In addition to a reduction in bone mass, the trabecular spatial microstructure is altered during estrogen deficiency (37). Bone microstructure is strongly associated with the mechanical properties of bone (35), and the measurement of bone microstructure is an important predictor of fracture risk (13). Estrogen deficiency for two years has been shown to influence the bone microstructure primarily by affecting the SMI (36,38). However, previously used osteoporosis models have been studied for no longer than 18 months following the ovariectomy (35). In the present study, the observation time was increased to 24 months to further investigate the effect of estrogen deficiency on bone microstructure. After 24 months, the BV/TV decreased by 37.1% (P<0.05), while the DA decreased by 16.5% (P<0.001), as compared with the SHAM group. The mechanical property of bone is mostly determined by the BV/TV and DA. When the BV/TV and DA decrease, the mechanical properties also decrease. Furthermore, the axial compressive load and elastic modulus of the vertebral body were found to be significantly lower in the OVX group when compared with the SHAM group (P<0.05), with the maximum compressive load reduced by ~24%. The important characteristics of trabecular bone degeneration include changes to the rod-like structure and the appearance of perforations (40). In the present study, the trabecular bone structure became significantly rod-like at 24 months after the ovariectomy. In addition, for the vertebral body, the SMI in the OVX group increased by 40%, and

the BS/BV increased by 37.7% (P<0.001). Furthermore, the Tb.N decreased by 36.7% (P<0.01), the Conn.D increased by 17.3% (P<0.01). In the femoral head, only the BV/TV (P<0.05) and DA (P<0.05) decreased significantly in the OVX group when compared with the SHAM group; this change was proportional to a change in the maximum axial compression load. When comparing the structure of the femoral neck between the OVX and SHAM groups, only the BS/BV, Tb.Th and Conn.D were significantly different, whereas the mechanical test results revealed no statistically significant differences. The parameters of the trabecular microstructure changed unevenly, which may have been the result of adaptive changes triggered by the decrease in BMD and alterations in mechanical properties (41). By reorganizing the trabecular direction, the trabecular bone is able to maintain mechanical properties. Results of the three-point bending test revealed that the maximum tibial bending loads were lower in the OVX group when compared with the SHAM group, showing a decrease of 7%; however, the difference was not statistically significant (P>0.05). The ultimate strength in the OVX group decreased by 4% (P>0.05) when compared with the SHAM group, while the elastic modulus decreased by ~7% (P>0.05). These decreases may have been caused by the high rate of bone turnover triggered by the estrogen deficiency, which subsequently increased the porosity of the trabecular bone.

In conclusion, the present study investigated the osteoporosis outcome in goats after extending the estrogen deficiency time to 24 months. After 24 months, the OVX goats exhibited features of osteoporosis (osteopenia). Therefore, based on the results, the following hypotheses can be concluded. Firstly, 24 months after an ovariectomy in goats, a pathological state similar to osteoporosis is produced. Secondly, goats may be a suitable animal model for the study of osteoporosis. Finally, a reduction in the BMD, alterations in biomechanical properties and a change in the microstructure are associated with estrogen deficiency. These results may aid the study into the long-term effects of different therapeutic protocols for osteoporosis.

Acknowledgements

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