# Effect of simvastatin on osteogenesis of the lumbar vertebrae in ovariectomized rats

TENG LONG[1], TINGTING TANG[1], YONGQIANG HAO[1], ZHENAN ZHU[1], LITIAN SHAO[2] and MING LIU[1]

[1]Orthopedic Implant Central Lab, Orthopedic Department, Shanghai Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200011; [2]School of Mathematics and Information Science, Shanghai Lixin University of Commerce, Shanghai 201620, P.R. China

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**Abstract.** The aim of the present study was to assess the role of simvastatin on osteoporosis of the vertebrae by examining the effect of simvastatin on the osteogenesis of the lumbar vertebra in ovariectomized (OVX) rats. A total of 60 6-month-old female Sprague Dawley rats were divided into one sham group and five ovariectomized groups, consisting of four simvastatin groups and one control group. Four dosages of simvastatin (5, 10, 20 and 40 mg/kg/d) were administered by gavage for three months. L4 vertebrae were examined by dual-energy X-ray absorptiometry (DEXA) and peripheral quantitative computed tomography (pQCT) to determine the mineral apposition rate (MAR). L5 vertebrae were examined using a compression biomechanical test. Although the measurements from DEXA, pQCT and MAR, and the biomechanical parameters in the OVX + simvastatin rats were higher than those for the OVX + vehicle group, no significant differences were detected. Therefore, simvastatin may not improve osteogenesis of the lumbar vertebra in OVX rats or prevent osteoporosis of the spinal vertebrae.

## Introduction

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a liver microsomal enzyme, which are widely used for the treatment of hypercholesterolemia (1). In 1999, Mundy *et al* (2) first reported that simvastatin and lovastatin stimulated *in vivo* bone formation in rodents and increased nascent bone volume in cultures from mouse calvariae. Statins have been investigated during the development of bone anabolic agents (3,4). Simvastatin is the most widely used statin, which has been the subject of extensive research.

In recent years, simvastatin has been shown to promote *in vitro* osteoblastic differentiation and inhibit the adipocytic differentiation of pluripotent cell lines or marrow stromal cells in individuals of all ages (5-10). However, few studies have been performed to examine the *in vivo* effect of simvastatin on ovariectomized (OVX) animal models of osteoporosis, and the conclusions to date remain inconsistent (11-19). Previous studies have found that statins promote bone formation and mineralization and may inhibit bone resorption (11-15); other studies do not support the hypothesis that simvastatin is able to increase bone mineral density (BMD) and reduce the fracture risk (16-19). Potential reasons for the contradictory results among these experiments include: Large age range of the selected animal models (3-6 months); large drug dose range administered to the model animals (0.3-20 mg/kg/d), which exhibits a lack of clear criteria; and the bone sites examined varied, and included the tibia, femur and vertebrae.

Osteoporosis is a metabolic and systemic bone disease characterized by BMD and microarchitectural deterioration, which results in increased bone fragility and fracture risk (20). Fracture, which is the most severe consequence of osteoporosis, is associated with enormous costs and substantial morbidity and mortality (21); the risk of lumbar vertebral fractures in osteoporosis fractures is ~50% (21). Therefore, lumbar vertebrae were investigated in the present study.

To evaluate the effect of simvastatin on osteogenesis in the lumbar vertebrae, a postmenopausal osteoporosis model was created using 6-month-old OVX rats and various doses of simvastatin. The present findings in model rats may help to determine whether simvastatin is able to effectively prevent osteoporosis from bone loss in the axial skeleton in postmenopausal women. No analogous research has been reported in humans.

## Materials and methods

*Animals.* A total of 60 female 5-month-old Sprague Dawley rats (body weight, 382±20 g) were purchased (Sino-British SIPPR/BK Lab Animal, Ltd., Shanghai, China) and housed in pairs at 22.2˚C at 40-70% humidity with a 12:12 light/dark cycle and were allowed free access to water and food pellets

*Correspondence to:* Dr Ming Liu, Orthopedic Implant Central Lab, Orthopedic Department, Shanghai Ninth People's Hospital, School of Medicine, Shanghai Jiaotong University, 639 Zhizaoju Road, Shanghai 200011, P.R. China
E-mail: ming_li4717@aliyun.com

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consisting of a commercial natural diet (SIPPR/BK Lab Animal, Ltd.). Following 2 weeks of acclimatization to the research facility, rats were divided into six groups (n=10); one group comprised the sham group, and the remaining five groups were bilaterally OVX. From 2 weeks post-surgery, four OVX groups were treated daily with 5, 10, 20 or 40 mg/kg simvastatin (MSD Pharmaceutical Co., Ltd., Hangzhou, China) via oral gavage for 90 days. The remaining OVX group was the control group. Sham and control groups were administered a vehicle consisting of physiological saline for 90 days. Simvastatin dosage was adjusted every 2 weeks according to the weight of the rats. Rats were subcutaneously injected with 25 mg/kg tetracycline (Bio Basic Canada, Inc., Markham, ON, Canada) 15 and 5 days prior to sacrifice. All rats were sacrificed by cervical dislocation following administration of 0.4 g/kg chloral hydrate (Baomanbio, Shanghai, China) anesthesia.

*Bone densitometry*. L4 vertebrae were harvested and prepared by removing the appendix, including the vertebral lamina. Subsequently, total BMD was determined *ex vivo* using dual energy X-ray absorptiometry (DEXA; Hologic, Inc., Marlborough, MA, USA). The Hologic Discover A (version 3.3.0.1; Hologic, Inc.) small animal model scanning software used for small animal bones automatically selected a small X-ray source collimator and employed a high-resolution protocol to scan the vertebra from the proximal to the distal ends. Following scanning, all the vertebrae from the respective rats were fixed in 70% ethanol at 4˚C for subsequent analysis.

*Peripheral quantitative computed tomography (pQCT) analysis*. L4 vertebrae were removed from 70% ethanol and scanned via pQCT densitometry in increments (slices of 0.8-1 mm) with 0.09-mm resolution. The median coronal slice was chosen for detection. All vertebrae were subsequently fixed in 70% ethanol at 4˚C.

The following measurements were obtained: Total bone content (TOT_CNT), total bone mineral density (TOT_DEN), total area (TOT_A), trabecular bone content (TRAB_CNT), trabecular bone mineral density (TRAB_DEN), trabecular bone area (TRAB_A), cortical bone content (CRT_CNT), cortical bone mineral density (CRT_DEN), cortical bone area (CRT_A), cortical thickness of circumference (CRT_THK_C), periosteal circumference (PERI_C), endocortical circumference (ENDO_C), and bone strength and mechanical properties in the three axial planes X, Y, and Z (SSI X, Y, and Z).

*Bone mineral apposition rate*. Following pQCT measurement, L4 vertebrae were dehydrated and the fat was removed prior to embedding in methyl methacrylate and subsequent sectioning into 50-$\mu$m-thick sections. Sections from each specimen remained unstained for epifluorescence microscopy. The mineral apposition rate (MAR) was calculated by dividing the distance between the two labels by the interlabeling period in days.

*Bone biomechanics*. The lumbar spine was mechanically evaluated via a compression test of the L5 vertebral bodies, which were removed from the -20˚C freezer and thawed in steps. The vertebral arch and spinous and transverse processes were removed using a low-speed diamond wheel saw. Vertebral body specimens consisted of the cancellous bone core surrounded by the cortical rim. Prior measurement, bones were maintained in gauze with cold normal saline to avoid drying. Tests were performed using a Lloyd EZ20a system (Ametek GmbH, Munich, Germany). The ends of vertebrae were trimmed to provide two parallel surfaces and placed on the center of a stainless steel plate in the cranial-caudal direction. For each vertebra, a second stainless steel plate was lowered from above with a strain rate of 0.5 mm/min along its longitudinal axis until the vertebra was compressed to failure. The load-strain curve was recorded, which indicated the mechanical properties of the whole vertebral body specimen. Ultimate load (F) indicates the load-bearing capacity. Ultimate stiffness (S) indicates the maximum slope of the curve. Work to failure (W) indicates the area formed by the load-strain curve and coordinate axis. Other mechanical properties were calculated by normalizing the above mechanical data to the cross-sectional area (CSA) and height (H) of each specimen. Other properties included the ultimate stress ($\sigma = F / CSA$), Young's modulus [$E = S / (CSA / H)$], and toughness [$T = W / (CSA \times H)$]. The structural property index includes F, S and W; the material property index includes $\sigma$, E and T (Fig. 1).

*Statistical analysis*. Data were expressed as the mean ± standard deviation and analyzed by analysis of variance followed by the least significant difference test. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed with SPSS 11.5 statistical software (SPSS, Inc., Chicago, IL, USA).

## Results

*Bone densitometry*. Treatment with simvastatin resulted in a dose-related decrease in BMD with the highest values observed at 10 mg, but this difference was not significant among the groups. BMD in the OVX + simvastatin groups was higher than in the OVX + vehicle group, but the difference was not significant. Notably, although the BMD in the sham + vehicle group was higher than that of the OVX + vehicle group, the difference was not significant (Fig. 2).

*pQCT analysis*. Values of CRT_CNT, CRT_A, CRT_DEN, CRT_THK_C, TOT_DEN, TRAB_DEN and SSI (X, Y, and Z) in the OVX + vehicle group were lower than those in the sham + vehicle group. Differences in CRT_DEN and CRT_THK_C were statistically significant.

Values of TOT_CNT, TOT_A, TOT_DEN, CRT_CNT, CRT_A, CRT_DEN, TRAB_DEN, PERI_C, CRT_THK_C and SSI (X, Y, Z) in the OVX + simvastatin groups decreased in a dose-related manner and the maximum value was obtained at 10 mg simvastatin. TRAB_CNT, TRAB_A and ENDO_C in the OVX + simvastatin groups increased with dosage, but the differences were not significant among the groups. Although all these measurements were higher than those in the OVX + vehicle group, these increases were not statistically significant (Fig. 3).

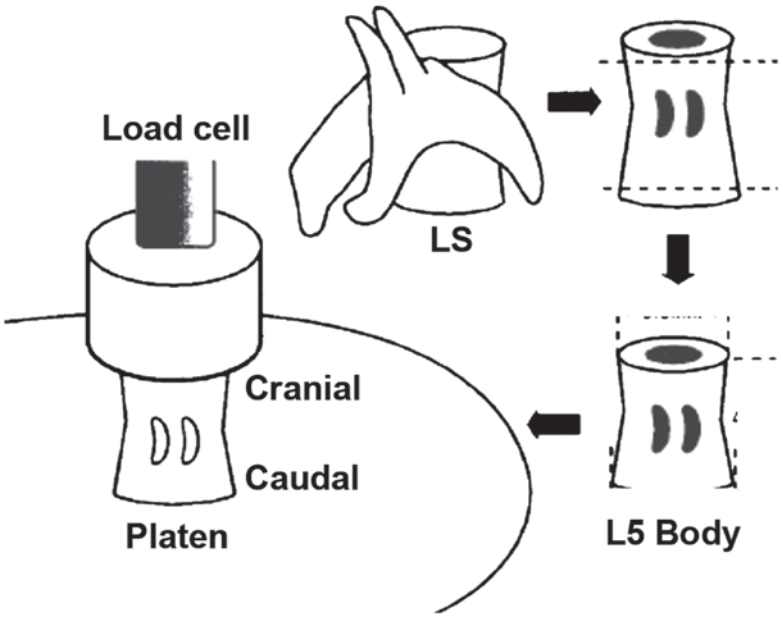


Figure 1. Schematic diagram of biomechanical experiments. LS, lumbar spine.

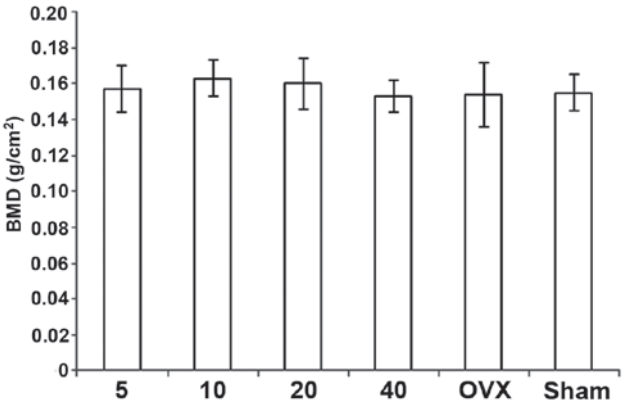


Figure 2. Bone densitometry was assessed in OVX rats treated with 5, 10, 20 and 40 mg/kg/day simvastin, and the OVX model and sham groups. OVX, ovariectomized.

*Bone MAR*. MAR was unaffected by simvastatin treatment. No significant changes were demonstrated between the OVX + simvastatin and OVX + vehicle groups (Fig. 4).

*Biomechanical measurements*. F, σ, s, E, W and T were increased in the OVX + simvastatin groups, as compared with those values in the OVX + vehicle group; however, no significant differences were detected among the groups. All of these measurements in the OVX + vehicle group were lower than those in the sham + vehicle group, and the differences in σ and T were statistically significant (Figs. 5 and 6).

## Discussion

Bone loss in OVX rats is similar to bone loss in postmenopausal women; therefore, the United States Food and Drug Administration and the World Health Organization recommend OVX rats as an animal model of postmenopausal osteoporosis (22). In previous studies, 3-month-old female rats have been used as an OVX model due to their sexual maturity (11-13,15-17); however, the skeletons of 3-month-old rats are not mature and continue to rapidly grow. The effect of anabolic drugs on osteogenesis may be unclear due to the effects of development on bone formation. Therefore, it is easy to obtain false positive or false negative errors, which influence the precise evaluation of drug efficacy in the treatment of osteogenesis and may be one of the primary reasons for inconsistent findings in previous studies (11-13,15-17). In contrast, 6-month-old rats have reached sexual maturity and peak bone mass (23). This is the optimal age for OVX rat modeling because the influence of bone development on osteogenesis can be avoided. Therefore, in the present study 6-month-old OVX rats were selected as a postmenopausal model (24).

Differences in drug tolerance between humans and animals are substantial; therefore, clinical doses of pharmacological agents must be converted into doses that can be safely administered to experimental animals to explore the novel functions of these agents. Currently, the maximum safe dose for oral simvastatin is 80 mg/day, and regular dosages include 10, 20, and 40 mg/day, which were provided in the simvastatin instructions issued by the U.S. FDA in 2007. Dosages were converted into rat gavage dosages according to the equivalent conversion of drugs between humans and animals (25,26) and the report issued by Illingworth *et al* (27), which investigated the clinical toxicity and equivalent analysis of simvastatin. Finally, simvastatin dosages administered to rats were divided into 5, 10, 20 and 40 mg/kg/day portions and administered by gavage. Therefore, the simvastatin dosages used in the present study were safe, reliable and clinically comparable to those in humans.

DEXA is primarily used to measure total BMD in animal experiments. It serves as a preliminary measurement for bone quantitative analysis and can quickly yield a macro impression of the bone quality of specimens. The present

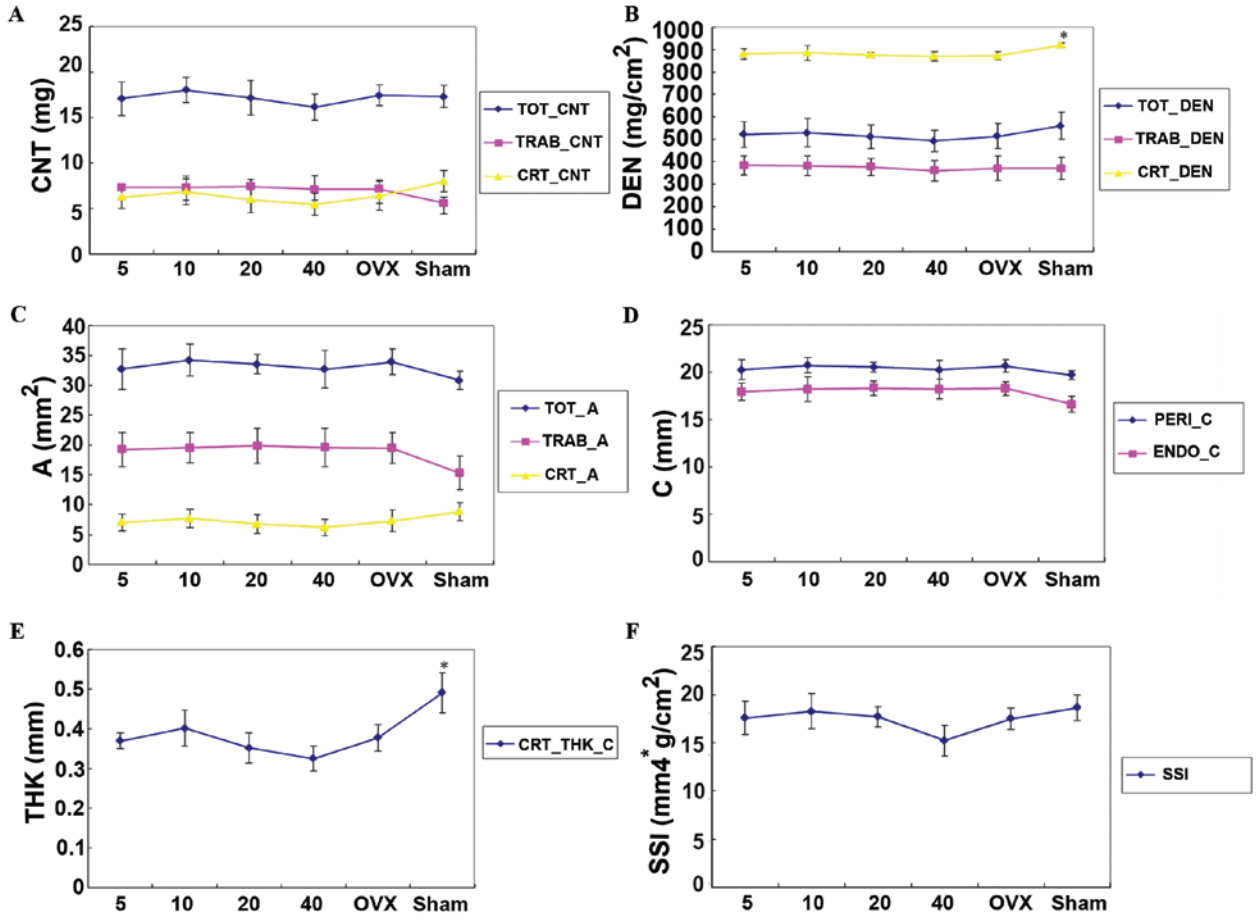


Figure 3. Analysis of the L4 vertebral body by pQCT in OVX rats treated with 5, 10, 20 and 40 mg/kg/day simvastin, and the sham and OVX model groups. *P<0.05 vs. the OVX group. (A-F) Effect of simvastatin on (A) CNT (bone content), (B) DEN (bone mineral density), (C) A (bone area), (D) C (bone circumference), (E) THK (bone thickness) and (F) SSI (bone strength and mechanical properties). pQCT peripheral quantitative computed tomography; OVX, ovariectomized; CNT, bone content; TOT, total; TRAB, trabecular; CRT, cortical; DEN, bone mineral density; A, area; C, circumference; PERI, periosteal; ENDO, endocortical; THK, thickness; CRT_THK_C, cortical thickness of the circumference; SSI, bone strength and mechanical properties.

results indicated that the total (T)-BMD of lumbar vertebrae in the OVX + simvastatin groups had a tendency to gradually increase as the simvastatin dosage was reduced and was higher than that in the OVX + vehicle group. Significant differences were not observed.

DEXA is a two-dimensional detection method. T-BMD from DEXA is predominantly determined by cancellous BMC. However, the loss rate of BMC in cancellous bone exhibits marked variation after ovariectomy depending on the skeletal site measured. It has been reported that significant cancellous bone loss in the lumbar spine typically occurs by 180 days post-ovariectomy (24,28). The post-ovariectomy period in the present study was 90 days. This may explain why the initial BMD analysis by the DEXA did not produce a positive result. pQCT is a relatively novel and more precise 3D bone measurement method than DEXA. It is advantageous as it accurately distinguishes between cortical bone and trabecular bone and separately calculate cancellous bone histomorphometry, cortical bone structure and the relevant BMC and BMD; simultaneously, it can also be used to deduce bone strength and other biomechanical indices (29-32). For peripheral bones and small animal skeletons, pQCT is more sensitive than DEXA and more beneficial in evaluating the effect of therapeutic interventions on different skeletal sites (33). Therefore, it is necessary to apply pQCT to scan the lumbar vertebrae by layers, which allows for separate assessment of the effect of simvastatin on the cortex and cancellous bones and helps to further confirm the experimental results.

The results of the present study indicated that the cortical PERI-C and ENDO-C values in the OVX + vehicle group were increased compared with the sham + vehicle group; however, the CRT-THK-C and CRT_DEN values in the OVX + vehicle group were decreased compared with the sham + vehicle group. These decreases were statistically significant. One reason for this may be that bone absorption in the endosteum prevailed over osteogenesis in the periosteum post-ovariectomy. The characteristics demonstrated were similar to the 'pencil line' or 'picture-framing' sign of the human vertebrae in postmenopausal osteoporosis, which indicates cortical thinning (34). This finding demonstrated that the CRT-THK-C and CRT_DEN values of lumbar vertebrae may be effective markers in the OVX rat model of osteoporosis when ovariectomy is performed <180 days prior and may be used as reliable indices for evaluating drug effects. The results of the present study showed that CRT-THK-C and CRT_DEN values in the OVX + simvastatin groups were increased compared with the OVX + vehicle group; however,

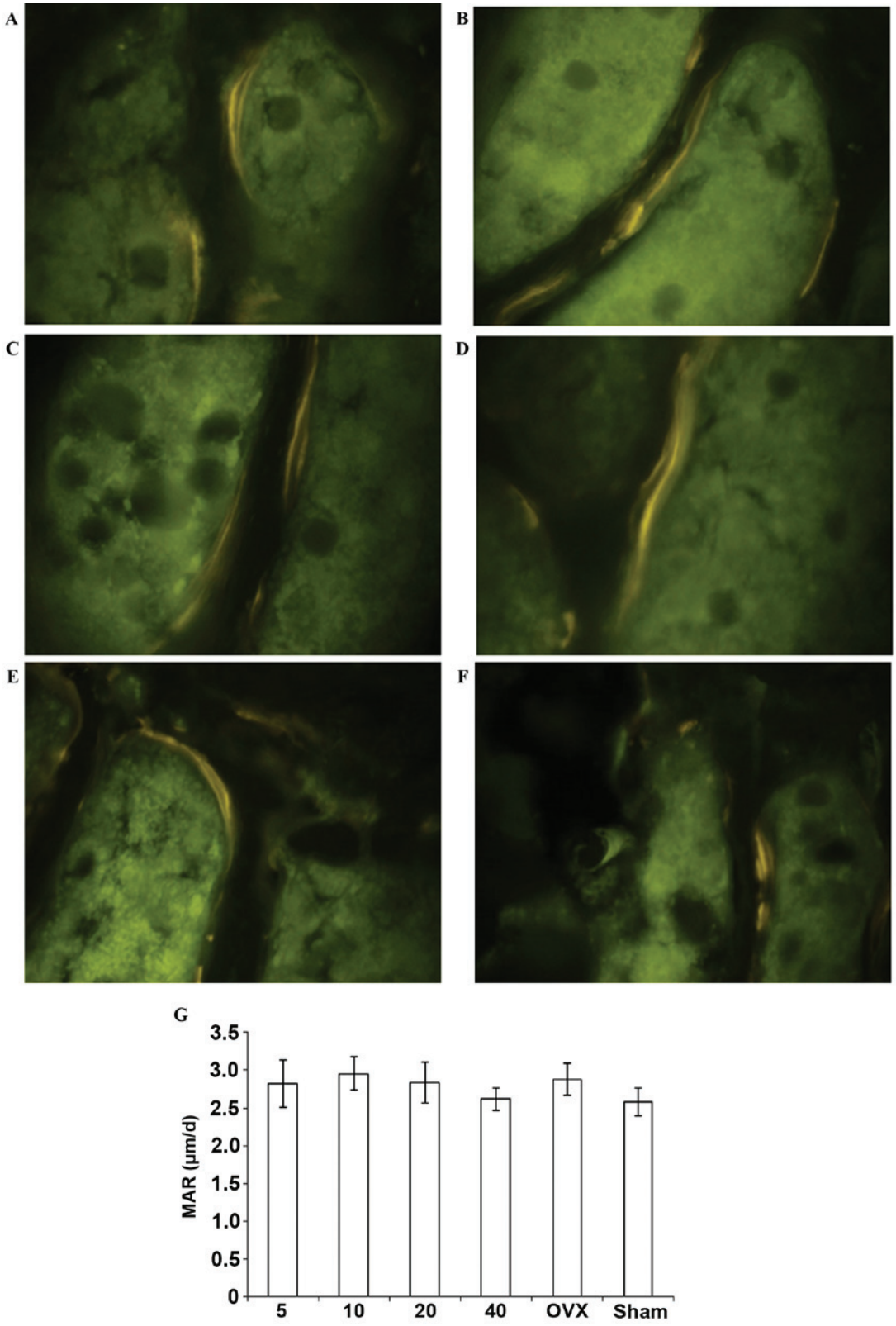


Figure 4. Tetracycline labels in epifluorescence microscopy (magnification, x400). (A) 5 mg/kg/day simvastin + ovariectomy; (B) 10 mg/kg/day simvastin + ovariectomy; (C) 20 mg/kg/day simvastin + ovariectomy; (D) 40 mg/kg/day simvastin + ovariectomy; (E) vehicle + ovariectomy; and (F) vehicle + sham. (G) Effect of simvastatin on MAR. MAR, mineral apposition rate; OVX, ovariectomized.

these differences were not significant. MAR is a dynamic measurement that complements the statistical measurements of pQCT. However, no changes were produced by simvastatin treatment.

Although simvastatin did not improve BMD of the vertebral body in OVX rats according to the results of DEXA, pQCT and MAR, skeleton biomechanical properties are the ultimate indices of the assessment of bone quality that reflect

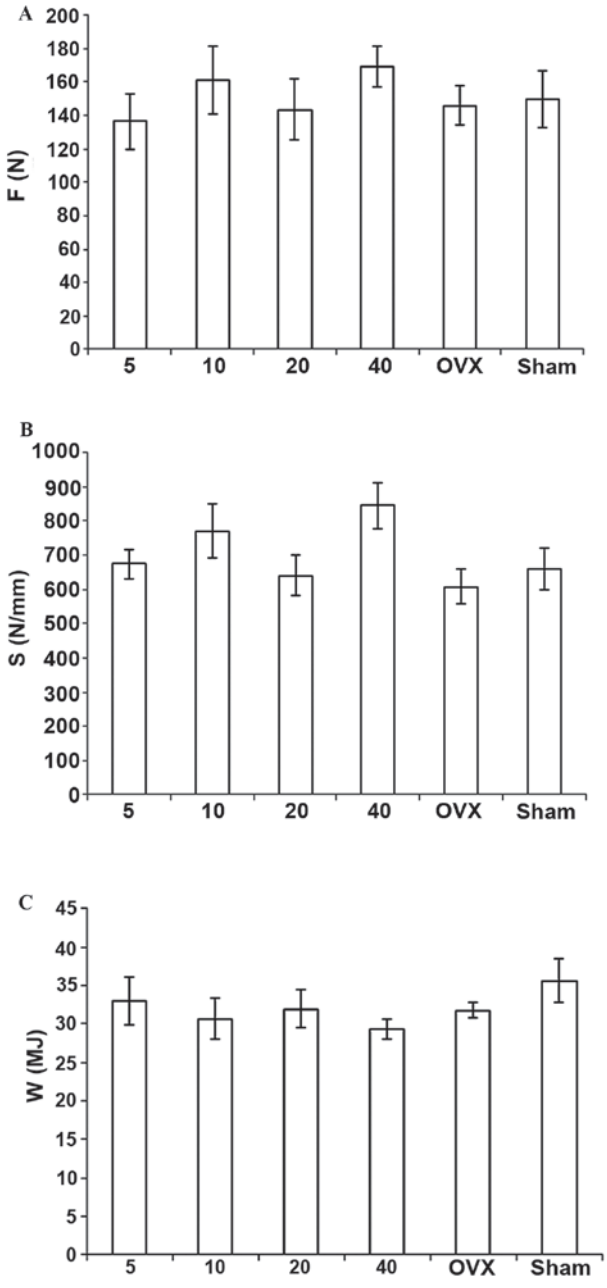


Figure 5. Effects of simvastatin on the structural biomechanical property index in OVX rats treated with 5, 10, 20 and 40 mg/kg/day simvastin, and the sham and OVX model groups. (A-C) Effect of simvastatin on (A) F (ultimate load), (B) S (ultimate stiffness) and (C) W (work to failure). F, ultimate load; S, ultimate stiffness; W, work to failure; OVX, ovariectomized.

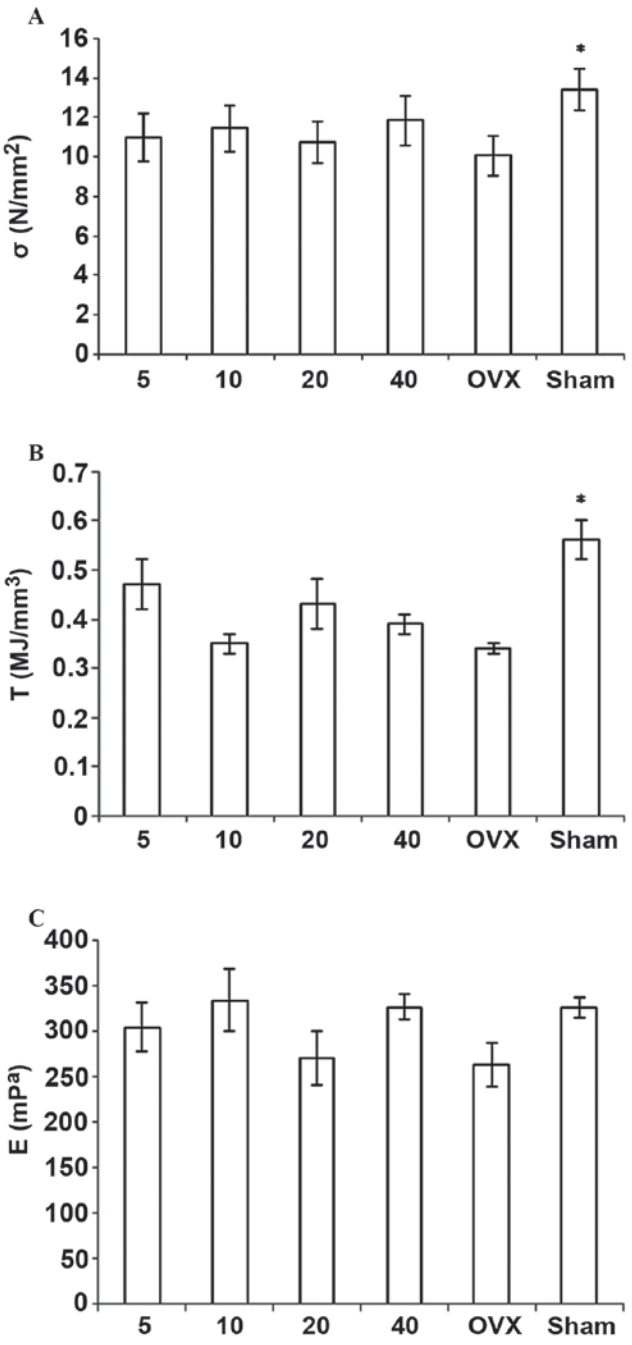


Figure 6. Effects of simvastatin on the material biomechanical property index in OVX rats. *P<0.05 vs. the OVX group. (A-C) Effect of simvastatin on (A) σ (ultimate stress), (B) T (toughness) and (C) E (Young's modulus). OVX, ovariectomized; E, Young's modulus; T, toughness; σ, ultimate stress.

the changes in bone structure (35). BMD may only represent 60-80% of bone mechanical strength, and the accuracy of BMD is <70% for the evaluation of the effects of drugs on osteoporosis. However, if the BMD value is combined with bone biomechanics, the accuracy of the assessment of the effects of drugs on osteoporosis may be as high as 90% (36,37). Therefore, it is necessary to use biomechanical analysis as the final measurement in the evaluation of the effect of drugs on osteoporosis.

Bone biomechanical characteristics can be divided into two parts: Structural mechanical properties and material mechanical properties (38). Structural mechanical properties include F, S and W, which reflect the changes in bone architecture and are predominantly affected by the geometric shape and size of the bone (36,39). Material mechanical properties include σ, E, and T, which represent bone mechanical characteristics that are not relevant to the geometrical shape of the bone, are calculated by the appropriate formula, and include bone area, bone height, bone diameter, structure mechanical index, and calibration factors (cross sectional moment of inertia) (36,37). These results showed that all of the biomechanical measurements in the OVX + simvastatin groups were increased compared with the OVX + vehicle group; however, statistically significant differences were not observed. Therefore, the biomechanical

result was also consistent with the DEXA, pQCT and MAR results.

The findings of the present study indicate that simvastatin did not promote osteogenesis of the lumbar vertebrae in OVX rats, suggesting it has no effect on the prevention and treatment of postmenopausal osteoporosis in the vertebrae.

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