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Osteoporosis influences the late period of fracture healing in a rat model prepared by ovariectomy and low calcium diet

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Abstract

To elucidate the influence of osteoporosis on the fracture healing, we produced a rat osteoporosis model by ovariectomy and by maintaining a low calcium diet; and monitored the healing process radiographically, histologically, and biomechanically for 12 weeks. Radiologic, histologic and biomechanical findings of the fracture areas 6 weeks after making the fractures were almost identical in both the osteoporosis group and the control group. However, 12 weeks after making the fractures, newly generated bones in the osteoporosis group showed histological osteoporotic changes and their bone mineral density on the fracture site decreased. These findings show that estrogen-deficient and low calcium conditions greatly affect the bone in the later period of the healing process, but do not affect remarkably the early healing period. This is clinically important when we consider fracture treatments for patients with osteoporosis due to menopause. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Osteoporosis easily causes bone fractures, and current therapies aim at the prevention of bone fractures. However, in a fractured osteoporotic bone, it is important to know whether healing ability is influenced by osteoporosis. Several studies have been conducted on the influence of osteoporosis on fracture healing [1,4–6], but its long-term influence is not well understood. For example, Lindholm [5] prepared a bone fracture model using rats fed with a low calcium diet, and reported that bone mineral density in the repaired tibial bone was as low as in the nonfractured bones. Blythe and Buchsbaum [1] and Langeland [4] examined the tensile strength of fractured tibial bone in female rats 5 or 2 weeks after producing fractures, and reported the strength did not differ significantly between ovariectomized rats and normal controls. Nordsletten et al. [6] produced tibial fractures in rats with and without sciatic neurectomy and immobilized the lower extremities with casts. They examined the fracture healing 25 days later, and found that callus formation was accelerated and bone mineral density was high in the neurectomy legs, but tensile strength did not differ significantly between the legs with sciatic neurectomy and those without. These are important findings about fracture healing in osteoportic bone, though still not sufficient to fully elucidate the influence of osteoporosis on fracture healing. In addition, the long-term healing process has not been investigated.

In the present study, we produced a rat osteoporosis

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model by performing ovariectomy and by maintaining a low calcium diet; and radiographically, histologically and biomechanically examined the healing process of fractured bone in this osteoporotic model for 12 weeks.

2. Methods

Subject animals were 90 30-week-old Wister female rats and their mean body weight was 450 g. This study was approved by the Experimental Animal Committee and performed according to the rules and regulations regarding animal research of Kyoto Prefectural University of Medicine.

2.1. Rat osteoporotic model

In this part of the study, 25 rats were used. Fifteen rats underwent bilateral ovariectomy after intraperitoneal injection of ketamine hydrochloride 40 mg/kg (Ketalar®, Sankyo, Japan), and they were given special solid food (a low calcium diet containing 0.1% Ca and 0.5% P, Oriental Bio-Service, Japan) and tap water (OVX group). The other 10 rats received a sham-operation under the same anesthesia, and were fed with a normal diet (non-OVX group). Immediately before, and 4, 8 and 12 weeks after the ovariectomy or sham-surgery, we measured body weight and bone mineral density of the body by using dual-energy X-ray absorptiometry (DXA; XR-26, Norland, USA) [7], and confirmed the usefulness of the OVX group as an osteoporosis model.

2.2. Fracture preparation

The osteoporosis model was prepared in 40 rats (including the 15 OVX group rats) by ovariectomy at 30 weeks of age and by maintaining a subsequent low calcium diet for 12 weeks. At 42 weeks of age, the rats were anesthetized with ketamine hydrochloride, a lateral longitudinal incision was made on the unilateral femur to produce a transverse fracture on the diaphysis, and they were fed with a low calcium diet throughout the study period (OVX + F group). As the controls, the same fracture was produced in another 40 rats at 42 weeks of age without ovariectomy and without a low calcium diet (control-F group). To stabilize the fracture site of all animals, 1.2 mm of stainless steel wire was inserted from the distal part of the femur to the medullary space.

Thirty rats each from the OVX + F group and the control-F group were used for examinations (2.2.1.–2.2.3.); and the remaining 10 rats were used for examination (2.2.4.).

2.2.1. Soft X-ray evaluation

Fractured femurs of the OVX + F and control-F groups were extracted 6 weeks ($n = 15$) and 12 weeks ($n = 15$) after making the fractures, and soft X-ray images (distance: 50 cm, voltage: 30 kVp, electric current: 3 mA, time: 50 s) were obtained. On the images, continuity and the maximum width of the callus were evaluated as described elsewhere [6].

2.2.2. Measurement of bone mineral density of the fracture site

After soft X-ray evaluation, an area of 1.5 cm height and 2.0 cm width was set centered on each fracture site, and the bone mineral density in this area was measured with a DXA method (small subject mode).

2.2.3. Biomechanical evaluation

After measuring the bone mineral density, the maximum tensile strength (maximum torque, N) was measured on the same femurs by the static tension test using a universal tester (Shimadzu Autograph AG500B, Shimadzu Corp, Japan). Both ends of the extracted femur were embedded and fixed in a metal cylinder by using methacrylate, and the cross-head speed was set at 0.5 mm/min.

2.2.4. Histologic evaluation

Ten femurs each were obtained from the OVX + F group and the control-F group 6 weeks ($n = 5$) and 12 weeks ($n = 5$) after making the fractures, and the tissue blocks of the fracture areas were fixed in 10% formalin for 24 h, decalcified in EDTA, and embedded into paraffin. The block was then cut into 4-μm slices along the sagittal plane passing through the longitudinal axis of the femur. They were stained with hematoxylin and eosin (HE), and examined for morphology under a light microscope.

Significant differences in the mean values between the OVX group and the non-OVX group, and the OVX + F group and the control-F group, were examined by using Student's *t*-test.

3. Results

3.1. Body weight, and bone mineral density of the body

Immediately, 4, 8 and 12 weeks after either the ovariectomy or the sham-surgery, body weight did not differ significantly between the OVX group and the non-OVX group.

Bone mineral density of the body in the OVX group was 0.167 ± 0.011 g/cm^2 at 8 weeks after ovariectomy and 0.159 ± 0.011 g/cm^2 at 12 weeks after ovariectomy; whereas in the non-OVX group, it was 0.181 ± 0.005 g/cm^2 and 0.177 ± 0.008 g/cm^2, respectively. For both

Table 1
Bone mineral density of the body after ovariectomy, measured by dual-energy X-ray absorptiometry. OVX group: rats received ovariectomy and low calcium diet. Non-OVX group: rats received a sham-operation and normal diet. *$p < 0.01$ (OVX vs. non-OVX)

Weeks	Bone mineral density (g/cm^2)	
	OVX group ($n=15$)	non-OVX group ($n=10$)
0	0.168 ± 0.016	0.168 ± 0.013
4	0.166 ± 0.013	0.167 ± 0.011
8*	0.167 ± 0.011	0.181 ± 0.005
12*	0.159 ± 0.011	0.177 ± 0.008

the 8 and 12 week results, the density was significantly lower in the OVX group than in the non-OVX group ($p < 0.01$, Table 1).

3.2. Soft X-ray analysis for the fractured femurs

Formation of the callus which bridges fractured bones was observed in the OVX + F group and the control-F group at 6 and 12 weeks after making fractures. Pseudoarthrosis was not observed in any animals. Calculated callus width at 6 weeks after making fractures was 6.13 ± 0.13 mm in the OVX + F group and 5.84 ± 0.82 mm in the control-F group; and at 12 weeks after making fractures, it was 7.29 ± 1.06 mm in the OVX + F group and 6.33 ± 0.42 in the control-F group. There was no significant group difference at this time point (Table 2).

3.3. Bone mineral density in the fractured area of the femur

Six weeks after making the fractures, bone mineral density on the fracture site of the femur was 0.120 ± 0.018 g/cm^2 in the OVX + F group and 0.135 ± 0.025 g/cm^2 in the control-F group; and there was no significant group difference (Table 3). On the other hand, 12 weeks after making the fractures, bone mineral density was 0.103 ± 0.015 g/cm^2 in the OVX + F group, and this was significantly lower than the density, 0.142 ± 0.023 g/cm^2, in the control-F group ($p < 0.01$).

3.4. Tensile strength of the femur

On the fractured femur, the maximum tensile strength at 6 weeks after making the fractures was 2.85 ± 1.98 N in the OVX + F group and 2.74 ± 2.37 N in the control-F group; and at 12 weeks after making the fractures, it was 4.29 ± 2.15 in the OVX + F group and 6.30 ± 3.46 N in the control-F group (Table 4). The maximum torque of the OVX + F group was lower than that of the control-F group at 12 weeks after making the fractures.

3.5. Histological findings

At 6 weeks after making the fractures, clear formations of the callus and periosteal bone at the fracture areas were found in both the OVX + F group and the control-F group. At a distal area from the fracture, the wire surface in the marrow space was surrounded with fibrous granulation tissues and some irregular-shaped bone trabeculae. At this time point, there were no group differences in histologic findings (data not shown).

In the control-F group at 12 weeks after making the fractures, the wire surface was covered with relatively thick fibrous tissues, and it was surrounded with the irregular-shaped newly formed bone trabeculae (Fig. 1A). The endosteal surface of the cortical bone was lined with appositional layers of lamellar bone (Fig. 1C). The endosteal surface was lined with flat cells. On the other hand, in the OVX + F group, the wire was surrounded with a thin fibrous tissue layer and a thin lamellar bone sheath (Fig. 1B). Clusters of osteoclasts with resorptive lacunae were present on the endosteal surface of the cortical bone (Fig. 1D). The newly formed lamellar bone layer was partially present on the endosteal surface. At this time point, the histological findings were markedly different between the groups.

4. Discussion

This study examined the influence of osteoporosis on fracture healing in a rat osteoporosis model which

Table 2
Callus width measured by soft X-ray analysis. OVX + F group: fracture was made after producing osteoporosis by ovariectomy and low calcium diet. Control-F group: fracture was made on rats without ovariectomy or low calcium diet

Weeks after making fractures	Callus width (mm)	
	OVX + F group ($n=40$)	control-F group ($n=40$)
6	6.13 ± 0.13	5.84 ± 0.82
12	7.29 ± 1.06	6.33 ± 0.42

Table 3
Bone mineral density in the fracture area. OVX + F group: fracture was made after producing osteoporosis by ovariectomy and low calcium diet. Control-F group: fracture was made on rats without ovariectomy or low calcium diet. $^{*}p < 0.01$ (OVX + F vs. control-F)

Weeks after making fractures	Bone mineral density (g/cm^2)	
	OVX + F group ($n = 40$)	control-F group ($n = 40$)
6	0.120 ± 0.018	0.135 ± 0.025
12*	0.103 ± 0.015	0.142 ± 0.023

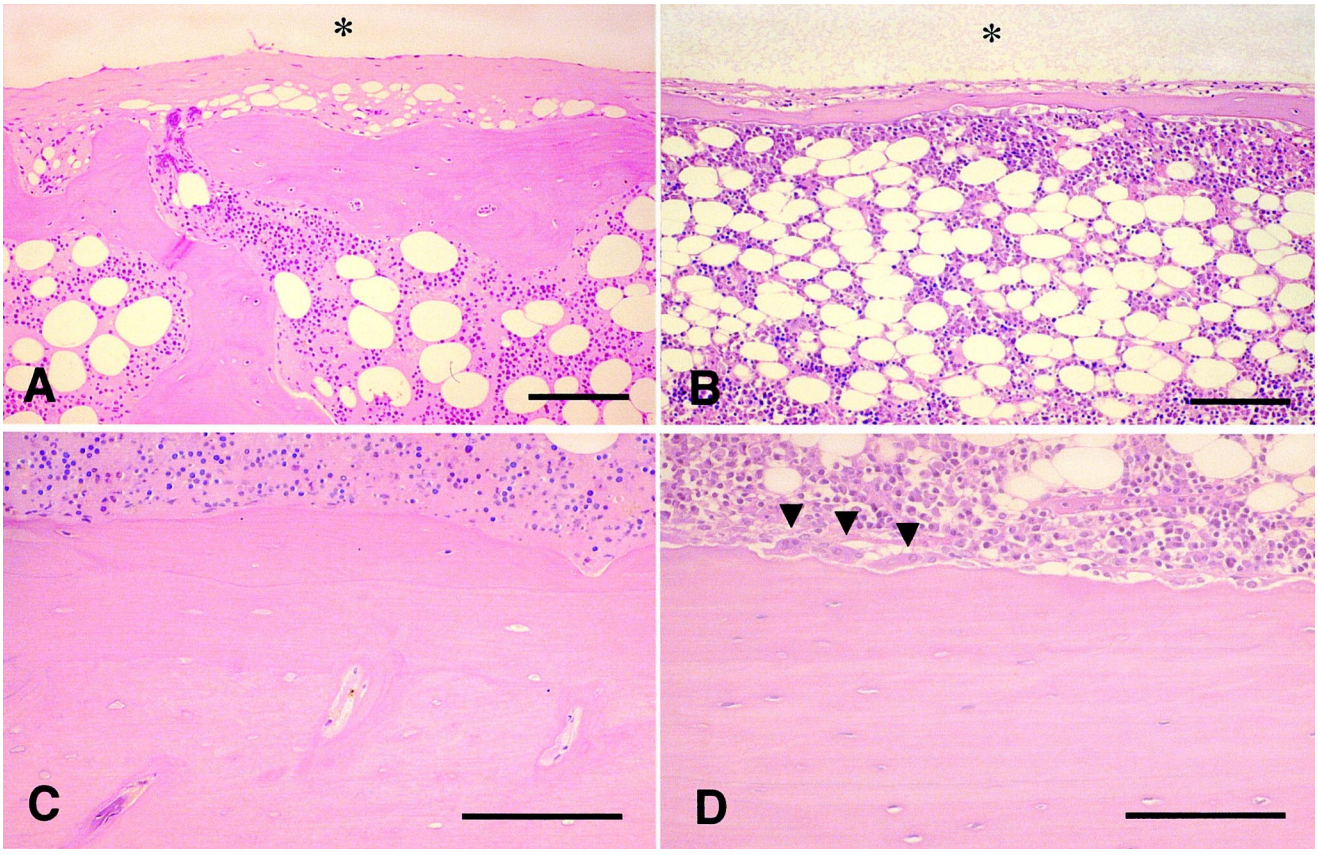

Fig. 1. Histologic findings at 12 weeks after making fracture. The newly formed bone on the wire, which location is shown with *, was relatively thick and there were irregular-shaped bone trabeculae in the control-F group (A), while there was a thin lamellar bone sheath on the wire in the OVX + F group (B). Endosteal surface of the cortical bone was covered with newly formed lamellar bone layers in the control-F group (C), while clusters of osteoclasts (arrow heads) with lacunae formation were occasionally present in the OVX + F group (D). HE staining. Scale bar: 0.5 mm.

Table 4
Tensile strength of fractured femurs. OVX + F group: fracture was made after producing oestoporosis by ovariectomy and low calcium diet. control-F group: fracture was made on rats without ovariectomy or low calcium diet

Weeks after making fractures	Maximum torque (N)	
	OVX + F group ($n = 40$)	control-F group ($n = 40$)
6	2.85 ± 1.98	2.74 ± 2.37
12	4.92 ± 2.15	6.30 ± 3.46

was prepared by ovariectomy and by maintaining a low calcium diet [8,10]. Eight and 12 weeks after ovariectomy, bone mineral density of the model rats became significantly lower than the control rats. This shows that our rat osteoporosis model is useful to evaluate the healing process of fractured bone under osteoporotic conditions.

Femur fracture was produced 12 weeks after ovariectomy, and the healing process was monitored for the subsequent 12 weeks. As a result, the healing rate in the 12th week after making the fractures was 100% in all animals. This shows that bone fracture itself can be healed even under such conditions as low bone mineral density, low estrogen and low calcium.

The healing process of fractures can be classified into three phases, i.e. inflammation phase, reparative phase and remodeling phase [2]. In our model, conditions in the 6th week after making the fractures corresponded to the reparative phase, when cartilaginous callus is the major component of healing. Our conditions at the 12th week after making the fractures corresponded to the remodeling phase.

Bone mineral density in the fracture areas at 6 weeks after making the fractures did not differ significantly between the OVX + F group and the control-F group. In addition, in this phase, there were no group differences in respect to the maximum callus width calculated on the soft X-ray images, tensile strength and histologic findings on the fracture area. By using ovariectomized rats, Blythe and Buchsbaum [1] examined the tensile strength of tibial fracture areas 5 weeks after making fractures and found there were no significant differences between the experimental rats and normal rats without ovariectomy. Langeland [4] investigated tensile strength of tibial fractures 2 weeks after making fractures and also found no significant differences between the experimental animals and the controls without ovariectomy. Furthermore, Cesnjaj et al. [3] examined bone induction ability in an ovariectomized rat model by transplanting decalcified matrix prepared from normal rats to the model rats and they reported that the bone induction ability of decalcified matrix in the model did not change. Our findings at the 6th week after making fractures agreed with these previous findings.

On the other hand, 12 weeks after making the fractures, bone mineral density in newly generated bones of the OVX + F group was significantly lower than the control-F group. Histologically, there was a remarkable difference between the groups. The newly formed bone of the OVX + F group showed osteoporotic changes. These findings show that under such conditions as produced by ovariectomy and a low calcium diet, osteoporotic changes occur not only in the existing bones but also in the newly generated bones and these changes occur in the period between 6 and 12 weeks after making the fractures. In addition, tensile strength in the fractured femurs of the OVX + F group was lower than that of the control-F group even though the difference was not significant. Wronski et al. [9] showed that osteoporotic changes due to estrogen deficiency were associated with the imbalance between bone regeneration and bone absorption. Kalu et al. [11] showed calcium restriction in the OVX group animals caused an increase of bone sensitivity to OVX, and resulted in a greater bone loss in a relatively short period. In the present study, because it is quite difficult to distinguish the effect of estrogen from that of the calcium restriction in our animal model, we did not separate them, but examined the two as a combined effect on osteoporotic changes in newly formed bones. Though we were unable to discuss each effect separately, our findings are useful when we consider the long-term effects of osteoporosis on the quality of bone which is repaired after a fracture.

Six weeks after making the fractures, radiologic, histologic and biomechanical findings on the fracture areas in the OVX + F group which received ovariectomy and a low calcium diet, and in the control-F group were almost identical. At 12 weeks after making the fractures, histological osteoporotic changes were observed on newly generated bones in the OVX + F group, and bone mineral density of these new bones decreased. These findings show that estrogen-deficient and low calcium conditions do not markedly affect the early healing process, but largely affect the bones in the later period of the healing process. This point is clinically important when we consider treatments for fractures in patients with osteoporosis due to menopause.

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