Effect of Dietary Calcium on Spinal Bone Fusion in an Ovariectomized Rat Model

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Objective: To evaluate the effect of calcium supplementation on spinal bone fusion in ovariectomized (OVX) rats.

Methods: Sixteen female Sprague Dawley rats underwent bilateral ovariectomy at 12 weeks of age to induce osteoporosis and were randomly assigned to two groups: control group (n=8) and calcium-supplemented group (OVX-Ca, n=8). Autologous spinal bone fusion surgery was performed on both groups 8 weeks later. After fusion surgery, the OVX-Ca group was supplemented with calcium in drinking water for 8 weeks. Blood was obtained 4 and 8 weeks after fusion surgery. Eight weeks after fusion surgery, the rats were euthanized and the L4-5 spine removed. Bone fusion status and fusion volume were evaluated by manual palpation and three-dimensional computed tomography.

Results: The mean fusion volume in the L4-5 spine was significantly greater in the OVX-Ca group (71.80±8.24 mm³) than in controls (35.34±8.24 mm³) (p<0.01). The level of osteocalcin, a bone formation marker, was higher in OVX-Ca rats than in controls 4 weeks (610.08±10.41 vs. 551.61±12.34 ng/mL) and 8 weeks (502.05±22.76 ng/mL) after fusion surgery (p<0.05). The level of C-terminal telopeptide fragment of type I collagen, a bone resorption marker, was significantly lower in OVX-Ca rats than in controls 4 weeks (77.07±12.57 vs. 101.75±7.20 ng/mL) and 8 weeks (69.58±2.45 vs. 77.15±4.10 ng/mL) after fusion surgery (p<0.05). A mechanical strength test showed that the L4-5 vertebrae in the OVX-Ca group withstood a 50% higher maximal load compared with the controls (p<0.01).

Conclusion: Dietary calcium given to OVX rats after lumbar fusion surgery improved fusion volume and mechanical strength in an ovariectomized rat model.

Key Words: Osteoporosis · Ovariectomized rat · Calcium · Spinal bone fusion.

INTRODUCTION

Osteoporosis is a bone metabolic disease characterized by low bone mineral density, which leads to bone fragility and an increased risk of fracture. Osteoporosis causes a difficult environment for spinal bone fusion surgery in patients with a degenerative spinal disease or traumatic fracture. In senile or postmenopausal osteoporosis patients, spinal bone fusion surgery is associated with complications such as nonunion, malunion, pseudoarthrosis, and instrument failure. Due to these complications, alternative treatment or improvements with surgical technique are currently under investigation.

Recently, a large number of osteoporotic patients with bone fragility have been treated with antiresorptive drugs such as bisphosphonates and selective estrogen receptor modulators after spinal arthrodesis. However, there exists debate about the actual effects of these drugs and their use is limited to patients with osteopenia or underestimated osteoporosis due to bony spur or calcified structures around bone. Recent studies have reported on the positive effect of calcium supplementation on fracture healing of osteoporotic bone in ovariectomized rats. Calcium is required for normal skeletal growth and mineralization, and plays an important role in regulating bone remodeling and bone mass. However, to our knowledge, no controlled studies have been undertaken to determine whether increased calcium intake promotes bone fusion volume and mechanical strength in spinal fusion surgery.

We evaluated the effect of calcium supplementation on spinal...
bone fusion in ovariectomized rats. We measured the fusion volume using three-dimensional microcomputed tomography (3D-μCT), the levels of biochemical bone metabolism markers (osteocalcin, C-terminal telopeptide fragment of type I collagen (CTX)), and bone mechanical strength in ovariectomized rats.

MATERIALS AND METHODS

Experimental design

All animal experiments were performed in accordance with the National Institute of Health guidelines on animal care and were approved by the Institutional Animal Care Committee. Sixteen female Sprague Dawley rats aged 11 weeks were purchased from Samtako Bio Inc. (Osan, Korea) and acclimated to the laboratory conditions for 1 week before the experiment. The rats were housed in an air-conditioned room with a 12-hrs light/dark cycle at a room temperature of 22±2°C and humidity of 45-65%, and given free access to food and tap water. At 12 weeks of age, they underwent bilateral ovariectomy to induce osteoporosis. We used the double dorsolateral approach described in detail by Park et al.34). Initially, the rats were anesthetized intraperitoneally with a mixture of xylazine (10 mg/kg) and ketamine (60 mg/kg). After bilateral skin incision just medial to the most bulging part of the back, the peritoneal cavity was entered by dissecting through the muscles to reveal the adipose tissue surrounding the ovary. After the surgery, muscle, fascia, and skin were sutured using silk sutures.

Throughout the experimental period, the body weight was monitored once a week. Blood samples were obtained by cardiac puncture at baseline, 8 weeks after the ovariectomy, and 4 and 8 weeks after spinal bone fusion surgery (represented in Fig. 1 as 8+4 and 8+8 weeks). Serum was separated by centrifugation at 15000g and stored at -80°C for later measurement of bone metabolic marker levels.

The entire experimental schedule is represented in Fig. 1.

Spinal posterolateral bone fusion model

Anesthesia was induced with ketamine intraperitoneally (90 mg/kg i.p.). After the surgical site was shaved and prepared, the rat was placed prone on the operating table. An L4-5 posterolateral intertransverse and translaminar arthrodesis was performed as described by Abe et al.1). Briefly, a posterior midline incision was made over the lumbar spine. The laminar-transverse space of the L4 and L5 vertebrae was exposed by splitting the back muscles (modified Wiltse approach). Once exposed, the laminar-transverse space of the L4-5 vertebrae was decoricated with an electric burr until a blush of cancellous bone was observed. About 0.3 g of autologous bone was harvested from both iliac crests through fascial incisions. The harvested iliac bone was morselized and implanted bilaterally on the decoricated fusion beds to bridge the L4 and L5 interlaminar and intertransverse space. Postoperative antibiotics were given subcutaneously (gentamicin, 0.5 mg/kg).

Calcium supplementation

After fusion surgery, the rats were divided into two groups: a control group and a calcium-supplemented group (OVX-Ca group). Calcium was supplemented by mixing 1% lactic acid hemicalcium salt (Sigma, St. Louis, MO, USA) in the drinking water, which was provided ad libitum. This method of calcium supplementation does not stress the rats compared with oral gavage or intramuscular injection and has been shown to be optimal for bone growth37). We measured serum calcium concentration to ensure adequate calcium intake.

Fusion assessment

Eight weeks after fusion surgery, the rats were sacrificed and the L4-5 segments were removed. Fusion at the bone-grafted segment was assessed by manual palpation10,22) and 3D-μCT scanning at the L4-5 segment, as described by Abe et al.1). Briefly, the harvested lumbar spine was palpated gently, and lateral side-bending motion at the L4-5 level was compared with the motion at the adjacent levels above (L3-4) and below (L5-6). Two independent neurosurgeons who were blinded to the grouping of animals tested the stability of lateral side-bending motion at the operative spinal segment.

μCT scanning (eXplore Locus SP, GE Healthcare, USA) of the lumber spine was performed under consistent conditions. The formation of bridging bone between the L4-5 laminar-transverse space and consolidation of the grafted...
bone were evaluated. Based on the results of manual palpation testing and 3D-µCT scanning, each specimen was classified as a solid union when no motion was observed by the two neurosurgeons and bony continuity between the L4 and L5 laminar-transverse vertebrae was observed and as a nonunion when motion was detected or discontinuity was observed[22-41).

Fusion volume analysis
Bone morphometric parameters of the fusion mass in the L4 and L5 vertebrae were assessed using µCT. The scanning protocol was set at X-ray energy settings of 80 kV and 80 µA, and the sample was scanned over one entire 360° rotation with an exposure time of 3000 ms/frame. An isotropic resolution of 15-40 µm voxel size that displayed the microstructure of the rat lumbar vertebrae was selected, and the angle of increment around the sample was set to 0.40, which resulted in the acquisition of 900 two-dimensional (2D) images. A modified Feldkamp cone-beam algorithm was used to reconstruct the 2D projections into a 3D volume.

For the bone analysis, bone tissue from the region of the fusion mass in the L4-5 vertebrae was selected as the region of interest, and image information was obtained based on the automatic domain value yielded by the computer. Bone volume fraction was applied to perform quantitative analysis using software provided with the 2.0+ ABA Microview of the µCT system.

Serum markers of bone metabolism
The serum level of osteocalcin, a sensitive biomarker of bone formation, was estimated using an osteocalcin EIA kit (Biomedical Technologies, Stoughton, MA, USA). The effects of the treatments on bone resorption were evaluated using a RatLaps ELISA kit (Nordic Bioscience Diagnostics, Herlev, Denmark) to detect CTX generated by osteoclasts.

Mechanical strength test
Mechanical spinal strength was assessed using a three-point bending test[2-13]. Each bone was positioned on the two lower supports of the anvil of a Universal Testing Machine (Instron 4202; Instron, Canton, MA, USA). Load was applied to the midportion of the fusion mass in the L4-5 vertebrae using a crosshead speed of 1.5 mm/min for all tests. The load versus displacement data were recorded automatically by the Instron software (INSTRON series IX Automated Materials Tester, version 8.04.00), which calculates the mechanical parameters from the load-displacement curves.

Statistical analysis
All statistical comparisons were computed using SPSS 17.0 software. The data were expressed as mean±standard deviation. Repeated measure analysis of variance was used to compare body weights between the two groups. A two-sample t test was used to identify differences between the groups. A p value <0.05 was considered significant.

RESULTS

Body weight
Body weight was measured once a week throughout the 16-week experimental period. Changes in the mean body weights between the two groups over time are illustrated in Table 1. Body weight did not differ between two groups throughout the experimental period.

Serum calcium level
The serum calcium concentrations in both groups are presented in Table 2. The serum calcium concentration was significantly higher in the OVX-Ca group than in the control group at both 4 and 8 weeks after spinal bone fusion surgery (p<0.05). This shows that the intake of calcium was appropriate in the OVX-Ca group.

Fusion assessment
The manual palpation testing showed that the fusion rates, defined as the percentage of solid union, at 8 weeks after fusion surgery were 85% in the OVX-Ca group and 42% in the control group, although this difference was not significant (p>0.05). The 3D-µCT scanning indicated that the OVX-Ca group had a greater and denser fusion mass compared with the control group 8 weeks after fusion surgery. This was a significant different fusion rate between the two groups (OVX-Ca : 85%, control : 28%; p<0.05).

Fusion volume
Eight weeks after fusion surgery, the fusion masses comprised a mixture of dispersed grafted bone fragments and newly formed bone in the OVX-Ca group. The fusion volume differed between the two groups (OVX-Ca : 85%, control : 28%; p<0.01) (Fig. 2). At that time, the fusion masses had matured with the development of a cortical shell around the trabecular bone (Fig. 3).

Table 1. Temporal changes in body weight between the Control and OVX-Ca groups (g-mean±SEM)

|                   | After fusion surgery |
|-------------------|----------------------|
|                   | 0 week    | 8 weeks | 8+4 weeks | 8+8 weeks |
| Control           | 204.00±1.95 | 321.00±6.77 | 324.29±4.29 | 348.57±4.04 |
| OVX-Ca            | 206.00±2.27 | 321.63±7.01 | 327.50±2.44 | 351.25±5.07 |

OVX-Ca : calcium-supplemented group, SEM : standard error of the mean

Table 2. Serum calcium concentration in Control and OVX-Ca groups (mmol/L-mean±SEM)

|                   | After fusion surgery |
|-------------------|----------------------|
|                   | 0 week    | 8 weeks | 8+4 weeks | 8+8 weeks |
| Control           | 1.44±0.03 | 1.42±0.03 | 1.39±0.02 | 1.41±0.04 |
| OVX-Ca            | 1.43±0.04 | 1.42±0.05 | 1.99±0.04* | 1.67±0.05* |

*<p<0.05 for OVX-Ca vs. control. OVX-Ca : calcium-supplemented group, SEM : standard error of the mean
Serum bone metabolism markers

The serum osteocalcin level in the OVX-Ca group remained high 4 and 8 weeks after fusion surgery. By contrast, the osteocalcin level decreased gradually in the control group from 4 to 8 weeks after fusion surgery. The serum osteocalcin level was higher in the OVX-Ca group than in the control group 4 and 8 weeks after fusion surgery \((p<0.05)\) (Fig. 4A).

The serum CTX levels increased in both the OVX-Ca and control groups in the first 4 weeks after bone fusion surgery but had decreased by 8 weeks after fusion surgery. Although the CTX level increased in both groups in the first 4 weeks after bone fusion surgery, the CTX level remained significantly lower in the OVX-Ca group than in the control group throughout the postoperative course \((p<0.05)\) (Fig. 4B).

Mechanical strength

Biomechanical testing was performed to measure the strength of the fused bone mass. The two groups had similar stress values. The three-point bending test showed that the fused vertebrae in the OVX-Ca group could withstand a 50% greater maximal load compared with the control group \((p<0.01)\) (Fig. 5).

DISCUSSION

After menopause, depletion of estrogen increases bone turnover, and the rate of osteoclastic resorption exceeds the rate of osteoblastic formation, resulting in a loss of bone mass\(^8\). Based on this observation, patients with osteoporosis and osteopenia may not be good candidates for fusion surgery due to instrument or fusion failure following surgery for traumatic fracture and degenerative spinal disease. It is estimated that more than 200000 spine fusion procedures are performed each year in the United States\(^3\). Posterior lumbar arthrodesis is the most common procedure performed, and failure to achieve a solid bony union (nonunion) occurs in 10-40% of patients with a single-level fusion and more frequently when multiple levels are attempted\(^{11,12,27}\).

Various kinds of drugs used in the treatment of osteoporosis, such as bisphosphonates, parathyroid hormone, estrogen, selective estrogen receptor modulators, calcitonin, and vitamin D, are options for inducing osteoporotic fracture healing\(^{14,17,20,29,36,37}\). However, whether these drugs are effectively help the patients with osteoporosis or osteopenia is unclear. Lehman et al.\(^{28}\) reported that alendronate sodium inhibited or delayed bone fusion after intertransverse process spinal fusion in a rabbit model. Presumably, this resulted from uncoupling of the balance be-
subcutaneous injection in the abdomen or thigh, and the ex-
hormone has several disadvantages, such as the need for daily

take increases bone formation and decreases bone resorption ac-

tivation after fusion surgery in the osteoporotic condition. The
tegration6,15,19,37). Ahmad et al.37) studied the effects of calcium supplementation on spinal

tations will form the basis of our future studies.

These results are consistent with previous findings in experi-

mental osteoporotic-fracture models6,15,21,25,37). Ahmad et al.37)
studied the effects of calcium supplementation on fracture heal-
ing of osteoporotic bone in ovariectomized rats. Calcium sup-
plements appeared to improve fracture healing of osteoporotic bone. Half of the ovariectomized rats were given calcium sup-
plements throughout the healing phases, and this may explain the
similar radiological evidence of fracture healing in the sup-
plemented and sham-operated rats.

In ovariectomized rats, compared to the Sham group (non-
operated rats), serum ionized-calcium levels showed no signifi-
cant differences in other experimental study40). Therefore, we
did not compare the sham group with the calcium supplement-
ted group. In this study, after fusion surgery, the serum calci-

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Fig. 5. The loading force to the maximal load on the fusion mass in L4 and L5 vertebrae determined using a three-point bending test. The OVX-Ca group significantly increases the maximal load as compared with to the control group (p<0.01). *p<0.01 for OVX vs. control group comparison. OVX-Ca : calcium-supplemented group.

The results of this study indicate that calcium supplementa-

CONCLUSION

The results of this study indicate that calcium supplementa-

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tion in ovariectomized rats undergoing lumbar fusion surgery increases fusion volume and mechanical strength.

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