Bone formation during correction of vertebral rounding deformity in a rat model of pediatric spondylolisthesis

Hiroaki Manabe MD, Kazuta Yamashita MD PhD, Kosaku Higashino MD PhD, Masatoshi Morimoto MD PhD, Kosuke Sugiura MD, Yoshihiro Ishihama MD, Fumitake Tezuka MD PhD, Yoichiro Takata MD PhD, Toshinori Sakai MD PhD and Koichi Sairyo MD PhD

Department of Orthopedics, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, Japan

Corresponding Author: Hiroaki Manabe, MD

Assistant Professor

Department of Orthopedics, Institute of Biomedical Sciences,

Tokushima University Graduate School

3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

Tel.: +81-88-633-7240; Fax: +81-88-633-0178

Email: s52726362@yahoo.co.jp

Conflicts of Interest and Source of Funding: None declared.

Sources of Support: None

The manuscript submitted does not contain information about medical device(s)/drug(s).

Key Words: spondylolisthesis; vertebral rounding deformity; growth plate; endochondral ossification; mechanical stress; transchondroid bone ossification

Mini abstract

It is possible to correct the rounding deformity of the vertebral body associated with isthmus slippage by decrease the load on the vertebral body in pediatric patients. In addition to improved chondrocyte differentiation and promotion of redifferentiation, there is possible involvement of a third mechanism, namely transchondroid bone ossification.

Key points

- Radiological and histological evidence showed correction of vertebral rounding deformity and development of a normal cancellous bone structure after starting tail suspension.
- After tail suspension was initiated, the growth plate stained positive for type X collagen and the restored site stained positive for types II and X collagen, which were expressed in a mosaic pattern; chondrocytes expressing types I and II were also present.
- The mechanism for correction of rounding deformity was associated with not only redifferentiation of endochondral ossification but also transchondroid bone ossification.

Structured Abstract

Study design: A study using rat spondylolisthesis models.

Objective: To elucidate the mechanism for correction of vertebral rounding deformity.

Summary of Background Data: Vertebral rounding deformity is the strongest risk factor for high-grade slippage associated with spondylolisthesis in adolescents. We previously reported that inadequate endochondral ossification of the anterior upper corner of the vertebral growth plate in response to mechanical stress could be the pathological mechanism of vertebral rounding deformity.

Methods: We created a model of spondylolisthesis using 4-week-old rats. They were divided into a tail suspension group that underwent tail suspension to decrease mechanical stress starting at 2 weeks postoperatively and a ground control group with no intervention. Radiographs and micro-computed tomography scans were obtained once weekly for 6 weeks postoperatively. The lumbar spines were then harvested for histological analysis. Immunohistochemical studies detected types I, II, and X collagen in the growth plate cartilage. Bone histomorphometrical analysis was also performed.

Results: Radiological and histological evidence in the ground control group showed progress the rounding deformity with time as previously reported. Formation of normal cancellous bone was observed radiologically over time in the tail suspension group, indicating correction of rounding deformity. Histologically, the site showing radiological evidence of correction was derived from cartilage tissue. After starting tail suspension, the growth plate stained positive for type X collagen and the corrected site stained for types II and X collagen in a mosaic pattern. Chondrocytes expressing types I and II collagen and tartrate-resistant acid phosphatase-positive cells were also present at the corrected site. Histomorphometrically, more endochondral bone was detected at the corrected site than in the posterior aspect of the normal growth plate.

Conclusion: Correction of vertebral rounding deformity was associated with improvement of chondrocyte differentiation, furthermore, there is possible involvement of a third mechanism, namely transchondroid bone ossification.

Introduction

Lumbar spondylolysis develops secondary to spondylolisthesis in adolescents and may progress to cause chronic low back pain and neurological symptoms. In adults, L 5 spondylolisthesis was thought to be caused by the same mechanism that causes intervertebral disc degeneration. However, in 1976, Farfan et al. speculated that the slippage occurs at the epiphysis of the vertebral body, which is a structure that is present only in adolescence[1]. Furthermore, in the late 1980s, Ikata et al. proposed an endplate slip theory after finding evidence of dissociation between the intervertebral disc and the endplate on magnetic resonance imaging[2]. Sairyo et al. subsequently proved this theory by creating pars defects in an immature fresh calf spine as a model of spondylolysis and confirming that the growth plate slipped when subjected to an anterior shearing load[3].

Wedging of the L5 vertebral body and rounding of the sacral surface are well-known deformities associated with isthmus slippage, and high-grade slippage has been reported to be closely related to rounding deformity[2,4–6]. Our group has created a rat model of lumbar spondylolysis that successfully reproduces this deformity. In this model, it was clarified that the cartilage layer was enlarged in the deformed anterior corner, although the direct cause of the slip was the posterior damage of the vertebral growth plate[7,8]. Using histological methods, we clarified the pathology of the enlargement of this cartilage layer to be impaired differentiation of the proliferating chondrocyte layer from the hypertrophic chondrocyte layer in response to forward mechanical stress because of spondylolysis during endochondral ossification in the growth plate[9].

On the other hand, we experienced some cases in which vertebral deformity was improved in adolescent patients with lumbar spondylolysis by rest sporting activity, wearing of a trunk brace, and surgical treatment such as spondylolysis repair[10,11]. These experience made us speculate that the reduction in mechanical stress due to vertebral body braking enabled vertebral repair. Then, in order to investigate the mechanism in detail, we attempted to reduce mechanical stress by tail suspension in a rat model of lumbar spondylolysis and succeeded in correcting vertebral body deformation[12].

The aim of this study was to elucidate the pathogenesis of vertebral deformity with lumbar spondylolysis and the mechanism via which it can be improved

Materials and Methods

A total of 24 female Wistar rats (4 weeks old, weighing 80–90 g) were obtained from Charles River Japan Co., Ltd. (Yokohama, Japan). The rats were housed in individual cages in a temperature- and humidity-controlled room (23°C±2°C; 55%±10% on a 12:12-h light-dark cycle.

Surgical Procedure

A juvenile rat model of slippage was created following a previously described surgical procedure[7-9]. Each rat was anesthetized with sodium pentobarbital (50 mg/kg body weight) followed by a midline longitudinal skin incision made on the back at the L4–L6 level. After separation of the paravertebral muscles from the L5 and L6 laminae, L5 laminectomy and bilateral L5/6 facetectomy were performed to destabilize the posterior spine. The rats were divided into a ground control (GC) group and a tail suspension (TS) group. The GC group underwent observation only after destabilization surgery. In the TS group, mechanical stress was decreased at the anterior upper corner of the L6 growth plate using a cage with a clip (Yamashita Laboratory, Tokushima, Japan;) that was attached to the rat tail to suspend the lower body (Fig. 1)[12], thereby creating lumbar lordosis (i.e., tail suspension). Tail suspension was started 2 weeks after surgery; that is, at 6 weeks of age, and continued for 4 weeks. During this time, the rats could walk around freely in their cages using their forelimbs. All rats had free access to food and water. The study protocol conformed to the guidelines for the care and use of laboratory animals at our university.

Radiological and Histological Evaluation

Radiographs obtained weekly for 6 weeks after the surgery were evaluated for forward slippage and rounding deformity. The rats were euthanized at each week. The lumbar spines were harvested and micro-computed tomography (μCT) images were obtained. Although not completely blind, a total of 3 authors (H.M & M.M & K.Y) evaluated all images. The specimens were then fixed in 10% buffered neutral formalin solution and embedded in paraffin after adequate decalcification with 10% ethylenediaminetetraacetic acid. The paraffin blocks were cut into 4-μm-thick sections and stained with hematoxylin and eosin (H&E), Alcian blue, and tartrate-resistant acid phosphatase (TRAP). The histological analysis included histochemistry,

immunohistochemistry, and histomorphometric analysis with calcein as a marker.

Immunohistochemistry of the Growth Plate

Immunostaining for collagen types I, II, and X was performed using an ImmPRESS reagent polymer kit (Vector Laboratories, Inc., Burlingame, CA) at room temperature. After predigestion, the sections were washed 3 times for 5 minutes each with 1% Tween-20 in phosphate-buffered saline (PBS). Endogenous peroxidase activity was removed by incubation with 0.3% hydrogen peroxide in methanol for 30 minutes. The sections were then washed 3 times for 5 min each with 1% Tween 20 in PBS, incubated with proteinase K for 9 min, and again washed 3 times for 5 min each. Nonspecific binding was blocked by incubation with 2.5% normal horse serum for 1 hour. The sections were then incubated for 20 minutes in 1% PBS containing the collagen primary antibody; that is, type I (Rockland Immunochemicals Inc., Gilbertsville, PA), II (Rockland Immunochemicals), or X (Invitrogen, Grand Island, NY) at a dilution of 1:400. After washing the sections 3 times with 1% Tween-20 in PBS, the sections were incubated for 30 minutes with ImmPRESS polymer reagent, washed 3 times for 5 minutes each with 1% Tween-20 in PBS, and stained using the DAB peroxidase substrate kit (Vector Laboratories). Counterstaining was performed using H&E and observed with an optical microscope.

Histomorphometrical Analysis

To further define the abnormal bone growth at the anterior corner of L6, a modified histomorphometric analysis was performed using single labeling with calcein as the marker. After 4 weeks of tail suspension in the TS group, calcein was injected intraperitoneally and the rats were euthanized. The L6 vertebrae were harvested the following day. Toluidine blue staining (pH 9.0) was used to determine the sites where calcification was present. Calcein labeling was assessed using undecalcified, frozen, 5-mm-thick sections of tissue fixed previously in 10% formalin for 24 hours. Calcein is a calcium chelator that adheres to regions of new bone mineralization. A fluorescence microscope (Nikon Corporation, Tokyo, Japan) was used with an excitation wavelength of 485 nm and an emission wavelength of 510 nm for pattern analysis.

Results

Radiological Evaluation

The radiographs obtained 1 week after surgery in both groups showed slippage at L5/6 in all rats and those obtained 2 weeks after surgery showed rounding deformity of the anterior upper corner of the L6 vertebra. Vertebral body deformation progressed with time in the GC group whereas bone was gradually restored after tail suspension in the TS group (Fig. 2). The μCT images obtained at 6 weeks in the TS group showed bone with a normal trabecular structure (Fig. 3). It can be difficult to completely capture the restored bone on plain radiograph alone due to sample hardness and exposure angle. Thus, whilst in Figure 2, the 3wk tail suspension scan appeared to demonstrate as much rounding as the GC 3wk scan, Fig. 3 clearly showed detailed bone restoration using μCT.

Histological Study

Fig. 4 shows the histological examination of the sections stained with H&E at weekly intervals (Fig. 4). Vertebra deformity induced by surgery for posterior instability faithfully reproduced enlargement of the cartilage layer as previously reported[7-9]. At 1 week after surgery, there was evidence of deformity, decreased numbers of hypertrophic chondrocytes, and increased numbers of proliferating chondrocytes with a disturbed arrangement (Fig. 5). Two weeks after surgery, the rounding deformity had progressed and hypertrophic chondrocytes in the anterior upper corner had disappeared (Fig. 5). Although deformity remains during the observation period in GC group, there was histological evidence of bone tissue restoration in the TS group (Fig. 4). After starting tail suspension, bone was restored over time and the hypertrophic chondrocytes reappeared with gradual restoration of normal cell arrangement in the anterior upper corner (Fig. 6). In the repaired bone tissue, chondrocytes were observed in the same matrix as that containing osteocytes. The deformed anterior corner stained positively with Alcian blue, indicating that the site was filled with cartilaginous tissue (Fig. 7). Furthermore, positive staining remained at the restored site, indicating that the restored tissue was derived from cartilage (Fig. 7). TRAP-positive cells were mainly observed in the vicinity of the growth plate but some were found in the deformed anterior corner before starting tail suspension (Fig. 8). However, TRAP-positive cells were induced

again at 1 week after starting tail suspension (Fig. 8) and were present in high numbers at the restored site after 3 weeks of suspension (Fig. 8).

Immunohistochemistry at the Growth Plate

The entire growth plate stained positive for type II collagen. In contrast, only the hypertrophic layer stained positive for type X collagen. As previously reported[9], most of the anterior corner corresponding to the deformed site stained positive for type II collagen with no staining for type X collagen in GC group. However, the growth plate stained positive for type X collagen after starting tail suspension, and types II and X collagen were expressed in a mosaic pattern in the deformed site that had been restored in TS group. Furthermore, types I and II collagen were expressed in chondrocytes at the restored site (Fig. 9).

Histomorphometrical Analysis

The pattern of calcein labeling in the anterior corner differed markedly from that in the middle region (Fig. 10). At L6, fluorescence was observed at the periosteal surfaces in the middle region on the cranial side. However, fluorescence was also detected in the anterior corner, not only on the endosteal surface of new bone but also on the periosteal surface (Fig. 10), indicating extensive and rapid endochondral bone growth in the anterior corner in TS group.

Discussion

Lumbar spondylolysis is a relatively common condition that occurs in about 6% of the general population in Japan[13] and is often asymptomatic. However, lumbar spondylolysis may progress to spondylosis in children who develop the disorder at a young age[5,14–16]. Unlike an intact spine, a lumbar spine with spondylolysis lacks posterior stability because of increased mechanical stress on the inferior vertebral endplate via the intervertebral disc. Finite element modeling in a biomechanics study by Sairyo et al. showed that mechanical stress during movement at the growth plate in a lumbar spine with spondylolysis is up to 6 times greater than that in the intact spine[17,18]. Terai et al. suggested that wearing a brace to decrease the amount of mechanical stress at the growth plate would correct functioning of the growth plate and help to restore the

vertebral rounding deformity to its normal shape[10]. Consistent with their suggestion, a more recent study by Yamashita et al. found that bone was restored by full reduction and firm fixation for spondylolysis following repair of spondylolysis[11]. To examine this mechanism in more detail, we used a young rat model of spondylolisthesis that was first reported in 2002 by Sakamaki et al.[7]. This model mimics the slippage and rounding of the surface at the anterior corner of the vertebral growth plate observed in pediatric patients with spondylolysis. Use of this model has also confirmed that slippage and deformation occurs in young rats but not in adult rats and that the main site of damage is the growth plate with almost complete preservation of the disc[7]. This model has been used by several groups of investigators when performing basic research on lumbar spondylolisthesis, including Higashino et al. investigated rounding deformity at the anterior corner of the vertebral growth plate[9]. They demonstrated that the site with radiological evidence of rounding deformation was filled with cartilage tissue and immunostained positive for type II but not type X collagen. Histomorphometrical analysis revealed few TRAP-positive cells near the growth plate and that the amount of endochondral bone at the deformed site was smaller than that at a normal posterior growth plate. Therefore, rounding deformity was determined to have been caused by mechanical stress-induced impairment of endochondral ossification at the growth plate in pediatric patients with spondylolysis[9].

The main findings of the radiological, histological, and immunohistochemical assessments performed in our study were as follows:

- Radiological evidence showed correction of rounding deformity and development of a normal cancellous bone structure after starting tail suspension.
- Histologically, cartilage tissue comprised the site where radiological evidence showed correction of rounding deformity.
- Many TRAP-positive cells were detected at the site of bone restoration.
- After tail suspension was initiated, the growth plate stained positive for type X collagen and the restored site stained positive for types II and X collagen, which were expressed in a mosaic pattern; chondrocytes expressing types I and II were also present.

- Histomorphometrically, the amount of endochondral bone was greater at the restored site than at the normal posterior growth plate.

Staining with H&E showed suppression of the increase in number of proliferating chondrocytes after starting tail suspension and return of their arrangement to the original state with reappearance of hypertrophic chondrocytes, which improved the chondrocyte differentiation. Moreover, the results of immunohistochemistry indicated that the growth plate at the anterior corner functioned normally because type X collagen was again contained in the layer close to the vertebral body. This layer of chondrocytes induces mineralization of the cartilage matrix but eventually disappears and is replaced by bone during endochondral ossification. Osteoclasts play a central role in resorption of a pre-existing cartilaginous matrix. In our model, TRAP-positive cells, which indicate the presence of osteoclasts, were actively induced at the site being restored. Furthermore, morphometric analysis using calcein as a marker showed active ossification at the anterior corner. However, the pattern of expression of types I and II collagen and chondrocytes in the same matrix as that containing osteocytes suggested not only redifferentiation of endochondral ossification but also a different type of bone formation.

We then focused on the chondrocytes observed in the same matrix as the osteocytes that appeared in the restored bone tissue. Originally, in the process of endochondral ossification, type II collagen is not expressed at the same time as type I collagen because apoptosis occurs when chondrocytes are replaced with osteocytes. However, expression in both types of cell was confirmed in this study. This finding was reported by Yasui et al. as “chondrocyte-like” cells[19]. During bone lengthening using external fixation, they demonstrated endochondral ossification in the early stage of extension and membranous ossification in the later stage, with appearance of chondrocyte-like cells expressing both type I and type II collagen in the middle stage. They described this finding as “transchondroid” bone formation, which they attributed to a third type of bone forming mechanism. Several other studies have also reported that hypertrophic chondrocytes can transdifferentiate directly into osteocytes without apoptosis and capillary invasion during bone extension[20-22]. The tail suspension model has been used

in many studies as a model that reproduces the unloading to the intervertebral disc space, but it has also been reported that tensile strain is applied[23-25]. In our study, it is possible that the lumbar lordosis created by tail suspension applied traction to the damaged growth plate and caused the same phenomenon.

This study has several limitations. First, humans are bipedal whereas rats are quadrupedal. However, previous studies have reported that the rat model corresponds to the clinical features of spondylolytic spondylolisthesis in humans and is considered appropriate as a research model[7-9]. Second, it is unclear how adequately the decreased mechanical stress achieved by tail suspension in rats can be reproduced in humans. The conventional lumbar orthosis may not be fully reproduced and further research will be needed before its application in human studies.

Conclusion

The results of this study suggest that it is possible to correct the rounding deformity of the vertebral body associated with isthmus slippage by appropriate treatment that decreases the load on the vertebral body in pediatric patients. In addition to improved chondrocyte differentiation and promotion of redifferentiation, there is possible involvement of a third mechanism, namely transchondroid bone ossification. When the mechanism via which the deformity is corrected becomes clearer, it will be possible to develop appropriate conservative treatment. For example, wearing of a lumbar lordosis brace with an optimal shape according to a defined optimal duration of use in adolescents with spondylolisthesis.

References

1. Farfan HF, Osteria V, Lamy C. The mechanical etiology of spondylolysis and spondylolisthesis. Clin Orthop Relat Res 1976;117:40–55.

2. Ikata T, Miyake R, Katoh S, et al. Pathogenesis of sports-related spondylolisthesis in adolescents. Radiographic and magnetic resonance imaging study.
Am J Sports Med 1996;24:94–98.

3. Sairyo K, Goel VK, Grobler LJ, et al. The pathomechanism of isthmic lumbar spondylolisthesis. A biomechanical study in immature calf spines. Spine 1998;23:1442–6.

4. Fredrickson BE, Baker D, McHolick WJ, et al. The natural history of spondylolysis and spondylolisthesis. J Bone Joint Surg Am 1984;66:699–707.

5. Sairyo K, Katoh S, Ikata T, et al. Development of spondylolytic olisthesis in adolescents. Spine J 2001;1:171–5.

6. Yue WM, Brodner W, Gaines RW. Abnormal spinal anatomy in 27 cases of surgically corrected spondyloptosis: proximal sacral endplate damage as a possible cause of spondyloptosis. Spine 2005;30:S22–S26.

7. Sakamaki T, Sairyo K, Katoh S, et al. The pathogenesis of slippage and deformity in the pediatric lumbar spine: a radiographic and histologic study
using a new rat in vivo model. Spine 2003;28:645–50 [Discussion 650–1].

8. Komatsubara S, Sairyo K, Katoh S, et al. High-grade slippage of the lumbar spine in a rat model of spondylolisthesis: effects of cyclooxygenase-2 inhibitor on its deformity. Spine 2006;31:E528–E534.

9. Higashino K, Sairyo K, Sakamaki T, et al. Vertebral rounding deformity in pediatric spondylolisthesis occurs due to deficient of endochondral ossification of the growth plate: radiological, histological and immunohistochemical analysis of a rat spondylolisthesis model. Spine 2007;32:2839–45.

10. Terai T, Sairyo K, Goel VK, et al. Biomechanical rationale of sacral rounding deformity in pediatric spondylolisthesis: a clinical and biomechanical study. Arch Orthop Trauma Surg. 2011;131:1187-94

11. Yamashita K, Higashino K, Sakai T, et al. The reduction and direct repair of isthmic spondylolisthesis using the smiley face rod method in adolescent athlete: Technical note. J Med Invest. 2017;64:168-72.

12. Yamashita K, Higashino K, Manabe H, et al. Reducing mechanical stress restores the rounding deformity in a rat model of immature pediatric spondylolisthesis [abstract]. 45th *ISSLS* Annual Meeting, 2018.

13. Sakai T. Sairyo K, Takao S, et al. Incidence of lumbar spondylolysis in the general population in

Japan based on multidetector computed tomography scans from two thousand subjects. Spine 2009; 34:2346-50
14. Laurent LE, Einola S. Spondylolisthesis in children and adolescents. Acta Orthop Scand 1961;31:45–64.
15. Saraste H. Long-term clinical and radiological follow-up of spondylolysis and spondylolisthesis. J Pediatr Orthop 1987;7:631–8.
16. Seitsalo S, Osterman K, Hyvarinen H, et al. Progression of spondylolisthesis in children and adolescents. A long-term follow-up of 272 patients. Spine 1991;16:417–21.
17. Sairyo K, Goel VK, Masuda A, et al. Three-dimensional finite element analysis of the pediatric lumbar spine. Part I: Pathomechanism of apophyseal bony ring fracture. Eur Spine J 2006;15:923–9.
18. Sairyo K, Goel VK, Masuda A, et al. Three dimensional finite element analysis of the pediatric lumbar spine. Part II: Biomechanical change as the initiating factor for pediatric isthmic spondylolisthesis at the growth plate. Eur Spine J 2006;15:930–5.
19. Yasui N, Sato M, Ochi T, et al. Three modes of ossification during distraction osteogenesis in the rat. J Bone Joint Surg Br. 1997;79:824-30.
20. Furuta N, Yoshioka I, Fukuizumi T, et al. Morphological features of cartilage observed during mandibular distraction in rabbits. Int J Oral Maxillofac Surg. 2007;36:243-9.
21. Rundle CH, Wang X, Sheng MH, et al. Bax deficiency in mice increases cartilage production during fracture repair through a mechanism involving increased chondrocyte proliferation without changes in apoptosis. Bone 2008; 43:880–888.
22. Bahney CS, Hu DP, Taylor AJ, et al. Stem Cell Derived Endochondral Cartilage Stimulates Bone Healing by Tissue Transformation. J Bone Miner Res. 2014;29:1269-82
23. Maynard JA. The effects of space flight on the composition of the intervertebral disc. Iowa Orthop J. 1994;14:125-33.
24. Yasuoka H, Asazuma T, Nakanishi K, et al. Effects of Reloading After Simulated Microgravity on Proteoglycan Metabolism in the Nucleus Pulposus and Anulus Fibrosus of the Lumbar Intervertebral Disc An Experimental Study Using a Rat Tail Suspension Model. Spine 2007;32:734-40
25. Hutton WC, Yoon ST, Elmer WA, et al. Effect of tail suspension (or simulated weightlessness) on the lumbar intervertebral disc. Spine 2002;12:1286–90.

Figure 1. Mechanical stress in the anterior vertebral body is decreased by creating lumbar lordosis using the tail suspension method.

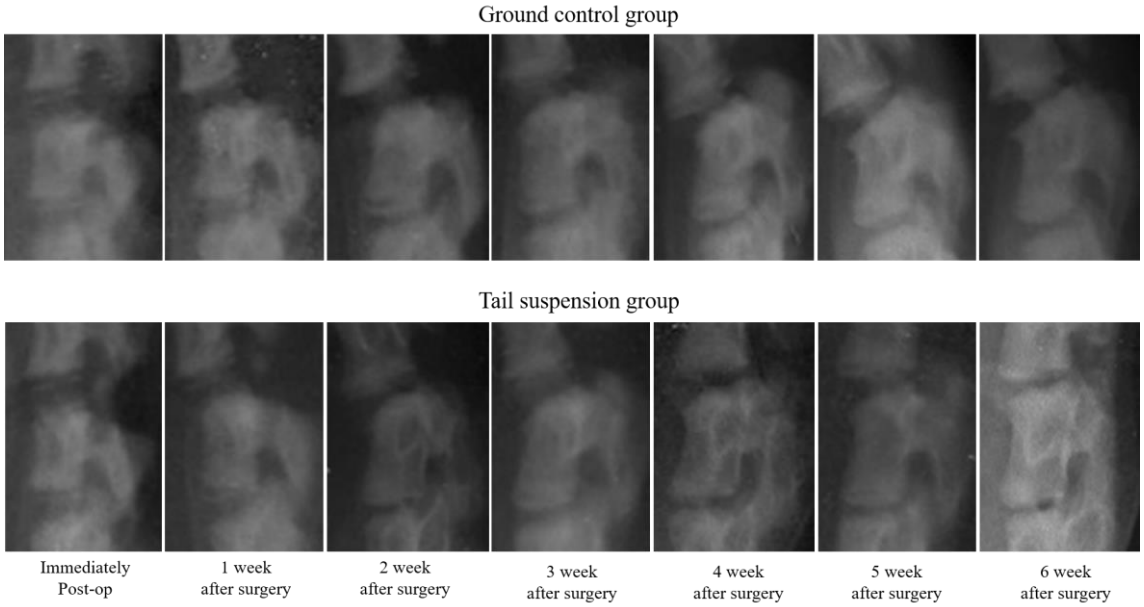


Figure 2. Radiological images show progression of the rounding deformity in the sacrum over time in a GC group. In contrast, remodeling of the upper corner of sacrum with restoration of bone tissue is seen in a TS group after starting tail suspension.

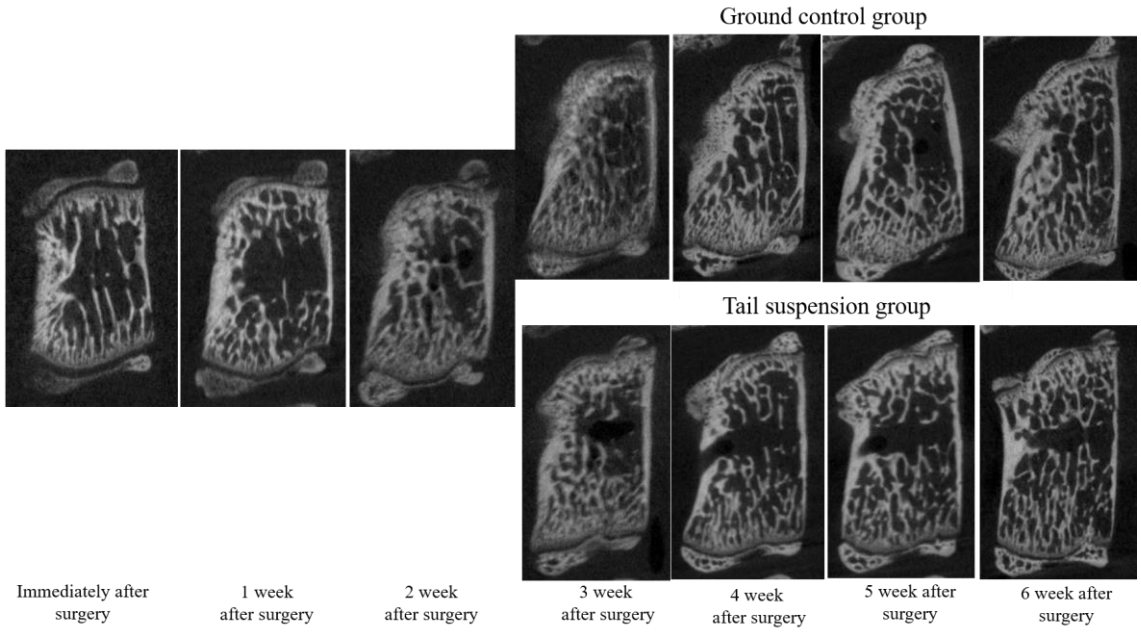


Figure 3. Micro-computed tomography images show progression of the rounding deformity in the sacrum with osteophytes over time in a GC group. Restoration at the upper corner of the vertebral growth plate with a cancellous bone structure is seen in a TS group.

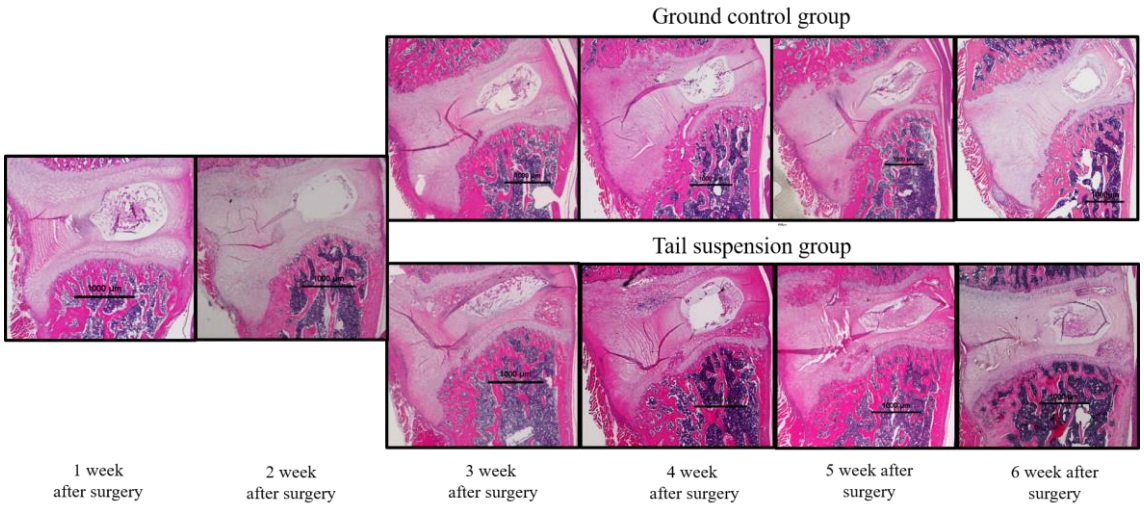


Figure 4. The deformity progresses with time in a GC group, whereas bone tissue is gradually restored after starting tail suspension in a TS group.

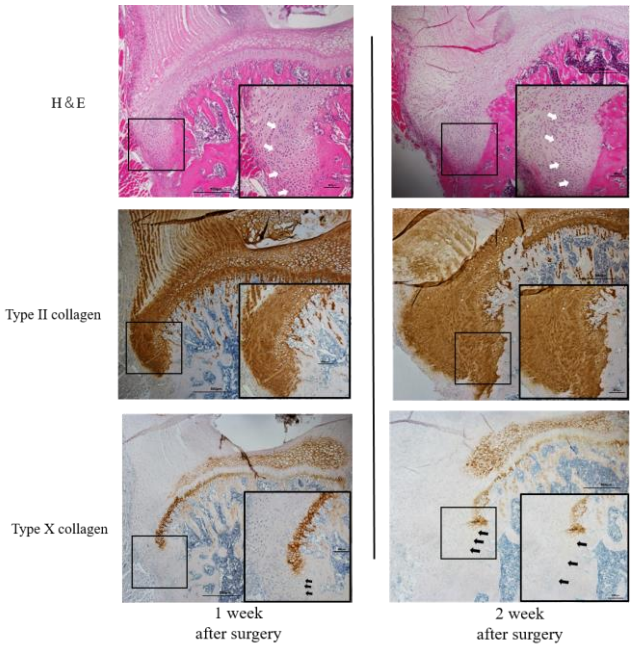


Figure 5. At 1 week after surgery, H&E-stained sections show an increase in the number of proliferating chondrocytes (white arrow) in addition to disturbance in the arrangement of cells in the growth plate. Immunohistochemical examination confirmed that expression of collagen type X disappeared (black arrow) and expression of collagen type II increased. This trend was more pronounced at 2 weeks after surgery. H&E, hematoxylin and eosin.

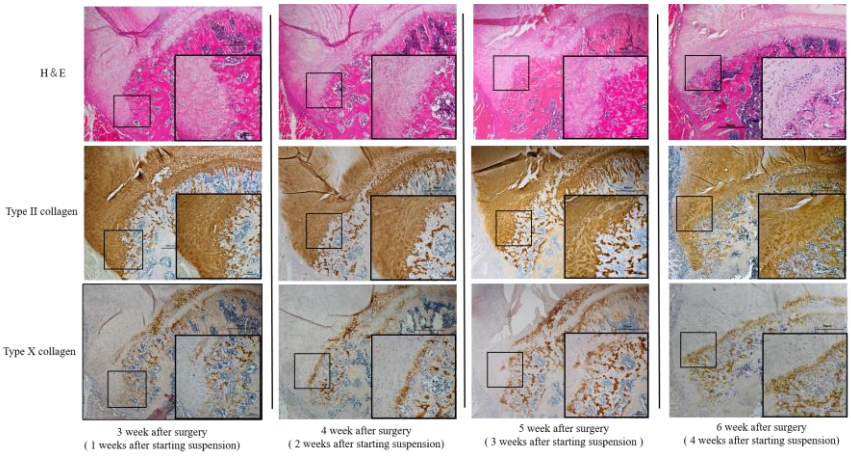
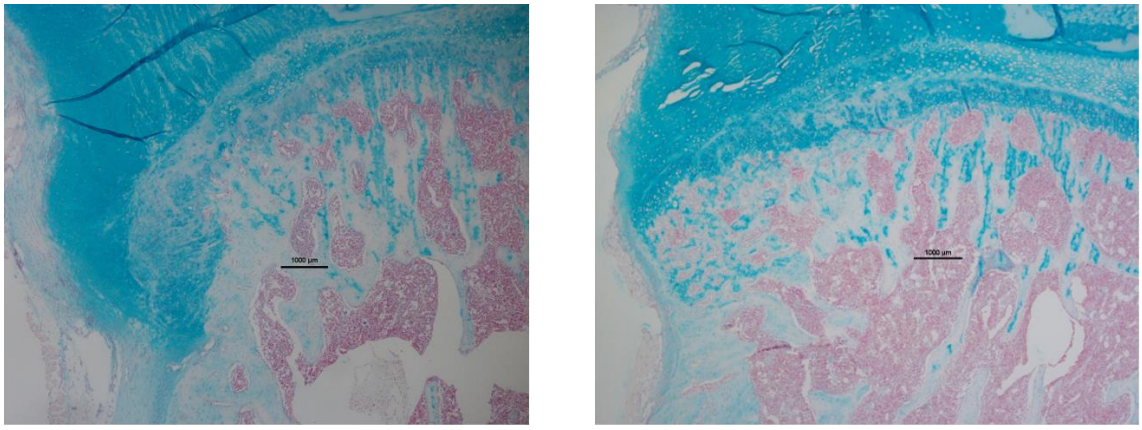


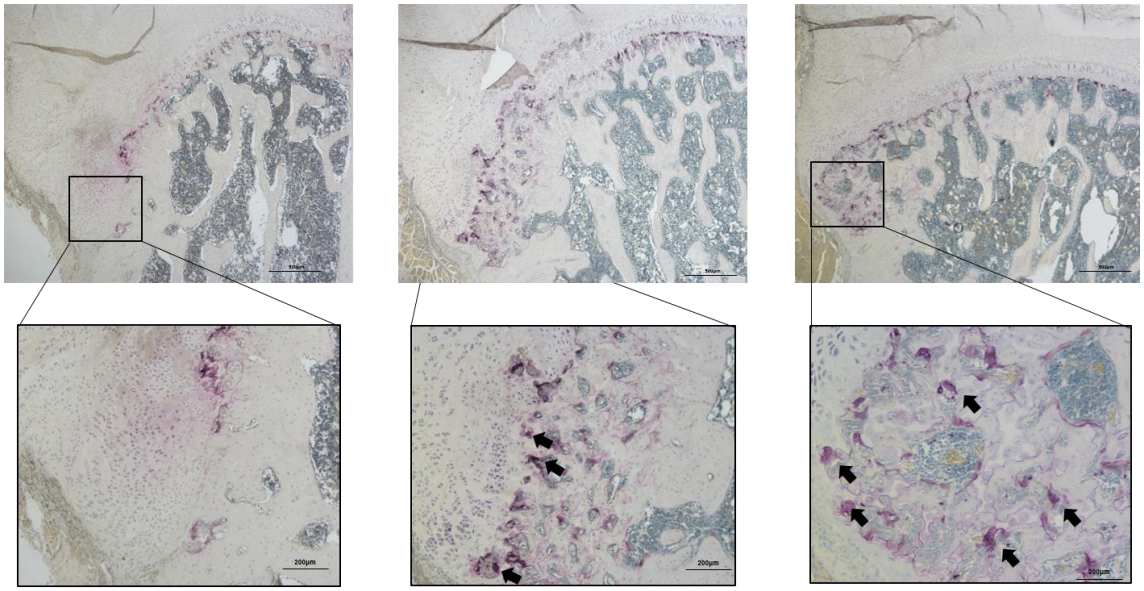
Figure 6. H&E-stained sections showing reappearance of hypertrophic cells after starting tail suspension and gradual cell rearrangement. Type II collagen was strongly expressed and then gradually settled, whereas type X collagen is re-expressed. The repaired site continued to stain for both two types of collagen. H&E, hematoxylin and eosin.



2 weeks after surgery

6 weeks after surgery
(4 weeks after starting suspension)

Figure 7. Before tail suspension (2 weeks after surgery), the deformity site is filled with cartilage tissue stained with Alcian blue. The repair site remains stained, indicating that the tissue is derived from cartilage.



1 week after surgery

3 week after surgery
(1 weeks after starting suspension)

6 week after surgery
(4 weeks after starting suspension)

Figure 8. Few tartrate-resistant acid phosphatase-positive cells (indicated by the arrows) were found at the anterior corner of the growth plate in a vertebral body with rounding deformity but were actively induced at the site of bone restoration after starting tail suspension.

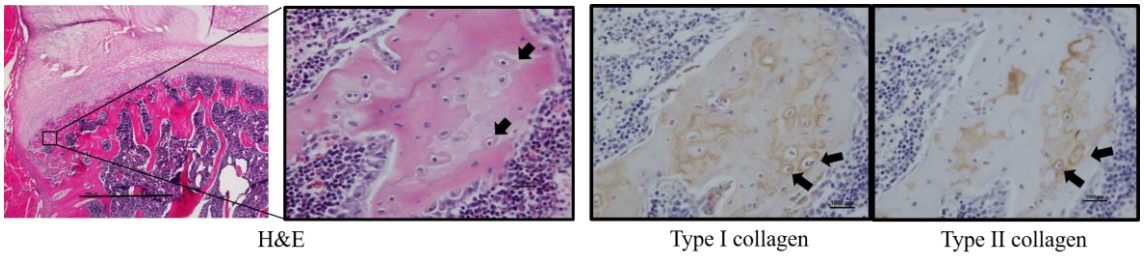


Figure 9. Chondrocyte-like cells (indicated by arrows) expressing types I and II collagen in a TS group H&E, hematoxylin and eosin.

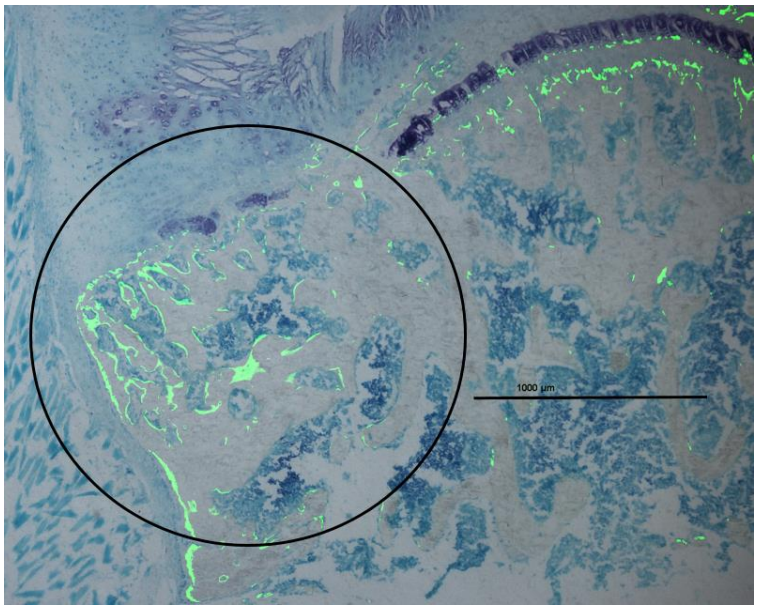


Figure 10. The circle shows calcein labeling at the periosteal and endosteal surfaces in newly formed bone.