Bone union-promoting effect of romosozumab in a rat posterolateral lumbar fusion model

（ラット腰椎後側方固定術(PLF)モデルにおける Romosozumab 投与による骨癒合促進効果に関する検討）

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Abstract

This study investigated the effect of romosozumab on bone union in a rat posterolateral lumbar fixation model. Posterolateral lumbar fixation was performed on 8-week-old male Sprague Dawley rats (n=20). For bone grafting, autogenous bone (40 mg) was harvested from the spinous processes of the 10th thoracic vertebra until the 2nd lumbar vertebra and implanted between the intervertebral joints and transverse processes of the 4th and 5th lumbar vertebrae on both sides. Rats were matched by body weight and equally divided into two groups: R group (Evenity®, 25 mg/kg) and control (C) group (saline). Subcutaneous injections were administered twice a week until 8 weeks after surgery. Computed tomography was performed at surgery and week 8 after surgery. The area and percentage of bone trabeculae in the total area of bone fusion were calculated. Statistical analysis was performed using an unpaired *t*-test ($P<0.05$). We found that the R group rats had significantly higher mean bone union rate and volume than did the C group rats at all time courses starting week 4 after surgery. The R group had significantly higher increase rates than did the C group at weeks 4 and 6 after surgery. The percentage of bone trabeculae area in the R group was approximately 1.7 times larger than that in the C group. Thus, we demonstrated that romosozumab administration has stimulatory effects on bony outgrowth at bone graft sites. We attribute this to the modeling effect of romosozumab.

1. INTRODUCTION

Because the proportion of aged people is increasing in the population, in recent years there has been an increase in the rate of spinal fusion surgery performed for osteoporotic patients. However, serious postoperative complications have often been observed, including postoperative vertebral fracture and pedicle screw (PS) loosening. According to a study on cadavers, osteoporotic patients tend to have a weak pull-out strength and PS loosening (1). As the number of spinal surgeries for patients with severe osteoporosis is expected to increase further, there is an urgency to prevent postoperative complications and achieve favorable postoperative outcomes. A variety of osteoporosis drugs (e.g., bisphosphonate, and a preparation of human parathyroid hormone) have been used thus far to effect bone remodeling and improve bone strength. The bone remodeling process consists of three stages: bone resorption stage, where bone resorption by osteoclasts is initiated; transitional stage, the intermediary stage between bone resorption and bone formation; and bone formation stage, where new bone is formed (2). This process is also consistent with bone union after spine surgery. Of interest is that various osteoporosis drugs have been recently reported to also promote bone union (3,4).

In particular, romosozumab, which has been recently introduced in Japan, is receiving increasing attention in this regard. Romosozumab is a human immunoglobulin monoclonal antibody that binds to sclerostin (a bone formation suppressor) to inhibit its action. Sclerostin is an extracellular inhibitor of the canonical Wnt signaling pathway and is secreted by osteocytes. As romosozumab specifically binds to sclerostin and prevents it from binding to lipoprotein receptor-related protein 5 (LRP5) and lipoprotein receptor-related protein 6 (LRP6), it thus inhibits the suppression of the canonical Wnt signaling in osteoblast lineage cells (5-7). Activation of the canonical Wnt pathway increases mass and strength of cortical and spongy

bone by promoting bone formation and inhibiting bone resorption. The pre-marketing clinical trial data have also demonstrated increased bone formation and decreased bone resorption. Thus, within one month of romosozumab administration, the bone resorption marker C-terminal telopeptide (CTX) in the blood decreased by approximately 35% (statistically significant) and a mean for the bone formation marker P1NP of approximately 95% (statistically significant) were reported (6).

Therefore, we propose that romosozumab exhibits a bone union effect. However, only a few studies on its therapeutic effects in clinical practice have been conducted because it has only recently been introduced in Japan. Therefore, this study aimed to conduct basic research to investigate the bone union-promoting effect of romosozumab in a rat spinal fusion model.

2. METHODS

2.1 Experimental animals

The study protocol was conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (2011 revision) and approved by the Ethics Committee of Chiba University (8). We used 8-week-old Sprague Dawley male rats (n = 20; 200-250 g, Japan SLC Inc., Shizuoka, Japan).

2.2 Posterolateral lumbar fusion (PLF) surgery

All rats were injected intraperitoneally with a mixture of three anesthetic agents, namely Domitor 0.15 ml/kg (Nippon Zenyaku Kogyo Co., Ltd., Japan), Dormicum 2 mg/kg (Astellas Pharma Inc., Japan), and Vetorphale 2.5 mg/kg (Meiji Seika, Ltd., Japan), or saline 1.45 ml/kg (Otsuka Pharma Inc., Japan). Subsequently, an antimicrobial agent (Ampicillin Sodium 20,000 U/kg,

Meiji Seika, Japan) was administered subcutaneously before surgery (9).

The vertebral arch and transverse process of the 4th/5th lumbar vertebrae on the left and right sides, and the 4th/5th lumbar intervertebral joint were exposed after performing a skin incision along the dorsal part of the median line and separating the fascia from the paraspinal muscle on both sides. We harvested 40 mg of graft bone from the spinous process of the 10th thoracic vertebra to the 2nd lumbar vertebra. We placed the graft bones between the intervertebral joint and the transverse process of the 4th/5th lumbar vertebrae on the left and right sides as autogenous bone graft (Figure 1) (8, 10, 11). The fascia and skin were sutured using 4-0 absorbable thread. Postoperatively, all rats were kept in cages where they could eat and drink freely (9).

2.3 Experimental groups

To avoid body size differences, we divided the rats sequentially into two equal groups based on body weight: the romosozumab group (herein, R group) and the control group (herein, C group). Rats in the R group were injected subcutaneously with romosozumab (Evenity®, sclerostin antibody 105 mg/1.17 mL, calcium acetate hydrate 2.41 mg/1.17 mL [13 mM], acetic anhydride 2.04 mg/1.17 mL [17 mM], sucrose 70 mg/1.17 mL (6%), polysorbate-20 0.070 mg/1.17 mL [0.006%] at pH 5.2, ©Amgen Inc., Thousand Oaks, CA), at a dose of 25 mg/kg twice a week (Tuesday and Friday mornings) for 8 weeks (Table 1). Animals in the C group were injected with an equivalent volume of saline (Otsuka Pharma Inc., Japan) (11,12).

2.4 Evaluation tests

2.4.2 Micro-computed tomography examination

Micro-computed tomography (CT) examination was performed under isoflurane inhalation

(1.5% isoflurane [Mylan, Canonsburg, PA,]) to evaluate the volume of bone union area (13). The bone graft site was scanned using a CT apparatus (in vivo micro-CT system, R_mCT2, Rigaku Co., Tokyo, Japan) before surgery and every two weeks until week 8 (resolution of 59 μm, tube voltage of 90 kV, tube current of 200 μA, field of view 30 mm, and an exposure time of 26 s) (Table 1) (9). Three spinal surgeons unrelated to the study evaluated the sagittal, coronal, and axial image data on the rate of bone union between the intervertebral joint and the transverse process in both groups, and the mean value was used (14). The reliability of the bone union rate results was then assessed by intraclass correlation coefficients (ICC) with intra-examiner error. A comparative review of the volume of bone union area using Ziostation2 (Ziosoft Inc., Tokyo, Japan) was conducted as a quantitative evaluation for bone union (Figure 2) (15).

2.4.3 Bone densitometry

Rats were euthanized by anesthetic overdose on week 8. The right femur from the right hip joint was collected and scanned using the CT apparatus mentioned earlier. The bone mineral density (BMD) (expressed as mgHA/cm^3) of each rat was measured from the obtained image using dedicated software (Bone analysis software, Rigaku Co., Ltd. Austin, TX) (12, 16, 17).

2.4.4 Histological examination

We collected the lumbar vertebrae after euthanasia (9). After paraffin block was prepared using 10% neutral buffer formalin (0.1 M, pH 7.4) by fixation, 2-μm-thick transverse sections were made and stained with hematoxylin and eosin (8).

The transected images of PLF region were generated using a fluorescence microscope (BZ-X800, Keyence Corp., Osaka, Japan). The total area of the PLF region was calculated using a dedicated microscopic measurement software (Hybrid Cell Count Module BZ-H4C Analyzer software,

Keyence Corp., Osaka, Japan). Six images (20-fold magnification) were randomly selected from PLF transverse images, and the area percentage of trabecular bone for each image was calculated and compared (Figure 3) (18).

2.5 Statistical Analyses

Data comparisons for each group were statistically analyzed using an unpaired *t*-test, and statistical significance was set at $P < 0.05$.

3. RESULTS

3.1 CT examination

Primary CT images for each group are shown in Figure 4a-f. There was no significant difference in the mean bone union rate between the intervertebral joint and transverse process in the two groups at week 2 after surgery; however, the mean bone union rate in the R group was significantly higher than that in the C group at all time points after week 4 (Group R vs. Group C: 47.0±3.3% vs. 37.0±2.8%, week 4; 79.0±3.1% vs. 61.0±3.6%, week 6; and 97.5±2.5% vs. 84.0±3.6%, week 8) (all $P<0.05$) (Figure 5). The ICCs with intra-examiner error were ICC(1,3)=0.996 and ICC(2,3)=0.931; there was no disagreement among examiners.

There was also no significant difference in the volume of bone union area between the period immediately after surgery and at week 2 after surgery in the two groups; however, the volume of bone union area in the R group was significantly higher than that in the C group at all time points after week 4 (R group vs. C group: 401.83±8.36 mm^3 vs. 361.36±13.29 mm^3, week 4; 483.11±11.81 mm^3 vs. 437.25±24.80 mm^3, week 6; and 547.46±14.10 mm^3 vs. 515.23±11.24 mm^3, week 8) (all $P<0.05$) (Figure 6).

We also divided the volume at each time point by the volume at the period immediately after surgery, defined as the increase rate. We found that the rate of increase in the R group was significantly greater than that in the C group at weeks 4 and 6 after surgery (R group vs. C group: 1.71±0.06x vs. 1.54±0.03x, week 4; 2.06±0.07x vs. 1.86±0.06x, week 6) (all $P<0.05$) (Figure 7). Conversely, there was no significant difference in the rate of increase between the two groups at week 8 after surgery (Figure 7).

3.2 Bone densitometry

The mean BMD of the distal femoral metaphysis was significantly larger in the R group than in the C group (R group vs. C group: 821.1 ± 9.6 mgHA/cm^3 vs. 731.1 ± 7.9 mgHA/cm^3) ($P < 0.05$).

3.3 Histological examination

The pathological images for the left vertebral arch of the 4th lumbar vertebra at week 8 after surgery in the two groups are shown in Figure 8a-d. At week 8 after surgery, there was no significant difference in the mean total PLF area between the two groups (R group: 3.7±0.7 mm^2 vs. C group: 4.4±0.8 mm^2). However, the percentage of bone trabeculae area was significantly larger by approximately 1.7 times in the R group (61.7±3.3%) than in the C group (37.2±1.5%) ($P < 0.05$) (Figure 9).

4. DISCUSSION

4.1 Bone union rate

We found that the mean bone union rate at week 8 after surgery was 82.5% in the C group. Similarly, Kamoda et al. (19) reported a bone union rate of 70% in the same model as assessed

using X-ray at week 8 after surgery. The bone union rate was significantly higher in the R group than in the C group at week 4 after surgery, and bone union was noted in almost all rats (97.5%) at week 8 after surgery. Therefore, favorable bone union was clearly achieved from an early stage following romosozumab administration, which can be attributed to the early effect of romosozumab. In a FRAME study for pre-marketing data (6), a statistically significant mean improvement rate in bone resorption marker (CTX) of approximately 35% and a statistically significant mean increase rate in bone formation marker (P1NP) of approximately 95% were reported within a month after administration. Osteoporosis patients also experienced an immediate effect of improvement of abnormal bone metabolism and a high increase in bone density from the early stage after administration (20-22). Thus, we assume that early and reliable bone union was also achieved in this study.

4.2 Volume of bone union area

Although we noted no significant difference in the mean volume of bone union area between the two groups before surgery, it became significantly higher in the R group at week 8. Similar results have been previously reported, although a different model (i.e., rat femur fracture model) was used. For example, Ominski et al. (23) reported that the fracture volume in the R group was significantly higher (41%) than that in the C group as shown by micro-CT scans at week 7 after surgery. McDonald et al. (24) found that the bone regeneration in the R group was significantly higher (26-38%) than that in the C Group as indicated by micro-CT analysis at week 8 after surgery. Thus, romosozumab administration possibly has a stimulatory effect on bony outgrowth at the bone graft site.

We attribute this result to the modeling effect of romosozumab. As romosozumab specifically binds to sclerostin and prevents sclerostin from binding to LRP5 and LRP6, it thus inhibits the

canonical Wnt signaling in osteoblast lineage cells. The activation of the Wnt signaling pathway leads to increased bone formation and decreased bone resorption, thereby increasing the bone mass of the cortical and trabecular bone (modeling) (6-7). We propose that a similar modeling mechanism occurred in the bone graft site, thereby increasing mean bone union volume following romosozumab administration.

4.3 Bone density

There was a significant increase in bone density of the distal femoral metaphysis following romosozumab administration, indicating that romosozumab increased bone density.

4.4 Pathological findings

We observed that the total PLF area was not significantly different between the two groups, but the percentage of osteophytes for PLF was significantly higher in the R group than in the C group. We also attribute this effect to the previously mentioned modeling effect of romosozumab. Clinical data on osteoporotic patients have revealed that Evenity increases bone density. When we checked the bone volume and microscopic structure during the 12th month as assessed by micro-CT examination of the FRAME study, the volume rate of the trabecular bone was significantly increased following Evenity administration (25). Thus, bone formation due to the active remodeling effect was observed not only in osteoporotic patients, but also in the bone graft site of spinal fusion surgery in this study.

Limitations of the Study

This study had some limitations. First, the dose of Evenity used in this study was much higher, by approximately 19-fold, than that used in clinical practice (26). Specifically, the adult dose of "210 mg/month" is 3 mg/kg of body weight equivalent to 70 kg, which may be considered an

approximately eight times higher dose, and "50 mg/kg subcutaneous injection once/week" in rats is around 19 times higher than the human equivalent (26). If the duration of administration is not taken into account, the exposure dose in this study would be the same as in a previous study (12). Therefore, we plan to conduct additional experiments in the future to examine whether different results would be obtained depending on the dose. Second, the effect of Evenity on BMD was only evaluated in the last observation. Thus, comparing with “before administration” is difficult, and sacrifice is required in our bone-density measuring equipment (because only the size of the femur can be measured). Additionally, most fusions that occur need to undergo a remodeling process to produce the highest functional quality bone in-vivo. We were unable to examine the bone metabolism markers in this study, so it is unclear whether this unionized bone will be remodeled in the future or not. Therefore, additional experiments should be conducted to examine before-and-after effects using a bone metabolism marker (Tracp 5b, etc.). Third, we did not examine bone strength (e.g., three-point bending test) in this study. This may be a limitation of the mechanical testing device (as the model used in this study is only for one intervertebral disc site due to the amount of grafted bone, we cannot conduct further studies using the mechanical testing device because of insufficient length); however, we plan to increase the number of intervertebral disc sites to examine bone strength using bone graft or artificial bone. Fourth, the rat model for posterior spinal fusion has been previously criticized due to its relatively high fusion occurrence rates compared with those in humans, in which it has been reported to have 40% or more non-union rates in non-instrumented fusion surgery (27). Therefore, we believe that the rat model used may be a limitation in this study. Additionally, romosozumab showed a modest improvement in fusion rates beyond 4 weeks (82.5% vs 97%). An important clinical question is how many of the rats would have had true "non-union" of the

fusion attempt that was avoided by this drug. If all of the rats eventually evolve a fusion, it would be difficult to assess clinical improvement. In human clinical practice, instrumentation is mostly used for spinal fusion, the bone fusion rate of PLF has been reported as approximately 74% 2-3 years after surgery, and there is no discernible difference in clinical outcomes between the union and non-union groups (14, 28, 29). For this reason, we believe that studies using rat models with instrumentation, or animal models described in the literature that document a clinical nonunion mimicking the human condition, should be considered in future research. Fifth, this rat model was not an osteoporotic model; therefore, the results may differ from those using the ovariectomy model. Since the PLF model established in our laboratory was based on this male rat model, we verified the results using the established model first. We plan to use this model for spinal fusion in non-osteoporotic patients. Of note, for the purpose of bone union, this drug is for off-label use; it promotes bone union as a secondary effect of administration in patients with osteoporosis. Therefore, we will consider using the ovariectomy model in the future. Sixth, in Figure 4d, it appears that the fusion is actually across the L3-4 interspace rather than at the L4-5 level. It is obvious that localization imaging is not performed in these small animal surgeries. In the surgeries in this study, we always exposed the superior margin of the sacroiliac joint from both iliac crests to confirm the L4-5 level. However, in three of 20 cases, the graft bone was also at the L3-4 level on immediate postoperative CT images. Because L3, L4, and L5 are functional lower lumbar vertebrae, we did not consider the difference in level in this study and determined that it was also one vertebra at the L4-5 level.

Conclusion

This study examined the effect of romosozumab administration on bone union in a rat lumbar PLF model. We showed that romosozumab administration clearly improved bone union from an

early stage and significantly increased the volume of bone union. Thus, these results suggest that romosozumab administration promotes bone union.

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We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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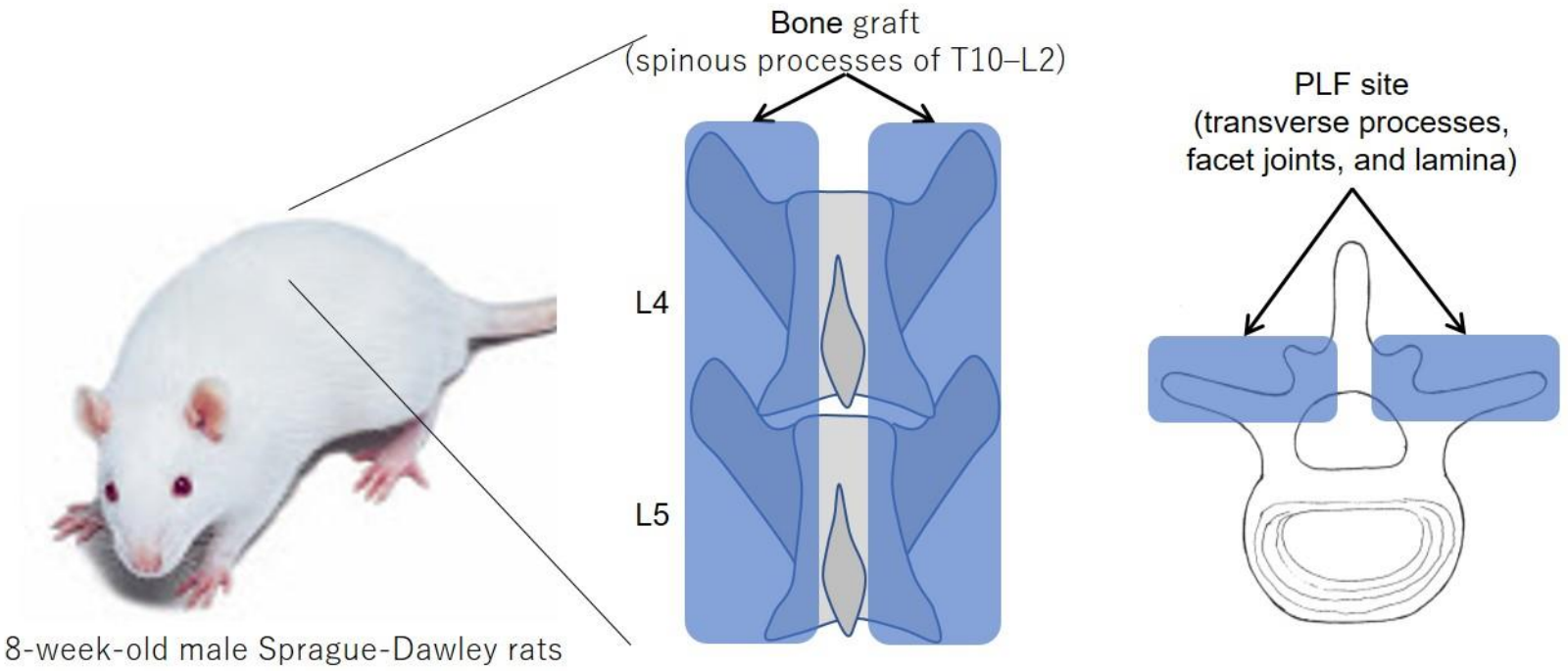
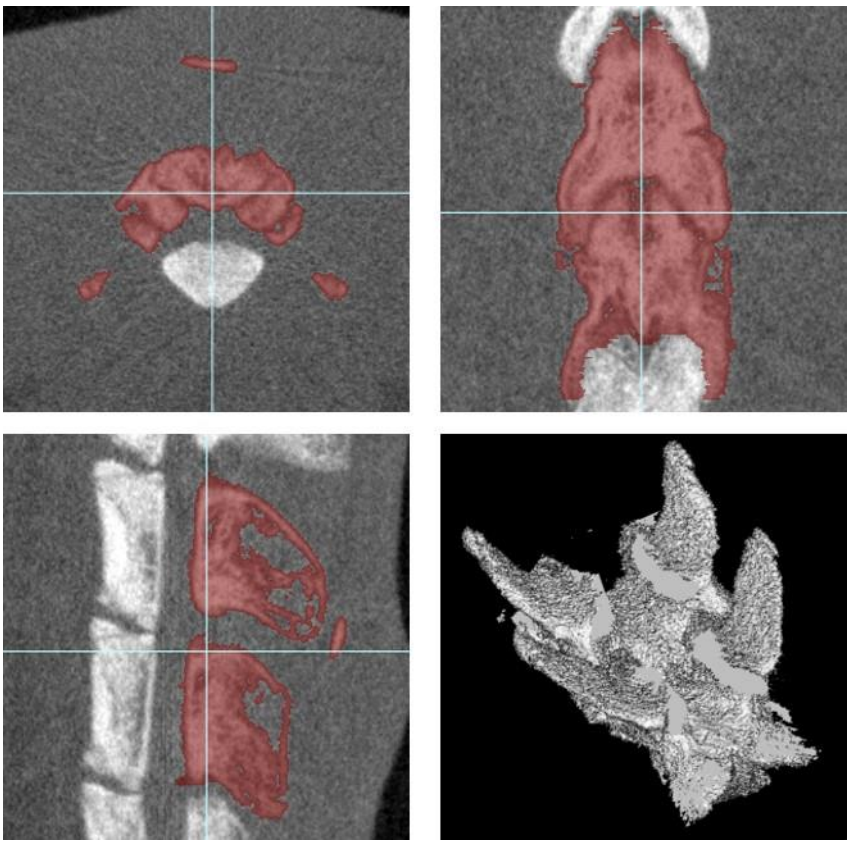
Figure Legends

Figure 1. The 4th/5th posterolateral lumbar fusion (PLF) surgery.



352 Figure 2. Measurement of the volume of the bone union area using Ziostation2 software.



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355 Figure 3. Calculation of the area of a trabecular bone using the hybrid cell count module of the

356 BZ-H4C Analyzer software.

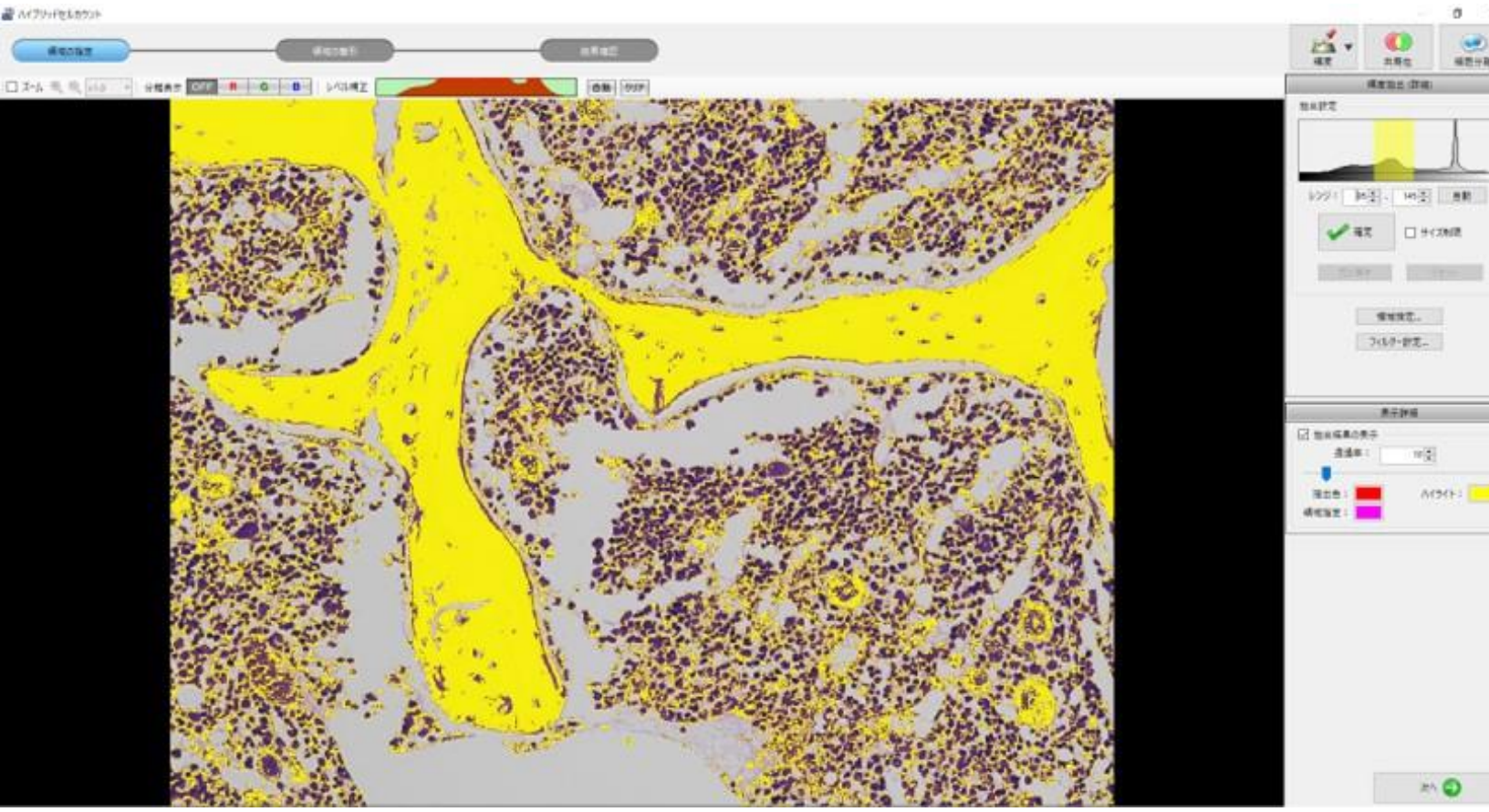

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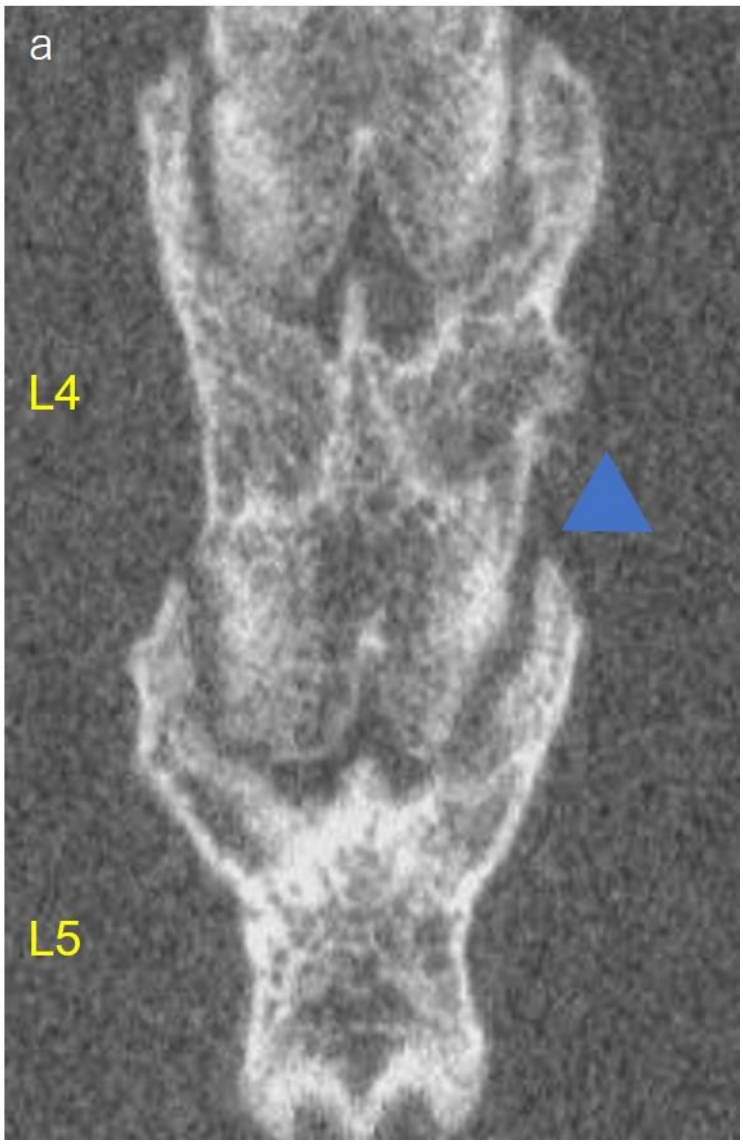
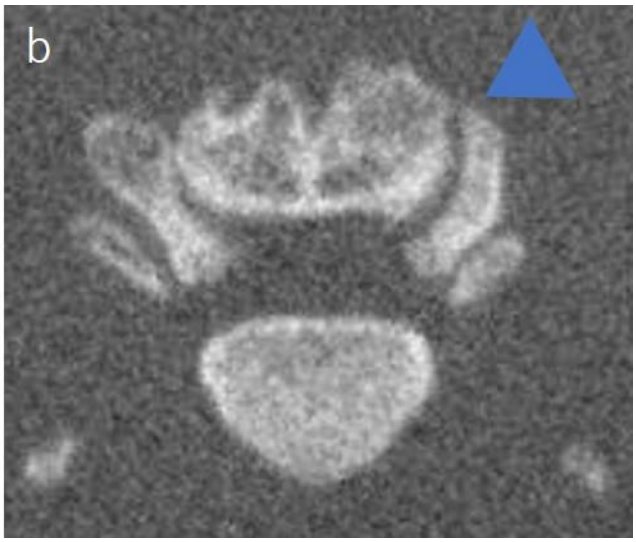
Figure 4. Representative CT sections at week 8 post-surgery. a. Coronal section, C group. b. Axial section of the 4th/5th lumbar intervertebral joint, C group. c. Axial section of the 4th lumbar vertebral arch, C group. d. Coronal section of bone union, R group. e. Axial section of the 4th/5th lumbar intervertebral joint, R group. f. Axial section of the 4th lumbar vertebral arch, R group.





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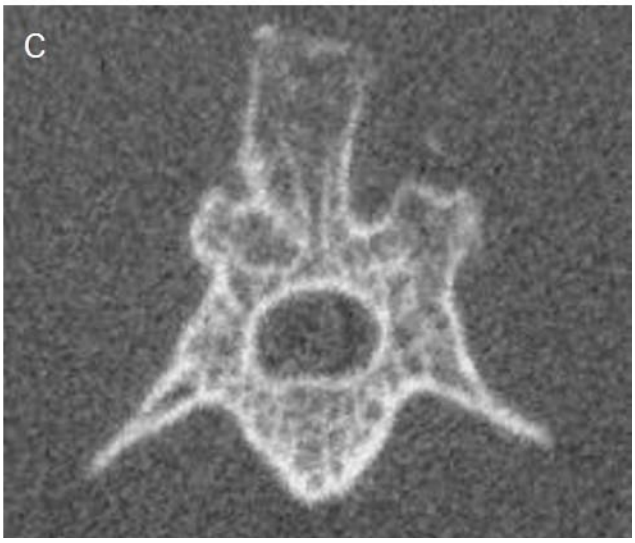

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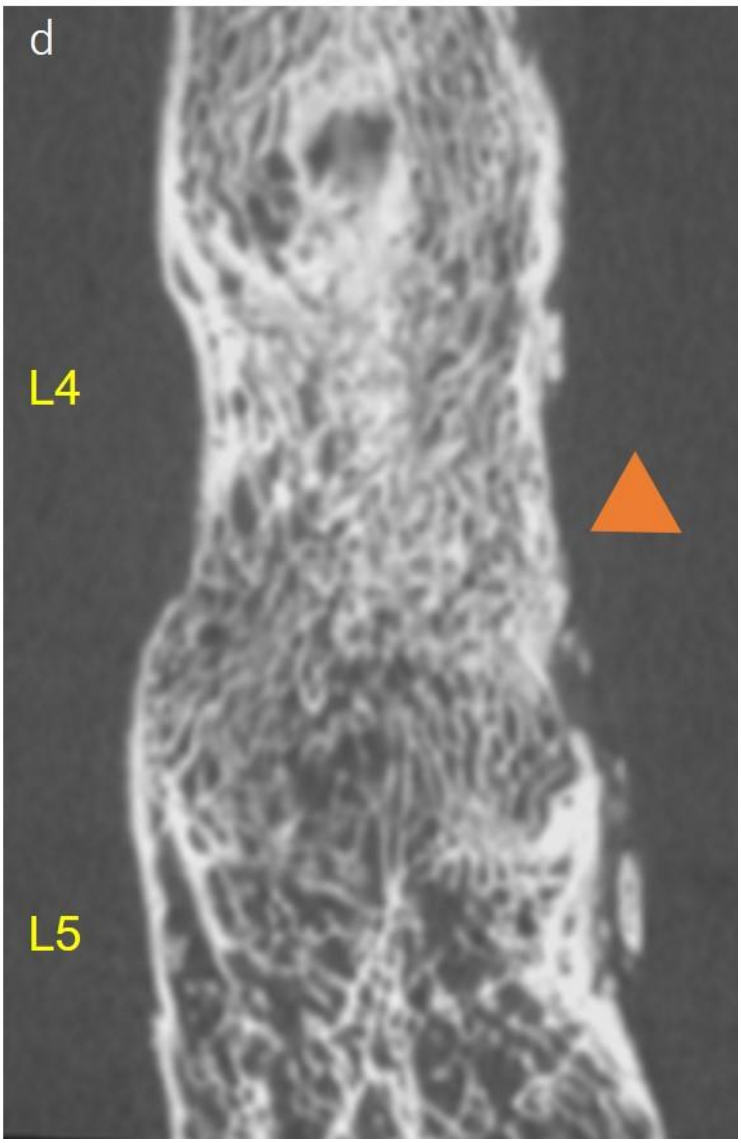

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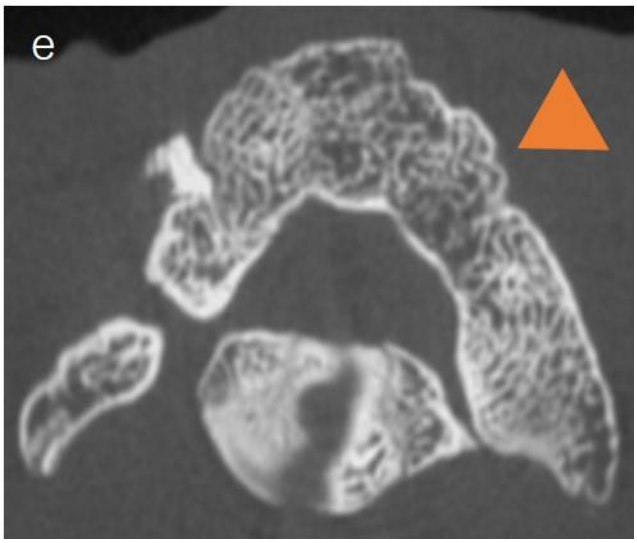

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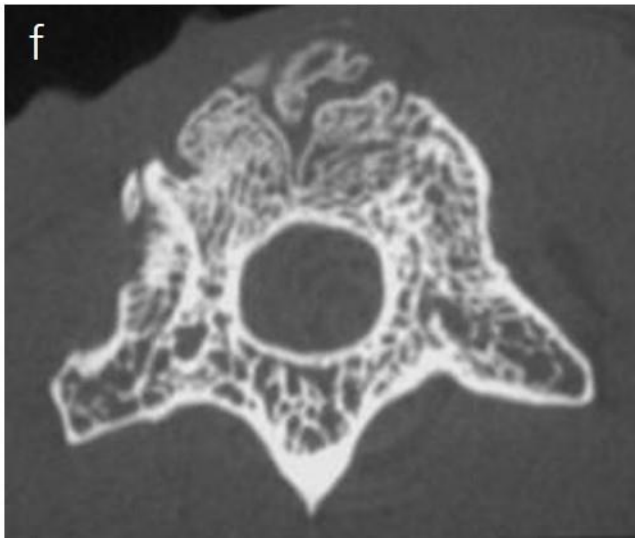
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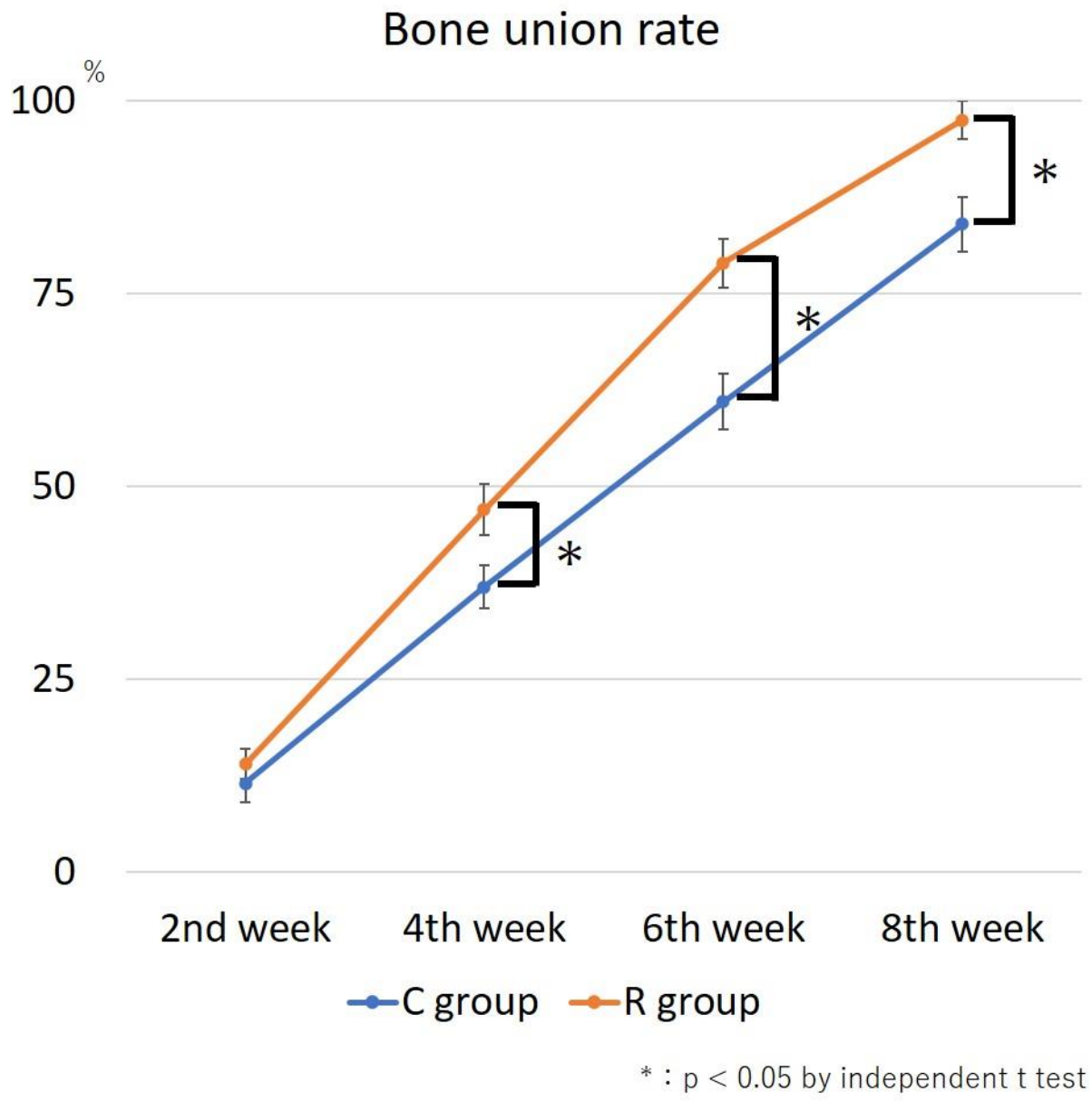
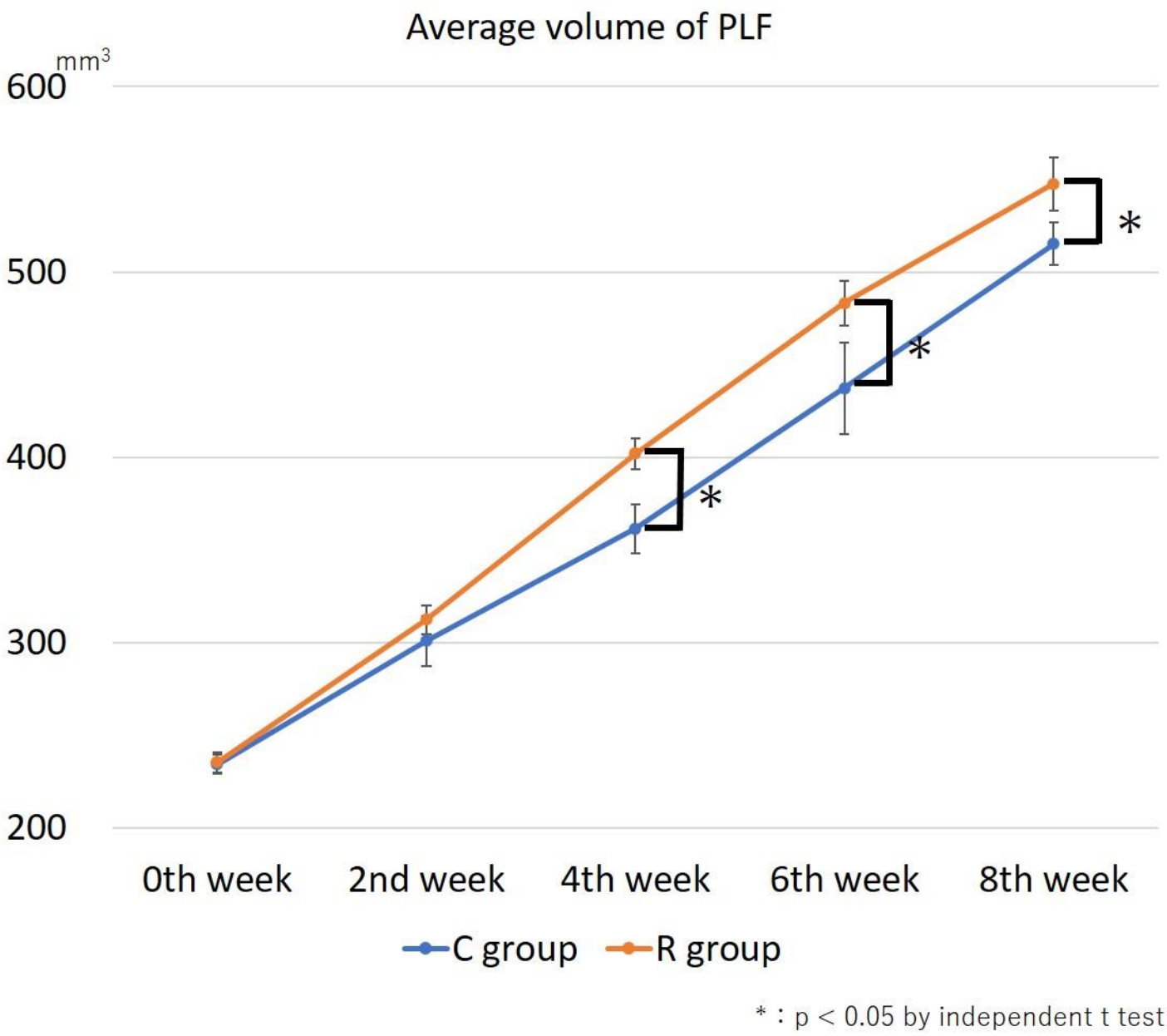
Figure 5. Bone union rate between the intervertebral joint and transverse process in the two groups after surgery.



376 Figure 6. Volume of bone union area after surgery.



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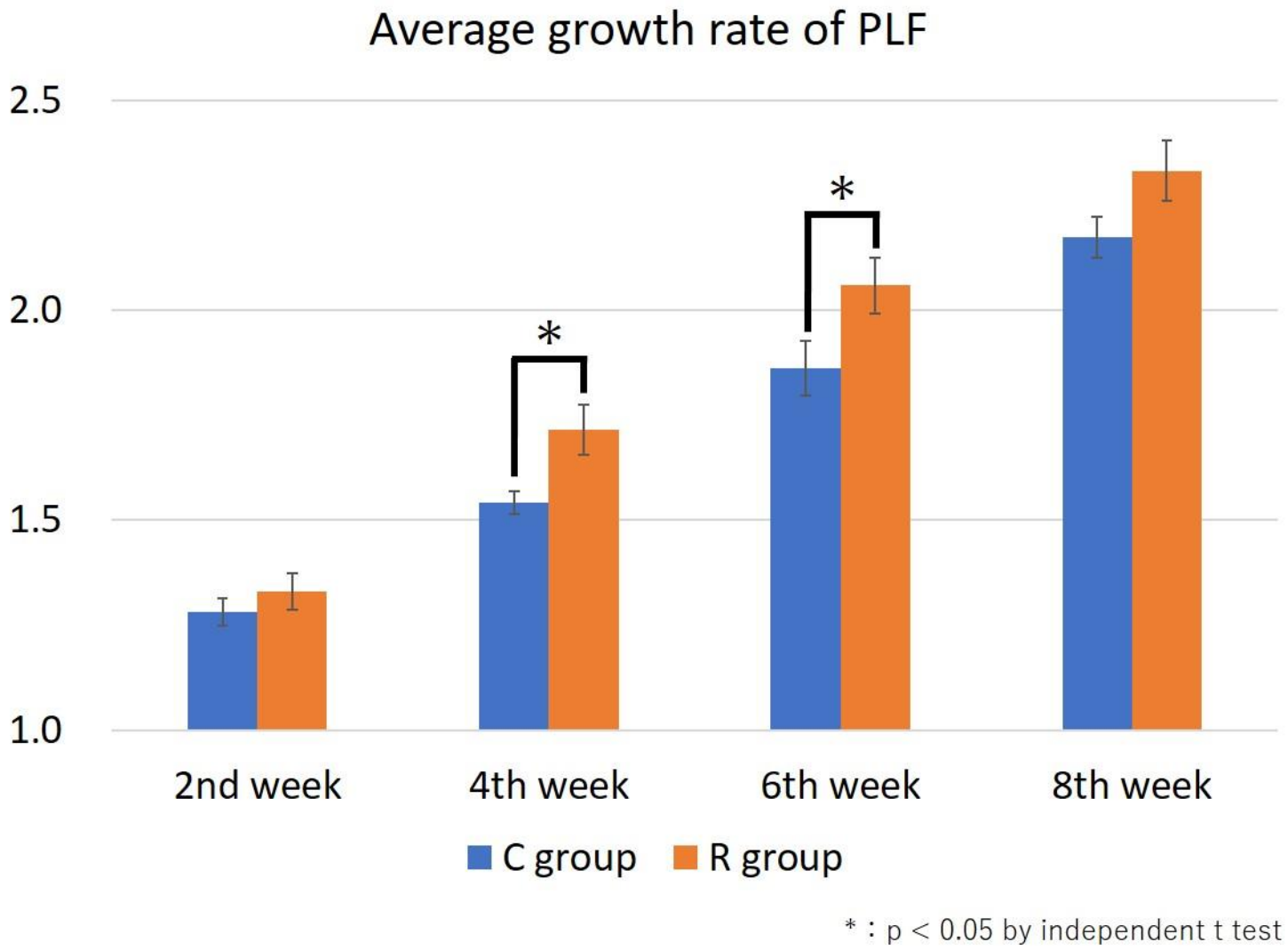
379 Figure 7. Rate of bone union area increase after surgery.



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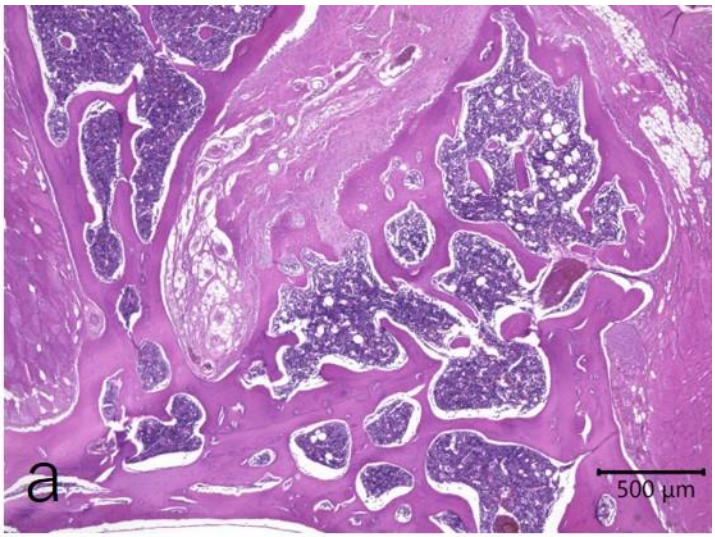
382 Figure 8. Pathological images. a-b. Left vertebral arch of the 4th lumbar vertebra, C group. c-d.

383 Left vertebral arch of the 4th lumbar vertebra, R group. a, c: 4× magnification; b, d: 20×

384 magnification.



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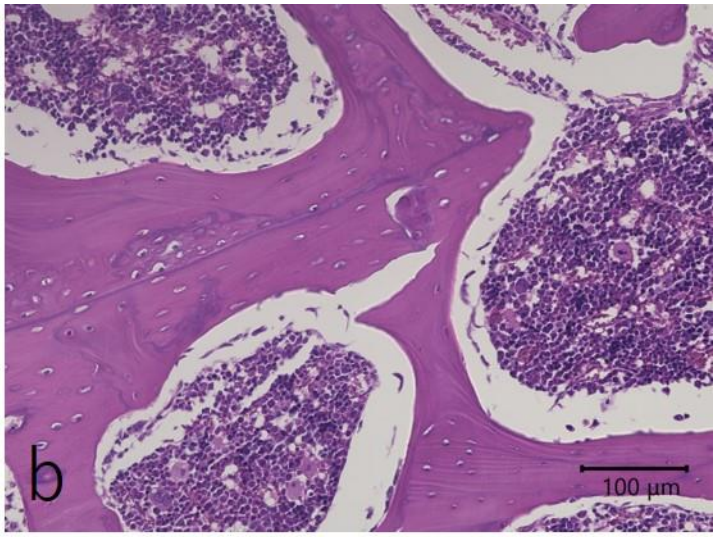

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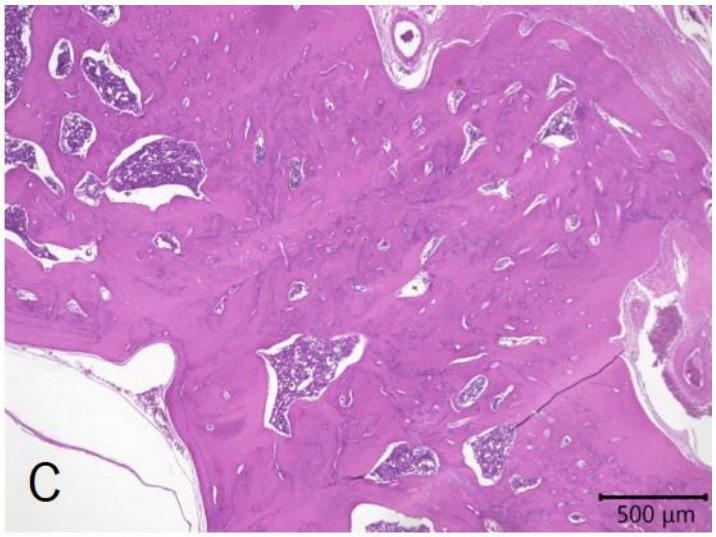

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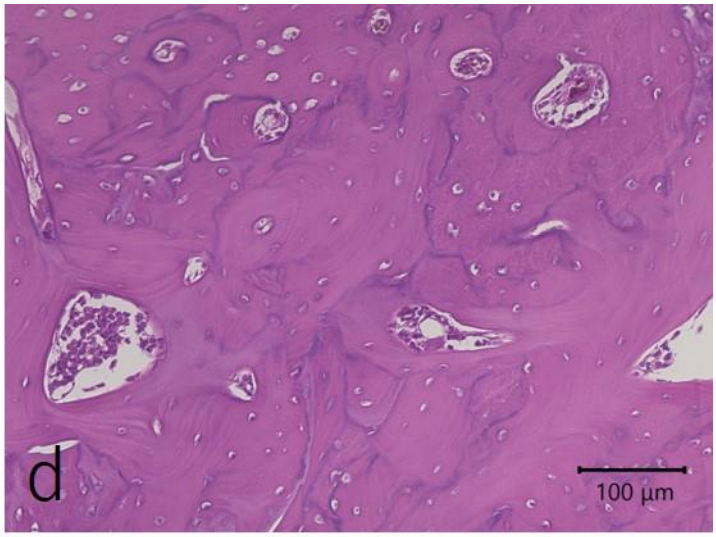

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390 Figure 9. Percentage of bone trabeculae area in the pathological images at 20× magnification.

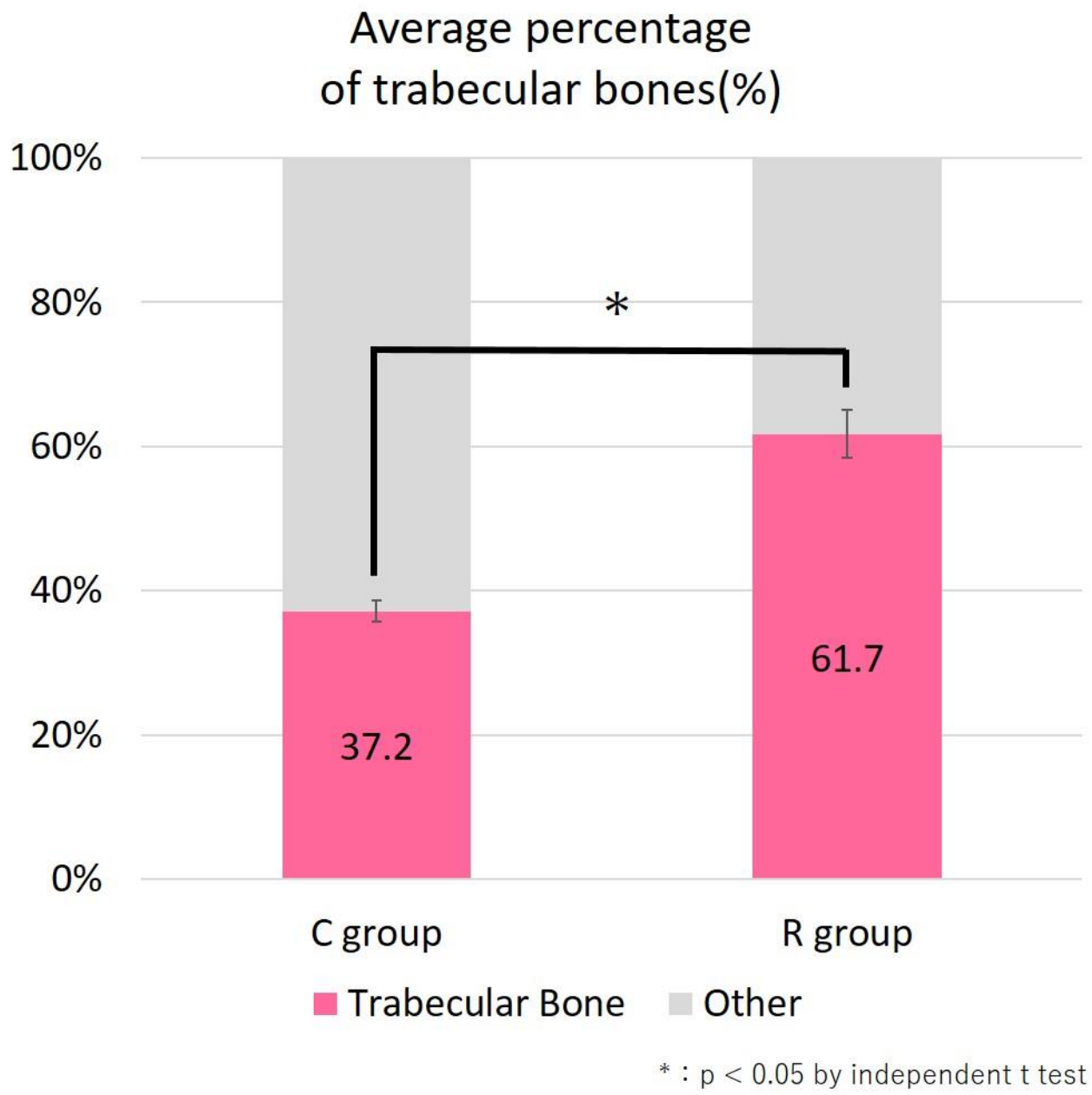

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Table

Table 1. Schedule of CT examination and drug administration