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β-Tricalcium Phosphate Combined with Recombinant Human Bone Morphogenetic Protein-2: A Substitute for Autograft, Used for Packing Interbody Fusion Cages in the Canine Lumbar Spine

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The use of β-tricalcium phosphate (β-TCP) as osteoconductive and bone morphogenetic protein-2 (BMP-2) as osteoinductive bone graft substitutes has recently gained considerable research interest in spine surgery. However, whether the combination can be extrapolated to a successful interbody spinal arthrodesis remains uncertain. In this study, β-TCP combined with recombinant human BMP-2 was examined in the canine lumbar spine model as a substitute for autograft used for packing interbody fusion cages. The discectomy and interbody cage fusion were performed at three disc spaces in 8 dogs. The examination of microradiographs and histological sections of the lumbar spine at 16 weeks post-surgery revealed three fusions in autograft group (A), three β-TCP group (B), and five in β-TCP-BMP-2 (C). The mean percentage of trabecular bone area in the cages was 51.9% in group A, 48.8% in group B and 65.6% in group C. Statistical analyses of the results could not give significance. The young animal model used in this study might suppress difference between the groups. As mentioned, however, actual data of mean percentage of trabecular bone formation and of mechanical stiffness were largest in the cages filled with β-TCP and BMP-2. In clinical settings, we expect that a cage filled with β-TCP and BMP gives beneficial effects especially for patients with poor fusion statuses.

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1. Introduction

The expected clinical advantages of cervical interbody fusion[1,2] after anterior cervical discectomy and osteophytectomy are the immediate stabilization of the cervical spine and preservation of an adequate interbody space and physiological lordosis. Since Cloward,[3] and Smith and Robinson[4] introduced the anterior approaches to the cervical spine, various methods have been introduced to stabilize the cervical spine and preserve the physiologically natural alignment. In most of these methods, autograft has been used to achieve cervical anterior interbody fusion. However, it does not give good results for multi-level fusions. And graft collapse, graft expulsion and pseudarthrosis, have sometimes been noted[5] even in the hands of experienced surgeons.[6] In addition, the use of autogenous iliac crest grafts induces donor-site related complications[1,5] such as wound infection, pelvic fracture, ureteral injury, and donor site pain. The morbidity rate has been reported to be as high as 30%.

Bagby[7] introduced a metallic cage for an interbody fusion. It has been improved to an autostabilizing cage, so additional fixation by an anterior cervical plate is not necessary. Autogenous bone is packed in its inner space to increase the rate of union between adjacent vertebral bodies. The cage demonstrates good clinical outcomes[8–10] and its usage in anterior cervical interbody fusion has been established. However, donor-site related complications still remain to be solved.

Several materials have been examined as a substitute for autogenous bone grafts, such as allograft,[11,12] ceramics,[13,14] and a variety of osteoinductive agents.[15] Some of them have been used clinically and have given acceptable surgical results. In our previous study,[16] synthetic beta-tricalcium phosphate ($Ca_3(PO_4)_2$) (β-TCP) was examined. It gave good outcomes as autogenous bone in the examinations of microradiographs and histological sections. Thus, we expect that β-TCP can be used as a substitute for autogenous bone for the interbody fusion cages. However, the rate of contiguous trabeculation spanning was still not satisfied in the fusion site from the adjacent vertebral bodies in either experimental group. In addition, the rate is expected to become lower in the clinical settings, because most patients who undergo these operations for cervical spondylotic disease are aged and so their natural osteoinductive capacity is decreased. More effective materials should be introduced in the interbody fusion cage surgery.

Recombinant human bone morphogenic protein-2 (rhBMP-2) can induce *de novo* formation of cartilage and bone in subcutaneous sites in the absence of marrow elements.[17] Various materials as a BMP carrier have already been examined, such as β-TCP,[18] hydroxyapatite,[19] biphasic calcium phosphate,[20] collagen,[21] polylactic acid-polyglycolic acid copolymer,[22] and titanium.[23] Several studies[18,24–26] indicate that β-TCP can be used as a slow-release delivery system for BMP and potentiates the activity of BMP. In this study, we examined cages filled with β-TCP and rhBMP-2 to improve the success rate for bony fusion. The biomechanical, radiographical and histological studies were done to examine the efficacy of interbody fusion cages filled with β-TCP and rhBMP-2.

2. Materials and Methods

2.1 Study design

Posterolateral lumbar interbody fusion of three consecutive levels was performed, using titanium cages in 8 adult

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male colony-reared hounds. The first cage was filled with autograft bone (group A), the second with β-TCP (group B) and the third with β-TCP combined with BMP-2 (group C). Mechanical, microradiographical and histological examinations of surgical sites were performed at 16 weeks post-surgery. This protocol was approved by the Animal Care and Use Committee of the Institute for Frontier Medical Sciences of Kyoto University.

2.2 Lumbar interbody fusion

A total of 8 adult male hounds (7-9 months old, 17-18 kg in body weight) from Nosan:NRB (Yokohama, Japan) were used in the current investigation. They were housed in an established animal facility for a minimum of 1 week before surgery to determine their condition and allow acclimatization. Each animal was placed under sedation with an intramuscular injection of ketamine (15 mg/kg body weight) and xylazine (2.5 mg/kg body weight). A venous route was then obtained and kept with a drip infusion of normal saline. Through the venous route, 3 mg/kg body weight of sodium pentobarbital was given. The dogs were then intubated, and intravenous sodium pentobarbital was intermittently administered to maintain a general anesthesia. Prophylactic antibiotics (1 g of intravenous cefalotin) were administered perioperatively. The dogs were shaved, placed in a left decubitus position and prepared in a standard surgical fashion. A dorsal paramidline incision was made on the right side. The ipsilateral lumbar mastoid articular processes were identified and exposed. This bony process is about 1 cm long, contains cancellous bone and is located at the base of the spinous process. Intraoperative fluoroscopy was performed to determine the adequate operating lumbar level. Three consecutive disc spaces (L1/2, 2/3, 3/4, 4/5) were then revealed more thoroughly. Titanium cages of 6-mm-diameter (CCM®) (Fig. 1) and a new type of instrumentation set were kindly donated by A-spine (Oakland, CA). At first, the disc spaces and vertebral endplates were reamed. The reamed channel was tapped, and an interbody fusion cage was inserted into three consecutive disc spaces. At the end of the insertion, the handle must be perpendicular to the spinal axis and parallel to the disc space, to have a well positioned hole in the cage. This cage is a threaded, hollow, porous, titanium-alloy cylinder, which has an open anterior and posterior end.

The animals were divided into three experimental groups. In group A, cages packed with autogenous bone graft were used, in group B, cages packed with TCP were used and in group C, cages packed with a mixture of TCP and rhBMP-2 were used. A disc space without any treatment, that is, a natural disc space, was used as a control. All four models were prepared in each animal and the order of the models was systematically rotated in the series experiment. Autogenous bone graft was collected from adjacent mastoid articular processes, which contained cancerous bone. Then, 0.3 g of β-TCP granules (OSferion®), purchased from Olympus (Tokyo, Japan) was used to pack the cage for groups B and C. Recombinant human BMP-2 (Batch Number 3A11K002; Genetics Institute, Cambridge, MA) was donated by Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan). The BMP-2 was suspended at 1 g/L in a pH 4.5 buffer of 5 mmol/L glutamic acid, 2.5 vol% glycine, 0.5 vol% sucrose and 0.01 vol% Tween 80, and conical tubes containing 0.2 mL of BMP-2 solution were preserved at a temperature of −80°C, and thawed at room temperature just before use. For group C, the β-TCP granules were soaked in 0.2 mL of the BMP-2 solution. Most of the BMP-2 was expected to be absorbed by the β-TCP granules in a few minutes. These granules suspended in the BMP-2 solution were packed into the cage and thus the BMP-2 remaining in the solution was also poured into the cage. So 200 μg of BMP-2 was applied to each cage in group C. Fascia and muscle layers were approximated with a 3-0 silk suture and the skin was closed with a 3-0 nylon suture. The amount of BMP-2 in this experiment was determined in consideration of previous studies using dogs[27] and goats.[28]

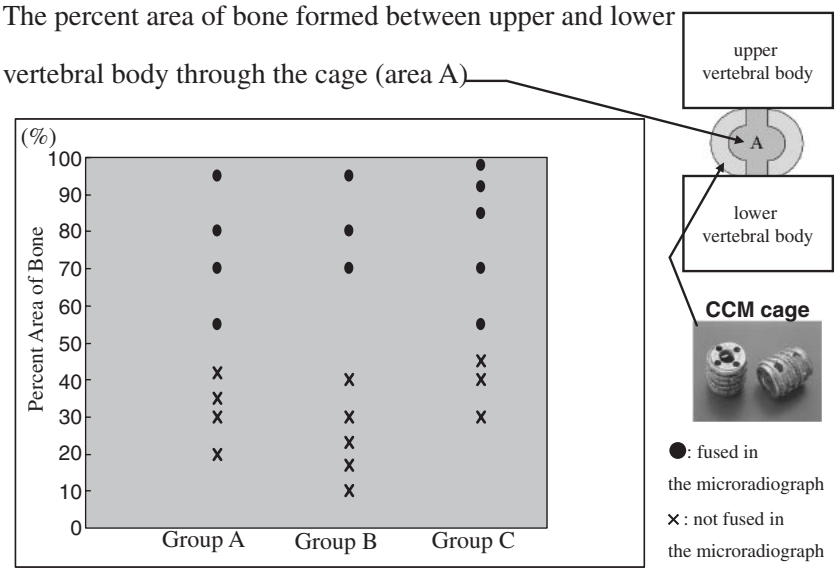


Fig. 1 The percent area (area A) of bone formed between upper and lower vertebral body through the cage (CCM (A-spine) cages of 6-mm-caliber) in three experimental groups.

2.3 Postoperative care

After surgery, the animals were observed until fully recovered from the general anesthesia. Activity was allowed in an indoor pen. Eating habits, ambulatory activities, and the wound condition were checked daily. At 16 weeks post-surgery, the animals were put down using an intravenous injection of an excess amount of sodium pentobarbital. The lumbar spine then was excised and the specimen was radiographed.

2.4 Radiographic analysis

Lateral and anteroposterior radiographs of the lumbar spine were obtained immediately after surgery and thereafter the location of the cages was examined radiographically at 2, 4, 8, 12 and 16 weeks. Radiographs of the lumbar spines excised at 16 post-operative weeks were analyzed for the absence or presence of lucent lines surrounding each cage. If a lucent line was seen on either the anteroposterior or lateral radiograph, it was judged that there is no calcification bridging between the cage and adjacent vertebral bodies. This interpretation is used commonly in the clinical settings. All final interpretations were reported by the attending radiologist.

2.5 Biomechanical testing

In preparation for nondestructive biomechanical testing, the removed lumbar spine was cleaned of soft tissues and disarticulated into two operative motion segments and one adjacent intact motion segment for a control. Care was taken to preserve all ligamentous attachments and not to disrupt the integrity of the fusion site. Both ends of the operative motion segments were fixed to a custom-made biomechanical testing device that was designed to measure the stiffness of axial rotation, flexion-extension, and lateral bending. Functional spinal unit stiffness was calculated as the peak value of the load (newton or newton-meter), which was sensed by a load cell unit (Kyowa Dengyo, Tokyo, Japan), divided by the corresponding segmental displacement (millimeter or degree). In total, 24 disc spaces for the experimental groups (8 in each group) and 8 disc spaces for the control group were biomechanically analyzed. The peak stiffness is used for quantifying the overall rigidity of the functional spinal unit. Although, it must be recognized that the nonlinear elastic behavior of the functional spinal unit can be further divided into neutral zones and a range of motion, these features were not included for discussion in this study.

2.6 Histologic and microradiographic analysis

After nondestructive biomechanical testing, specimens were removed from the testing device. They were fixed with 20% formaldehyde, embedded into polyester resin (Showa Koubunshi, Tokyo, Japan), and cut into sagittal sections of about 400 μm thickness at the level of the fusion site using a diamond saw (Maruto, Tokyo, Japan). These slices were then ground to a thickness of 90 μm using a micro-grinding device (Maruto, Tokyo, Japan). A sagittal microradiograph of the specimen was obtained in a HITEX type HX-100 unit (HITEX CO., LTD., Tokyo, Japan). The specimens were placed 30 cm from the beam source and exposed for 120 seconds, at a peak of 25 kV and 3 mA. The area of bone formed between upper and lower vertebral body through the cage (Fig. 1) was evaluated from the resultant high resolution microradiographs using a computerized histomorphometric system (NIH Image Analysis). It was also used for evaluation of the fusion status. A successful fusion was implied by the existence of contiguous trabeculation stretching through the cage from the adjacent vertebral bodies. Next, the specimens were stained with toluidine blue for histological examination.

3. Results and Discussion

3.1 Surgical procedure

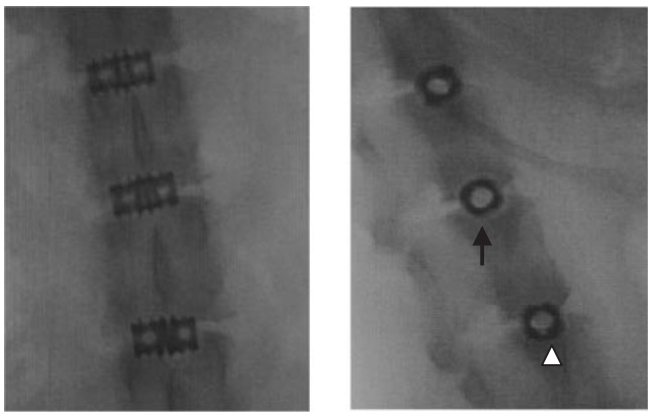
All 8 dogs successfully underwent an operation and survived without any difficulties during 16 post-operative weeks. Neither vascular nor neurologic complications were observed. No complication related with surgical site infection was detected.

3.2 Radiographic results

None of the cages were displaced during the post-operative observation period. Radiographs of lumbar spines excised at 16 post-operative weeks were analyzed for the absence or presence of lucent lines surrounding each cage as seen in Fig. 2. Lucency around the cage was seen in three cases (37.5%) in group A, four cases (50.0%) in group B, and only one case (12.5%) in group C. So, from the radiographic studies, the fusion rate (per disc space) was 62.5% for group A, 50.0% for group B and 87.5% for group C.

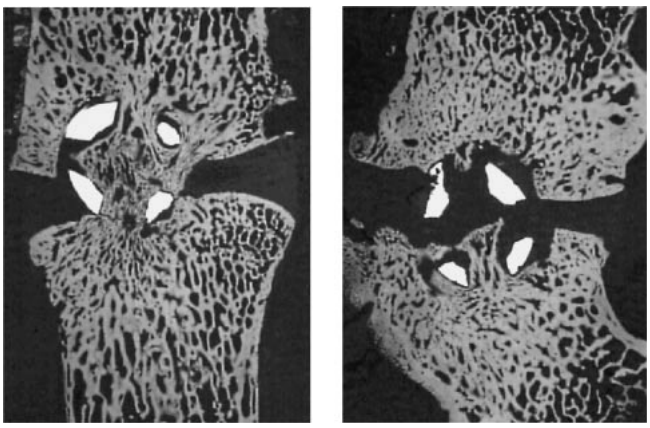
3.3 Microradiographic and histologic results

No TCP remnant was recognized in any cages after 16 post-operative weeks. The combined indications obtained from examinations of micro-radiographs (Fig. 3) and histologic sections (Fig. 4) demonstrated three fusions in eight operations (37.5%) in group A, three in eight (37.5%) in group B and five in eight (62.5%) in group C. The macroscopic radiographs of the lumbar spines shown in Fig. 1 gave higher fusion rates than the microscopic examinations in all of the experimental groups. Spatial resolution of the macroscopic radiographs limits finding of a



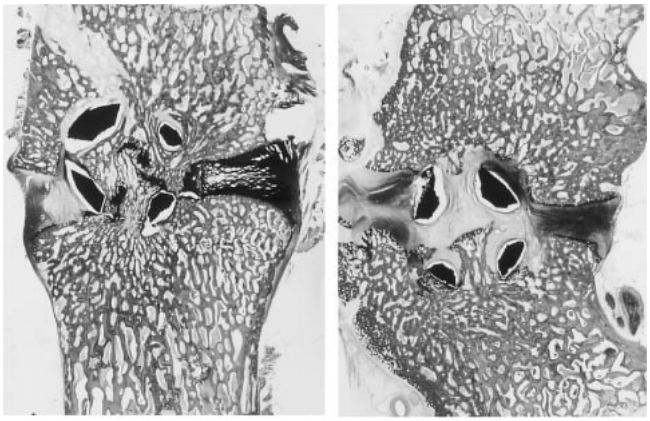
Anteroposterior View Lateral View

Fig. 2 Lateral and anteroposterior radiographs of the lumbar spine excised at 16 post-operative weeks. The radiograph showed lucency (arrow) and no lucency (arrow-head) around the cages.



(a) (b)

Fig. 3 Midsagittal microradiographs of the lumbar spine excised at 16 post-operative weeks. These images reveal examples of union (a) and nonunion (b). In all specimens defined as fused, there was definitive evidence of a continuous formation of trabecular bone from within the CCM cage itself to the adjacent vertebral bodies, as is shown.



(a) (b)

Fig. 4 Midsagittal histologic sections (magnification × 1; stained with toluidine blue) of the lumbar spine excised at 16 post-operative weeks. These images reveal examples of union (a) and nonunion (b). In all specimens defined as union, there was a well-organized network of woven trabecular bone through the CCM device and spanning the fusion site.

thin layer without bone formation on the cage. We anticipate that the absence of lucent lines surrounding a cage does not always mean sufficient bone formation between the cage and adjacent vertebral bodies. This discrepancy should be carefully paid attention, because we judge bone fusion from the examination of lucent lines in the clinical settings. In most cases, the cage that has lucency on the radiogram is thought to be a failed fusion from the microradiogram.

The mean percentage area of trabecular bone formed in the cage was $51.9 \pm 22.3\%$ in group A, $48.8 \pm 29.6\%$ in group B and $65.6 \pm 23.8\%$ in group C on histomorphometry. These results are well collated with the fusion rates obtained from the microradiographic and histologic examinations.

3.4 Biomechanical analysis

Figure 5 shows representative data of nondestructive

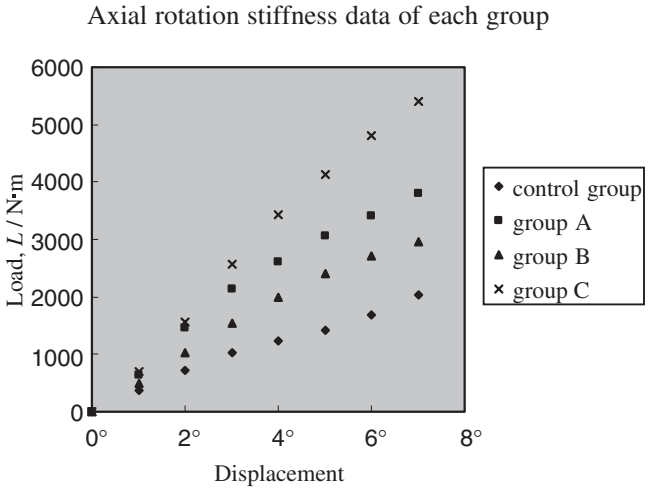


Fig. 5 Representative data of the axial rotation biomechanical test in each group.

biomechanical testing of the operated lumbar functional spinal units. With these nondestructive methods, fusion site disruption was not observed in any of the specimens tested. The results reveal that the three experimental groups (group A, B and C) demonstrated significantly higher mean values of functional unit stiffness than those observed in natural intact spine in all three testings (Fig. 6). The data of axial rotation and flexion-extention stiffness (Figs. 6(a) and (b)) showed that group C had a tendency of superior mechanical stiffness compared with group A and B. On the other hand, the data of lateral bending stiffness (Fig. 6(c)) revealed that there was no remarkable difference among three groups. We thought that it was because the stiffness of the cage itself might play a dominant role in the stiffness of lateral bending. The lumbar spines with a cage with a successful arthrodesis to be stiffer than those with a cage with less trabecular bone formation in all experimental groups as seen in Fig. 6.

In our previous study,[16] the interbody fusion cages filled with β-TCP demonstrated comparable efficacy to the cages with autogenous bone in the fusion rate of union between adjacent vertebral bodies. It has been reported that BMP can induce *de novo* bone formation in subcutaneous sites in the absence of marrow elements. Supplement of BMP to β-TCP in the cage is expected to give additional effect on bone formation. This study demonstrated that the cages filled with β-TCP and BMP-2 had a tendency of superior mechanical stiffness and higher fusion rates compared with the cages with autograft bone or the cages with β-TCP. However, statistical analyses could not give significance between any combination of these groups in all of examinations. Usage of young health dogs might suppress difference between the groups in efficacy of bone formation. In clinical settings, most patients are elder and some of them have additional poor fusion statuses, such as the smoking habit or those who have undergone hemodialysis for a long time. We expect that cages filled with β-TCP and BMP gives beneficial effects especially for such patients. It should be carefully examined using an appropriate animal model in future.

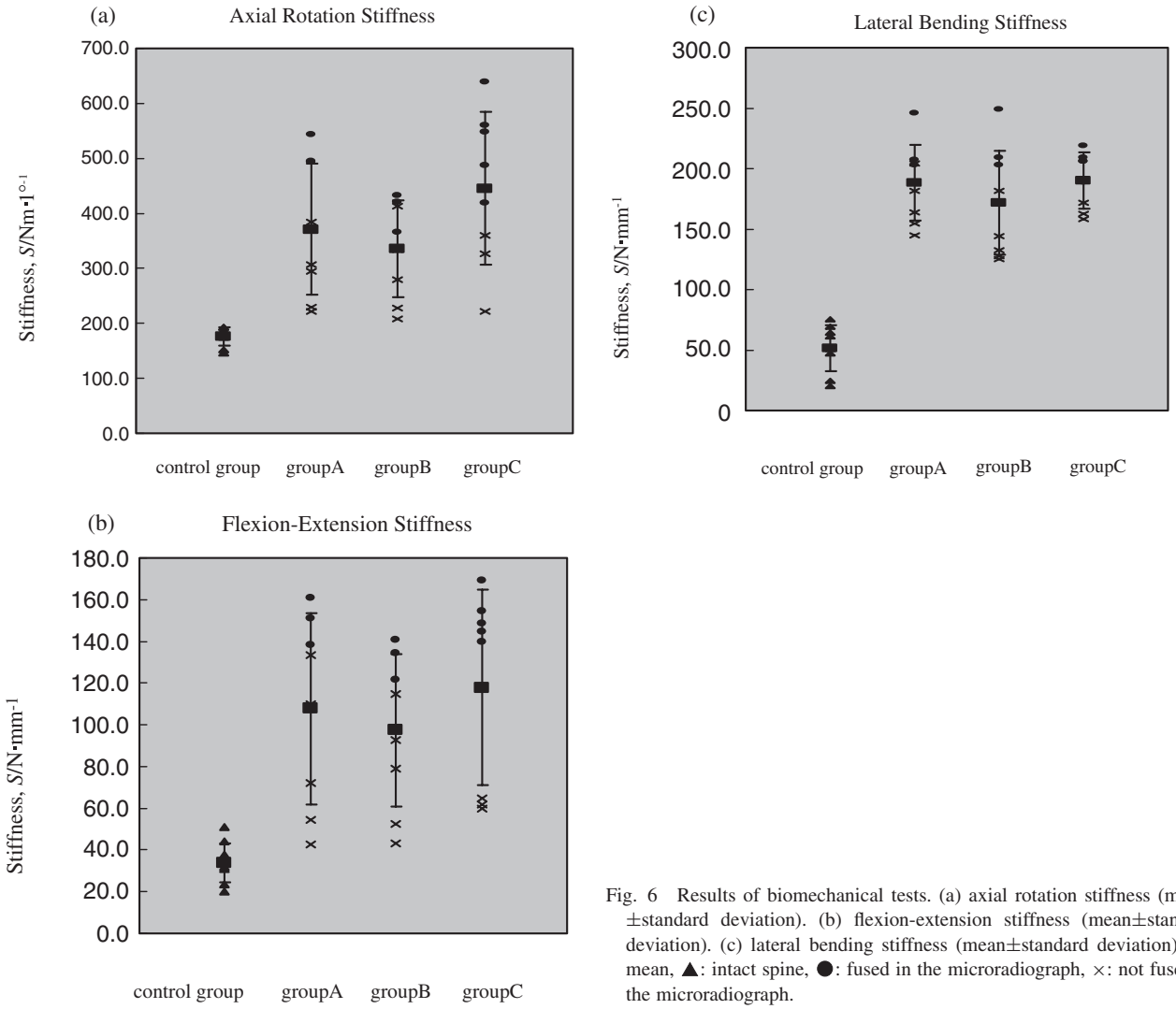


Fig. 6 Results of biomechanical tests. (a) axial rotation stiffness (mean±standard deviation). (b) flexion-extension stiffness (mean±standard deviation). (c) lateral bending stiffness (mean±standard deviation). ■: mean, ▲: intact spine, ●: fused in the microradiograph, ×: not fused in the microradiograph.

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