学位論文

Addition of a Vascular Bundle Accelerates Bone Union in Femoral Bone Defects

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Original Article

Addition of a Vascular Bundle Accelerates Bone Union in Femoral Bone Defects

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Abstract

Background The Masquelet method has become increasingly popular for the treatment of bone defects in recent years. In this method, an induced membrane (IM) with abundant blood circulation, stem cells, and osteogenesis-promoting factors is formed by implanting bone cement during the first surgery. This IM stimulates bone formation in the bone defect after implantation of the bone graft during the second surgery. However, the Masquelet method requires two surgeries and thus a longer treatment period. In the present study, we investigated whether bone defects could be reconstructed in a single surgery by introducing a vascular bundle into the bone defect as an alternative to the IM, in addition to bone grafting.

Methods Thirty-six 12-week-old female Sprague-Dawley rats were used. After creating a 5-mm long bone defect in the femur, a mixture of autologous and artificial bone was grafted into the defect, and a saphenous arteriovenous vascular bundle was introduced. The animals were divided into three groups: the control group (bone defect only), the BG group (bone grafting only), and the BG + V group (bone grafting + vascular bundle introduction). After surgery, radiological and histological evaluations were performed to assess osteogenesis and angiogenesis in bone defects.

Results In the BG + V group, significant bone formation was observed in the bone defect on radiological and histological evaluations, and the amount of bone formation was significantly higher than that in the other two groups. Furthermore, cortical bone continuity was observed in many specimens in the BG + V group. On histological evaluation, the number of blood vessels was also significantly higher in the BG + V group than in the other two groups.

Conclusion Our results suggest that the introduction of a vascular bundle in addition to bone grafting can promote bone formation in bone defects and allow for complete bone defect reconstruction in a single surgery.

Keywords
- Masquelet method
- bone defect
- vascular bundle
- diamond concept

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Fractures and nonunions associated with massive bone defects caused by high-energy trauma are often difficult to treat. Vascularized autologous bone grafts, allogeneic bone grafts, the Irizarov method, and the Masquelet method are among the strategies employed for these conditions.[1–4] In particular, several recent studies have highlighted the effectiveness of the Masquelet method for the treatment of bone defects.[4–11] The Masquelet method requires two stages of surgery. In the first stage, bone cement is implanted into the bone defect, and external or internal fixation is performed to form an induced membrane (IM) around the cement. The IM exhibits abundant blood flow and is considered to contain many osteogenesis-promoting factors and stem cells. In the second stage, the implanted cement is removed, and the vacant region surrounded by the IM is filled with cancellous bone chips, although sometimes artificial bone chips are also added to compensate for insufficient autologous bone volume. Bone formation is achieved using implanted cancellous bone and artificial bone as a scaffold. However, the necessity of two-stage surgery is one of the disadvantages of the Masquelet method because it requires a longer treatment period.

So far, we have investigated the value of allogeneic bone implantation in bone defect reconstruction. In the relevant experiments, we attempted to convert a non-vascularized allo-bone graft into live bone by introducing a donor's vascular bundle into the graft, which was indeed successful in stimulating vascularization and bone formation within the graft.[12–14] Based on our experience, we believe that this technique can be applied to the Masquelet method. Specifically, we hypothesized that introducing a vascular bundle instead of an IM would lead to vascularization of the cancellous bone implanted into the bone defect, stimulating bone formation in the defect. Verification of this hypothesis would suggest that bone defects can be reconstructed in a single surgery.

Therefore, using a rat model of a femoral bone defect, the present study aimed to verify whether such defects can be reconstructed in a single surgery by introducing a saphenous vascular bundle into the defect after filling it with autologous and artificial bone chips.

Methods

Animal Model

Thirty-six 12-week-old female Sprague-Dawley rats provided by Japan SLC (3371-8 Kotoh-cho,Nishi-ku,Hamamatsu, Shizuoka 431-1103, Japan) were used for the current study. Rats were kept in cages during the experiment (floor area: 988 cm^2; height: 18 cm) and allowed ad libitum access to water and feed (Oriental Yeast Co., Tokyo, Japan). This in vivo study was approved by the Institutional Animal Care and Use Committee of our institution and performed in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

Preparation of Autologous and Artificial Bone Grafts

Sources of cortical and cancellous bone for autologous bone grafts were obtained from a part of the femur, which was excised at the time of bone defect generation. A sponge-like hydroxyapatite/collagen (HAp/Col) source (Refit; HOYA Technosurgical, Tokyo, Japan) was used to create artificial bone grafts. In the dry state, 1 cm^3 of HAp/Col was cut into 16 equal pieces on a strip to make a block of $2.5 \times 2.5 \times 10\ mm^3$ for use during the experiment. The autologous and artificial bones were crushed, mixed together, and implanted into the bone defect.

Research Design and Surgical Method

In this study, we divided the animals into one control group ($n = 12$: bone defect only) and two experimental groups (BG and BG + V, $n = 12$ each) (►**Table 1**). The BG group underwent autologous and artificial bone grafting only, while the BG + V group underwent autologous and artificial bone grafting plus vascular bundle introduction.

Prior to surgery, rats were anesthetized via inhalation of sevoflurane (Japanese Pharmacopeia Sevoflurane FUJIFILM, Hyogo, Japan), which was administered using an inhalation anesthesia machine (MK-A100W; Muromachi Kikai Co., Ltd. Tokyo, Japan) designed for use in laboratory animals. Rats were placed in the introduction box, and anesthesia was introduced at a concentration of 5.0% and a flow rate of 1.5 L/min. Anesthesia was maintained by switching to a mouthpiece that provided a sevoflurane concentration of 2.5% at a flow rate of 1.5 L/min. A 3-cm skin incision was made on the medial side of the left thigh, the subcutaneous tissue was dissected, and the saphenous arteriovenous bundle was elevated with the surrounding fascia. The femur was then exposed by splitting between the vastus medialis muscle and the abductor longus, and a 5-mm length of bone was excised from the mid-portion of the femur using a bone saw. The divided femur was then fixed with an intramedullary nail using a 1.0-mm diameter partially threaded Kirschner wire (K-wire) while maintaining a 5-mm gap. The bone defect was wrapped with a silicone tube (diameter: 6 mm, thickness:

Table 1 X-ray results

Weeks	Group (12 each)	New bone formation	Cortical bone continuity
	Control	0(0%)	0(0%)
2	BG	0(0%)	0(0%)
	BG + V	0(0%)	0(0%)
	Control	0(0%)	0(0%)
4	BG	3(25%)	0(0%)
	BG + V	6(50%)	3(25%)
	Control	2(17%)	0(0%)
8	BG	5(42%)	3(25%)
	BG + V	9(75%)	7(58%)
	Control	4(33%)	0(0%)
12	BG	5(42%)	3(25%)
	BG + V	9(75%)	7(58%)

Abbreviations: BG, bone grafting only; BG + V, bone grafting + vascular bundle introduction.

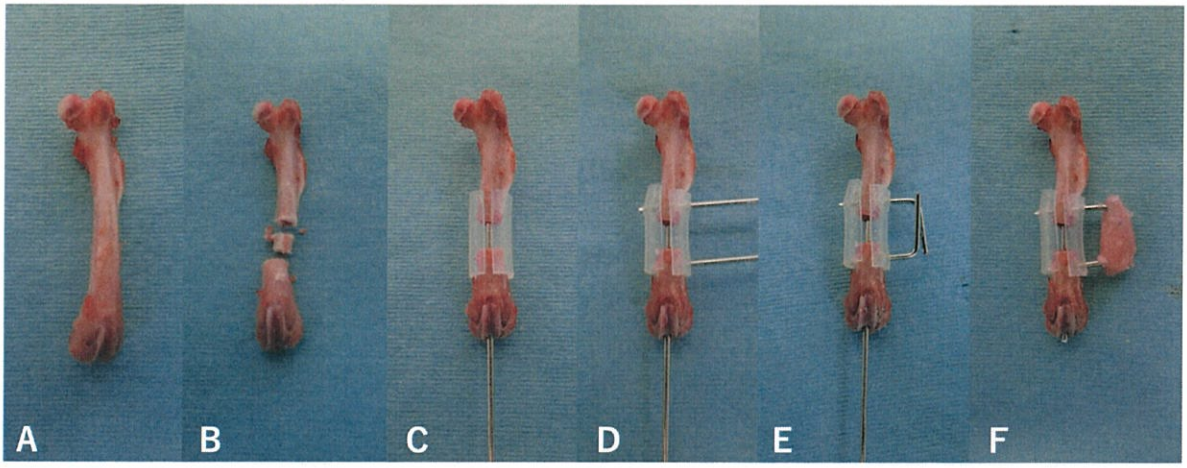

Fig. 1 Surgical procedure to create a bone defect model. (**A**) Left femur. (**B**) A 5-mm length of bone was excised from the middle-portion of the femur. (**C**) The divided femur was fixed with an intramedullary nail, and the bone defect was wrapped with a silicone tube containing a slit. (**D**) Two Kirschner wires (K-wires) were inserted vertically into the proximal and distal parts of the femur. (**E**) The K-wires were bent to 90 degrees and (**F**) connected with resin.

2 mm; AS ONE Corporation, Osaka, Japan) containing a slit, and two 1.0-mm diameter partially threaded K-wires were inserted vertically into the proximal and distal parts of the femur. The K-wires were then connected with resin (UNIFASTII, GC Corporation, Tokyo, Japan) after bending to 90 degrees (►**Fig. 1**). In the control group, only the above procedure was performed, and bone grafting was not performed. In the BG group, a mixture of autologous and artificial bone was implanted into the bone defect. In the BG + V group, autologous and artificial bones were similarly implanted, and a saphenous arteriovenous bundle was introduced into the slit of the silicone tube and fixed by suturing with 5–0 nylon thread (►**Figs. 2** and **3**).

X-Ray Evaluation

At 2, 4, and 8 weeks postoperatively, rats were anesthetized via inhalation of sevoflurane and fixed on the cassette, following which lateral plain X-ray radiographs of the left femur were obtained. The imaging conditions were 0.35 mV and 1 mA. Twelve weeks postoperatively, the whole left

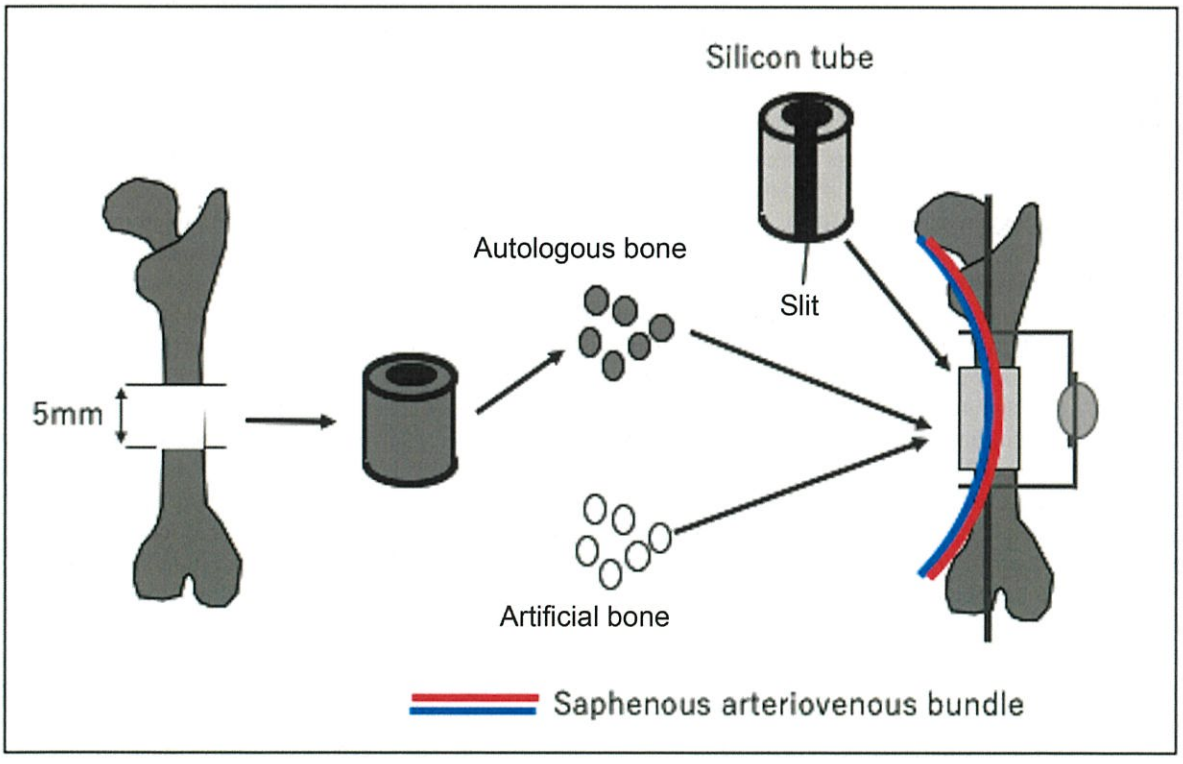

Fig. 2 Bone graft procedure and introduction of vascular bundle. Autologous and artificial bones were implanted into the bone defect, and a saphenous arteriovenous bundle was introduced into the slit of the silicone tube.

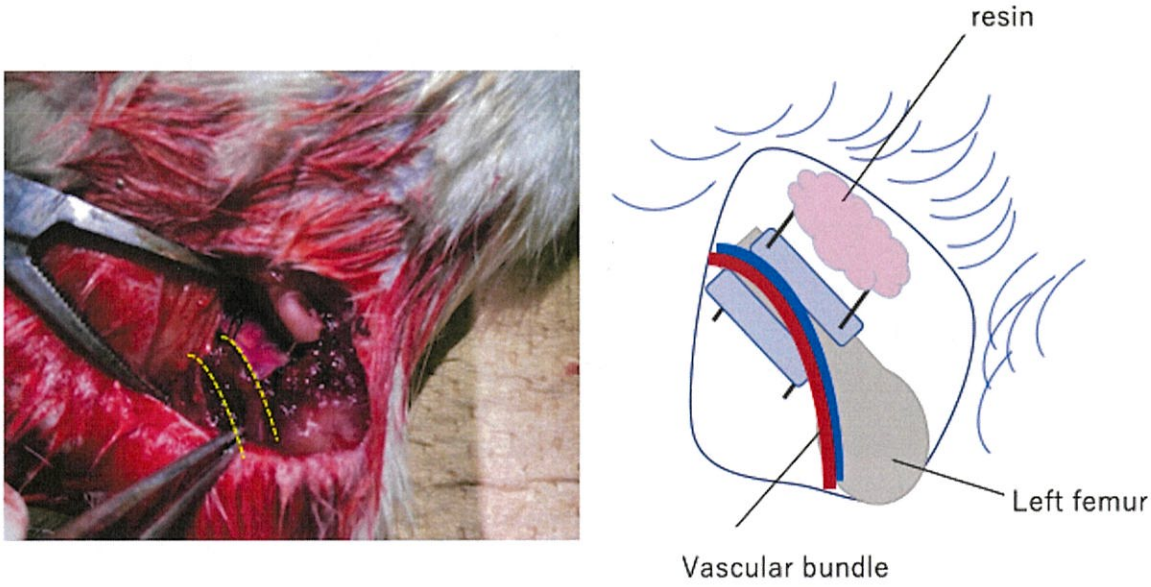

Fig. 3 Intraoperative findings. The dotted lines indicate the introduced saphenous vascular bundle.

femur, including the bone defect, was collected from the rats, and plain X-ray radiographs were taken under the same imaging conditions. The number of rats in which new bone formation and cortical bone continuity were observed was counted in each group.

Histological Preparation

After radiography was performed 12 weeks postoperatively, the whole left femur of the rat was cut at ~2 mm proximal and distal to the bone defect using a bone saw. The bones were fixed with a 10% formalin solution, decalcified with ethylenediaminetetraacetic acid for 4 weeks, and embedded in JB-4 glycol methacrylate (JB-4 Embedding Medium, Polysciences, Warrington, PA). The samples were cut to a thickness of 5 µm using a microtome in the long axis direction and stained with hematoxylin and eosin (H&E).

Histological Examination

Histological examination was performed using an all-in-one microscope (FSX100, Olympus Corporation, Tokyo, Japan) and histological morphometry software (Cell Sens Dimension Desktop 1.14; Olympus Corporation, Tokyo, Japan). The area of new bone formation was measured in the bone defect, and the percent bone area (%BA: new bone formation area/bone defect area × 100) was calculated. We also counted the number of newly formed blood vessels in the bone defect by determining the luminal structure with erythrocytes. The number of blood vessels (NBV: total number of blood vessels/bone defect area) was also calculated.

Statistical Analysis

Statistical analyses were conducted using SPSS Statistics Ver. 26 for MAC (IBM, Armonk, NY). Differences between the treatment groups were analyzed using a one-way analysis of variance, and Dunnett's test was used to determine the significance of differences between the two groups. Statistical significance was set at $p < 0.05$.

Results

Patency of Introduced Vascular Bundles

In the BG + V group, bleeding was observed from the distal portion of all introduced vascular bundles at the time of bone collection at 12 weeks postoperatively.

X-Ray Results

The detailed radiological results are presented in ►**Table 1**. Two weeks postoperatively, no shortening of the bone defect or new bone formation was observed in any of the groups. The implanted autologous bones remained intact in the BG and BG + V groups. At 4 weeks, the control group exhibited no new bone formation in the bone defects. However, new bone formation was observed in 25 and 50% of animals in the BG and BG + V groups, respectively. Cortical bone continuity was observed only in some animals in the BG + V group. Although some control animals exhibited a small amount of new bone formation in the bone defect at 8 weeks, no cortical bone continuity was observed. New bone formation was observed in 42 and 75% of animals in the BG and BG + V groups, respectively. Furthermore, cortical bone continuity was observed in 33 and 58% of animals in the BG and BG + V groups, respectively. At 12 weeks, although there was a slight increase in the number of animals with new bone formation in the control group, the amount of bone formation was still low, and no cortical bone continuity was observed. In the BG and BG + V groups, the number of animals with new bone formation and cortical bone continuity remained the same at 8 weeks, although the amount of bone formation increased (►**Fig. 4**).

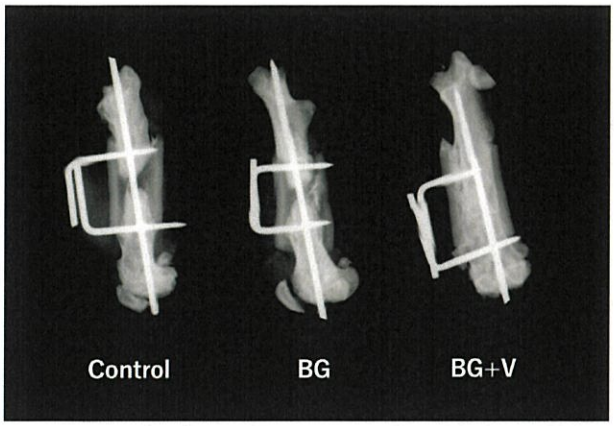

Fig. 4 X-ray images obtained 12 weeks postoperatively. In the control and BG groups, a small amount of bone formation was observed in the bone defect, but cortical bone continuity was not observed. In the BG + V group, abundant bone formation and cortical bone continuity were observed. BG, bone grafting only; BG + V, bone grafting + vascular bundle introduction.

Histological Results at 12 Weeks

In the control group, the bone defect was occupied mainly by fibrous tissue, and the %BA was 3.14%. In the BG group, residual autologous implanted bones were still observed in the bone defect and were surrounded by fibrous tissue. The % BA in this group was 5.38%. In the BG + V group, most of the implanted autologous bone had already been resorbed, and obvious cortical bone continuity was observed in 8 of 12 cases. Normal bone marrow-like tissues were also observed. The %BA in this group was 19.61%, which was significantly higher than that in the control and BG groups (►**Fig. 5**). NBV was 25.75/mm^2 in the control group, 34.75/mm^2 in the BG group, and 45.78/mm^2 in the BG + V group. The NBV in the BG + V group was significantly higher than that in the control and BG groups (►**Fig. 6**, **7**).

Discussion

Growth factors, osteogenic cells, osteoconductive scaffolds, and the mechanical environment are crucial factors for bone regeneration. Together, these four factors represent the diamond concept, the importance of which Giannoudis et al discussed for the treatment for nonunion.[15] Among the various techniques used to regenerate bone in patients with fractures exhibiting large bone defects, the Masquelet method is considered to incorporate these four factors.

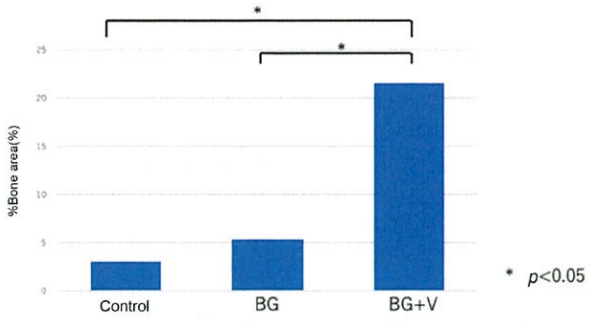

Fig. 5 Comparison of the percent bone area (%BA) among the groups. The %BA of the BG + V group was significantly higher than that of the control and BG groups BG, bone grafting only; BG + V, bone grafting + vascular bundle introduction.

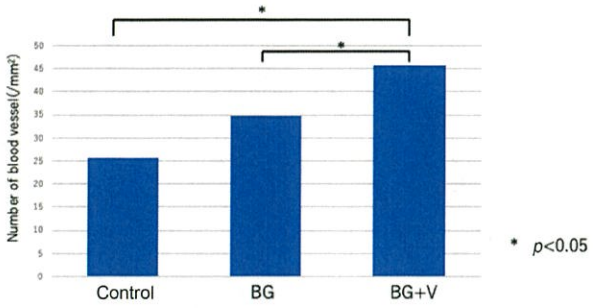

Fig. 6 Comparison of the number of blood vessels among the groups. The number of blood vessels was significantly higher in the BG + V group than in the control and BG groups. BG, bone grafting only; BG + V, bone grafting + vascular bundle introduction.

The hematoma formed after a bone fracture is known to contain cytokines, such as interleukins 1, 6, 8, and 10, and growth factors, such as tumor necrosis factor-α, receptor activator of nuclear factor-kappa-B ligand, and osteoprotegerin. Several of these cytokines, such as bone morphogenic protein β, fibroblast growth factor, and platelet-derived growth factor, are known to play important roles in bone formation.[16,17] In this study, bleeding from the osteotomy site resulted in the formation of a hematoma in the silicone tube, which was thought to contain osteogenesis-promoting factors. When this method is applied in actual clinical practice, it is expected that the bleeding at the fracture site will lead to hematoma formation.

Among osteogenic cells, multipotent stem cells (MSCs)—which are formed from bone marrow cells or vascular endothelial cells by the action of cytokines—are known to be involved in the formation of a hard callus in the cortical bone and soft callus in the medullary cavity.[18,19] Therefore, MSCs are important cellular components.[15–17] The IM used in the Masquelet method exhibits abundant blood flow, supplies cellular components, and releases cytokines. Therefore, when bone grafting is performed in the space surrounded by the IM, good bone formation occurs.[3] In our method, the saphenous vascular bundle was introduced into the bone-grafted site as an alternative to the IM, and this procedure resulted in good bone formation. This suggests that the saphenous vascular bundle used in our method supplied cellular components in a manner similar to the IM.

Necrotic bone at the fractured site plays a key role as scaffold material in normal fractures.[20] Allogeneic and autologous bone grafts are typically used as scaffolds in the treatment of bone defects.[20–22] In this study, a mixture of autologous bone and HAp/Col was inserted into the bone defect as a scaffold for bone formation. Our results indicated that bone formation in the BG group was superior to that in the control group without bone grafting, suggesting that the mixture of autologous bone and HAp/Col functioned to some extent as a scaffold for bone formation.

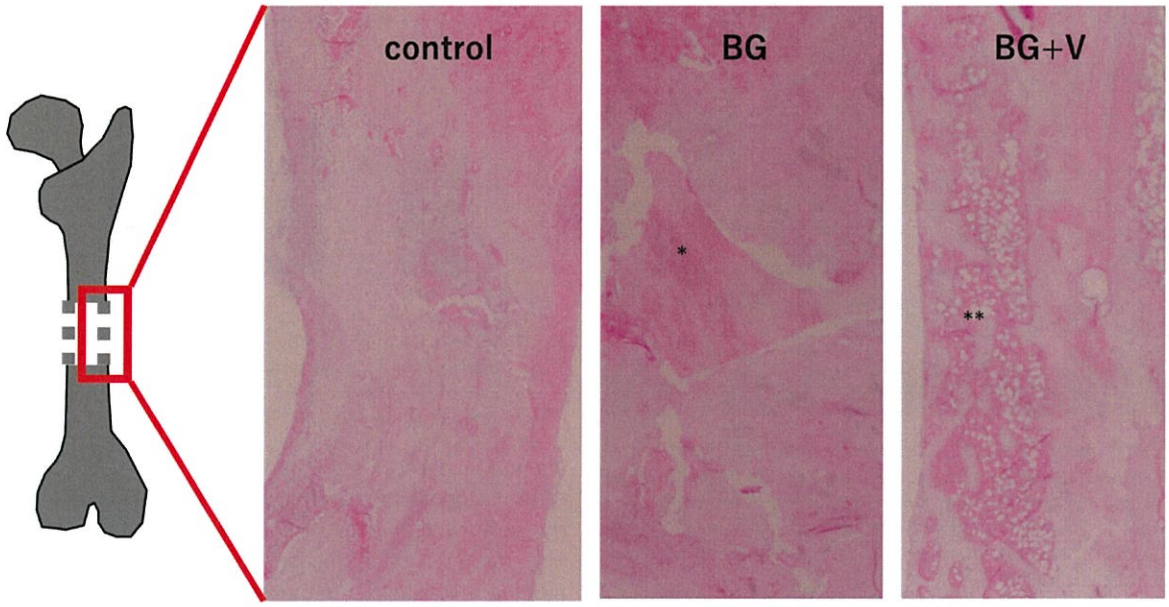

Fig. 7 Histological findings of the bone defects at 12 weeks postoperatively. No obvious bone tissue formation was observed in the control group. In the BG group, implanted autologous bone remained, and fibrous tissue formation was observed. In the BG + V group, abundant bone formation and cortical bone continuity were observed, along with normal bone marrow-like tissue. *implanted autologous bone. **bone marrow-like tissue. BG, bone grafting only; BG + V, bone grafting + vascular bundle introduction.

Secure fixation of the fractured site is a crucial component of the mechanical environment.[19] Since the model used in the present study included a 5-mm bone defect, we suspected that intramedullary nail fixation using K-wires alone would lead to instability in the longitudinal and rotational directions. Therefore, cross-linked fixation was performed using K-wires and resin. Given that this strategy maintained the bone defect length throughout the study period, it is thought to provide sufficient stability.

In the present study, good bone formation, cortical bone continuity, and formation of normal bone marrow-like tissue were observed in the BG + V group. In contrast, bone formation in the BG group was inferior to that in the BG + V group, highlighting the important role played by the introduction of vascular bundles. While the cytokine, scaffold, and mechanical environment components of the diamond concept were noted in the BG group, blood flow as a source of cellular components was lacking. In contrast, the introduction of the vascular bundle ensured that all elements of the diamond concept were incorporated in the BG + V group, which was thought to have promoted good bone formation. In addition, the BG + V group exhibited abundant blood vessel formation and regeneration of normal bone marrow-like tissues. This suggests that the introduction of vascular bundles creates a more physiological environment at the site of bone formation.

The advantage of the present method is that it can be used to reconstruct bone defects without sacrificing important bones or major blood vessels, similar to the Masquelet technique. In actual clinical applications, cancellous bone may be obtained from the bone marrow of the ilium or femur. In addition, the minor vascular bundles around the bone defects, which are used for various reconstructive microsurgeries, may be introduced into the bone defect. Another notable advantage of our method is that reconstruction of bone defects can be completed in a single surgery, in contrast to the Masquelet method.

In the present study, we used autogenous and artificial crushed bone grafting with vascular bundle introduction to reconstruct bone defects. We believe that if this method is applied in actual clinical practice, it will be useful for reconstructing bone defects caused by trauma or bone tumors. Similar methods, such as devitalized autograft associated with the vascularized fibula graft,[23] allogeneic bone graft associated with the vascularized fibula graft,[24–26] and prefabricated vascularized allogeneic bone grafts,[12–14] have been reported. Devitalized autograft associated with the vascularized fibula graft has been reported in several clinical studies, and it is reliable and useful; however, it requires sacrificing the healthy fibula.[23,24,26,27] Allogeneic bone graft associated with the vascularized fibula graft and prefabricated vascularized allogeneic bone grafts are also very useful if a suitable allogeneic bone can be obtained for the bone defect; however, if no suitable allogeneic bone can be obtained, another method should be chosen. As described above, our method requires relatively little sacrifice of healthy bone tissue if the grafted autologous bone is harvested from the bone marrow of the ilium or femur. In addition, since artificial bone is a readily available surgical material, our technique is easier to perform than other techniques.

Regarding the introduction of blood vessels, arteriovenous loops, arteriovenous bundles, or flow-through vascular axes have been used to induce angiogenesis in various tissues in the field of tissue engineering and regenerative medicine.[28–31] We previously used distal ligation type

arteriovenous bundles in a preliminary experiment, but abandoned their use due to frequent thrombus formation. Therefore, in our previous studies, we have used flow-through vascular axes, which prevented the occurrence of thrombus formation.[12–14,32] Tanaka et al reported that arteriovenous loops and distal ligation type arteriovenous bundles are more effective in achieving angiogenesis in the introduced tissues than flow-through vascular axes.[31] Although flow-through vascular axes may be inferior to other methods in their ability to induce angiogenesis, the technique is simpler to apply in clinical practice. In addition, since our previous studies[12–14] have shown that flow-through vascular axes also promote angiogenesis in allogeneic bone, we used flow-through vascular axes in the present study.

This study had several limitations. Silicone tubes are made of nonabsorbable materials. Therefore, it is necessary to replace the silicone with a bioabsorbable material to achieve a true single-stage surgery. In addition, depending on the location and extent of the trauma, it may be difficult to find a suitable vascular bundle around the bone defect. In such cases, it may be necessary to change to the conventional Masquelet method. Furthermore, some animals in the BG + V group did not achieve bone union. This suggests that not all bone defects can be reconstructed using our method. Future studies should consider the addition of osteogenic factors to further improve the rate of bone union. Another limitation is that we could not confirm the patency of the introduced vascular bundles in the collected bones histologically. In the present study, we used a simple H&E histological examination. Furthermore, osteogenesis and cortical bone continuity were assessed using long axis sections at the center of the bone defect. Therefore, it was difficult to include the vascular bundles, which were located on the surface of the bone defect in the section. To more reliably prove the patency of the vascular bundles, it is necessary to use angiography or other methods in future studies.

Conclusion

The present results obtained using a rat model of femoral bone defects demonstrate that such bone defects can be reconstructed in a single-stage surgery by introducing a vascular bundle into the bone defect, in addition to implanting autologous and artificial bone. Indeed, our findings suggest that the vascular bundle introduced in this method plays an important role in angiogenesis and bone formation at the site of bone defects. When bioabsorbable tubes can be incorporated, this procedure is expected to further improve clinical outcomes by introducing cytokines that promote bone formation.

Conflicts of Interest
None declared.

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