



Effect of difference in fixation methods of tendon graft and the microfracture procedure on tendon-bone junction healing

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Background: There are generally two methods of fixation for tendon grafts used in ligament reconstruction: bone tunnel fixation and anchor fixation. The microfracture (Mf) procedure is a technique to induce bleeding from the bone marrow, and the bleeding may contain cells with differentiation potential. However, few studies have compared the effects of the Mf procedure with those of the fixation methods. This study aimed to evaluate the effectiveness of the Mf procedure on two tendon graft fixation methods: histological, gene expression, tendon graft thickness, and mechanical. We especially focused our investigation on junction healing of tendon grafts and bone in the two fixation methods.

Methods: We used 20 rabbits to evaluate tendon and bone healing in a peroneal tendon graft model. The rabbit models were divided into five groups according to the combination of peroneal tendon graft fixation method and Mf technique as follows: control group (C, n = 4), bone tunnel fixation without Mf procedure group (BT – Mf, n = 4), bone tunnel fixation with Mf procedure group (BT + Mf, n = 4), anchor fixation without Mf procedure group (A – Mf, n = 4), and anchor fixation with Mf procedure group (A + Mf, n = 4). All animals were sacrificed at 4 weeks postoperatively. The specimens underwent histological evaluation, mRNA analysis, tendon graft thickness at the tendon-bone junction, and biomechanical testing.

Results: Histological evaluation of the BT + Mf and A + Mf groups showed healing with fibrocartilage formation at the tendon graft–bone junction. The mRNA expression showed significant increase in type 2 collagen, Scleraxis, and SRY-box9 in the BT + Mf and A + Mf groups. In biomechanical tests, the BT + Mf and A + Mf groups showed significantly increased tensile strength compared with the BT – Mf and A – Mf groups (BT + Mf group, 21.6 ± 1.7 N; A + Mf group, 22.5 ± 2.3 N vs. BT – Mf group, 12.3 ± 2.4 N; A – Mf group, 11 ± 2.3 N).

Conclusion: The Mf procedure resulted in fibrocartilage formation at the tendon-bone junction in the BT and anchor fixation and improved the fixation strength at 4 weeks.

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An enthesis exists in the site of attachment of a tendon, ligament, or joint capsule to the skeleton.^{4–6} It plays an important role in transmitting the various mechanical stresses on tendons and ligaments to the bone.^{3,5,22} An enthesis is classified as either fibrous

or fibrocartilage based on the structure of the attachment site, and fibrocartilage entheses consists of four layers.³⁶ The central structure of the fibrocartilage entheses comprises the noncalcified and calcified cartilage layers.^{1,10} The noncalcified cartilage layer is a nonvascular zone that consists mainly of chondrocytes, type 2 collagen fibers, and aggrecan, which cannot regenerate spontaneously once damaged.^{5,6,18,20,42} Recently, chronic stress accumulation due to repetitive exercise and inflammation has caused a problem in athletes with loss of entheses function.^{9,17,24,38} Moreover, ulnar collateral ligament (UCL) reconstruction using bone tunnels (BTs) is generally performed for UCL injuries of the elbow,

This animal study was approved by The Animal Care and Use Committee, Okayama University (OKU-2020769).

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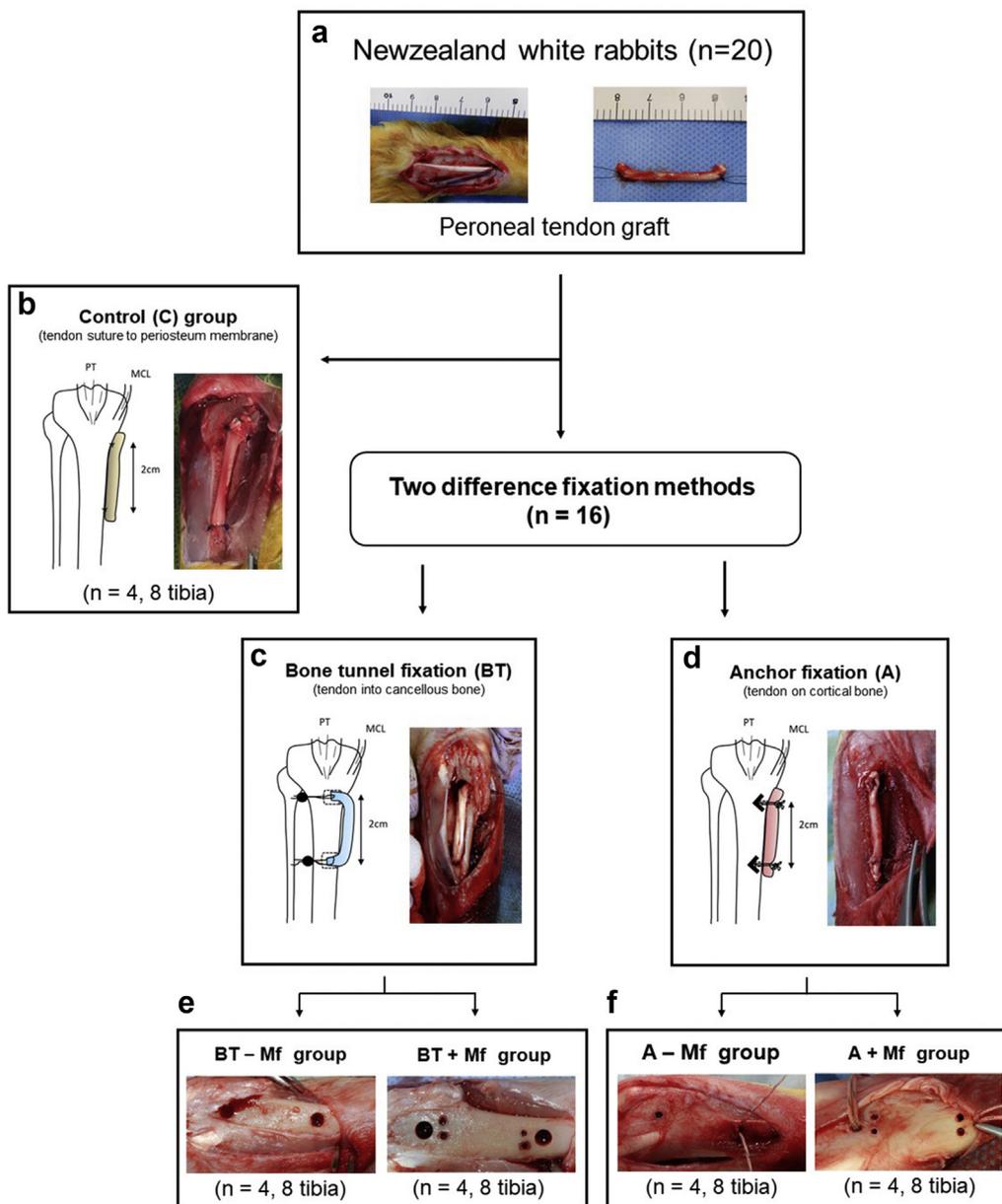


Figure 1 (a) Peroneal tendons were harvested in a posterolateral approach of the lower leg for grafting. Twenty rabbits were divided into three tendon graft fixation groups. (b) The control (C) group underwent simple suture fixation of the tendon graft to the periosteum membrane (n = 4, 8 tibias). (c) Bone tunnel (BT) fixation is the insertion of the tendon graft into the bone tunnel. (d) Anchor (A) fixation is the fixation of the tendon graft with an anchor. (e, f) The BT and A fixation groups were divided into two groups according to the presence or absence of microfracture procedures, respectively: BT without microfracture procedure was defined as the BT – Mf group (n = 4, 8 tibias), and BT with microfracture procedure was defined as the BT + Mf group (n = 4, 8 tibias). A without microfracture procedure was defined as the A – Mf group (n = 4, 8 tibias), and A with microfracture procedure was defined as the A + Mf group (n = 4, 8 tibias). Furthermore, BT – Mf and A – Mf groups were defined as no Mf groups, while BT + Mf group and A + Mf group were defined as Mf groups.

but anatomically created BTs caused damage of the original enthesis.^{8,13,34,41}

Conversely, ligament fixation method using anchor fixation is less invasive than BT fixation and allows better preservation of the footprint.¹² Hechtman et al performed a hybrid technique in UCL reconstruction of the elbow with the tendon graft passing through a BT on the sublime tubercle and being fixed with two anchors on the humeral side.¹⁶ The success of the surgery depends on a strong healing between the graft and bone, but the method of restoring the enthesis in ligament reconstruction surgery has not yet been established.^{2,6,33,36,39} Currently, several studies have reported that induction of autologous bone marrow bleeding containing stem cells at the tendon-bone junction site may enhance

healing.^{15,19,21,28,31,35,44} However, the advantage of inducing bone marrow bleeding by microfracture procedures in the neighborhood of the free tendon graft fixation site is not fully understood, and few studies have explained the differences in histological, gene expressive, and mechanical results that develop when the free tendon graft is fixed using different methods. Moreover, we expect that the bone marrow bleeding induced at the tendon graft-bone junction and differentiation of the stem cells it may contain can affect the initial fixation of the junction site and potentially shorten the rehabilitation period of baseball players after UCL reconstruction surgery. This study aimed to evaluate how BT and anchor fixation differ in the healing process of the tendon graft-bone healing using histological, biomechanical, and genetic analyses. Moreover,

Table 1
Modified histological scoring for the tissue engineering of the enthesis.

Score	Cell at bone-tendon junction	Type II collagen at bone-tendon junction	Collagen organization	Chondrocyte organization
1	No	<25%	No	No cells
2	Clusters	25 to 50%	Isolated patches around few cells	Isolated cells
3	Large areas	50 to 75%	Focal area	Clusters
4	Over the whole junction	>75%	Covering bone-tendon junction	In columns

to evaluate the effectiveness of the microfracture procedure in enhancing the repair process, we prepared an experimental animal model in which no stretching was applied to the grafted tendon to simply evaluate the difference between the fixation and microfracture techniques. The hypothesis of this study is that the insertion of the tendon graft into the bone marrow cavity will be affected by bleeding and that BT fixation will provide better histological and mechanical results than anchor fixation. The augmentation of the microfracture procedure was defined as a technique that leads to the formation of fibrocartilage tissue at the junction and promotes healing levels histologically, through gene expression, and mechanically.

Materials and methods

Animals

This animal study was approved by The Animal Care and Use Committee, Okayama University (OKU-2020769). New Zealand White male rabbits weighing 2.0 to 2.5 kg were purchased from Shimizu Laboratory, Japan. The rabbits were housed in individual cages of a size that allowed for minimal exercise in a temperature-controlled facility.

Experiment design

We used 20 rabbits to evaluate tendon and bone healing in a peroneal tendon graft model. The rabbits were divided into five groups according to the combination of peroneal tendon graft fixation method and microfracture procedure as follows: negative control group (C, $n = 4$), BT fixation without microfracture procedure group (BT – Mf, $n = 4$), BT fixation with microfracture procedure group (BT + Mf, $n = 4$), anchor fixation without microfracture procedure group (A – Mf, $n = 4$), and anchor fixation with microfracture procedure group (A + Mf, $n = 4$). BT – Mf group and A – Mf group were defined as no Mf groups (without microfracture procedure groups), while BT + Mf group and A + Mf group were defined as Mf groups (with microfracture procedure groups). The animals were sacrificed, and samples were collected at 4 weeks postoperatively. Each group used two animals (4 tibias) for biomechanical testing and two animals (4 tibias) for histological evaluation, mRNA evaluation, and tendon graft thickness at the tendon-bone junction. Because both ends of the tendon grafts were subjected to the same fixation and treatment, two junction samples were obtained from one tibia. Thus, eight tissue specimens were prepared in one group. Histological analysis, modified histological scoring, and mRNA evaluation were performed on one tissue sample. Outcome measures included histological analysis, modified histological scoring, mRNA analysis, and tendon graft thickness and biomechanical testing.

Operative procedure

The rabbits were anesthetized with intramuscular injection of ketamine hydrochloride (Ketalar for intramuscular injection, 500 mg, 50 mg/kg; Daiichi Sankyo Pharmaceutical, Tokyo, Japan),

followed by inhalation anesthesia with isoflurane (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cefazolin was injected intramuscularly into the thigh to prevent postoperative infection. A skin incision was made distally on the lateral side of the lower leg to expose the peroneal tendon. The tendon was sutured with 4-0 nylon thread in two places 3 cm apart, and two cuts were made (Fig. 1, a). Once the tendon graft was harvested, the skin was closed with 4-0 nylon thread. Next, a curved skin incision of approximately 4 cm was made proximally on the medial side of the ipsilateral lower leg, exposing the proximal medial aspect of the tibial diaphysis. The gracilis muscle was partially detached to prepare a space for the graft tendon transfer. The C group rabbits underwent simple suture fixation of the tendon graft to the periosteum (Fig. 1, b). The BT fixation was created in the monocortical bone using a 2.8-mm-diameter cannulated drill, and the contralateral cortex was penetrated with a 1.5-mm-diameter drill. The proximal BT was created slightly distal, anterior to the knee medial collateral ligament, and the distal BT was placed 2 cm distal to the proximal BT. A 4-0 nylon thread sutured to the tendon graft was passed through the medial BT and out of the lateral cortical bone to guide the grafted tendon into the tibial marrow cavity. The nylon thread was fixed at the lateral aspect of the tibia with a lead ball (Fig. 1, c). The anchor fixation sites were made with a 1.5-mm-diameter drill. The bone holes were located on the tibia's medial aspect, similar to that in the BT group. Two soft-tissue anchors (1.4-mm Juggerknot soft anchor; Zimmer Biomet, Warsaw, IN, USA) were inserted into the bone holes. The anchor thread was passed through the graft tendon for suture fixation (Fig. 1, d).

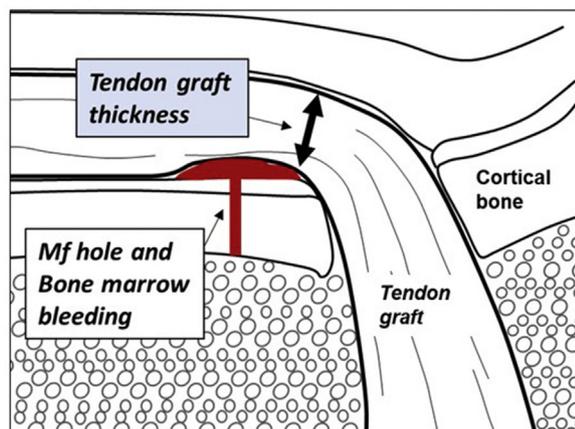
Microfracture procedure to induce bone marrow bleeding

The BT + Mf and A + Mf groups (Mf groups) underwent the same microfracture procedure to induce bone marrow bleeding. Two 1.5-mm-diameter holes were drilled 2 mm from the BT or anchor hole on the fixation-to-fixation side before tendon transfer (Fig. 1, e and f). After confirming the flow of blood from the drill holes, the tendons were fixed to cover the drilling holes.

Histological analysis

At four weeks postoperatively, each graft-tibia specimen was harvested. All specimens were fixed in 10% buffered formalin solution at room temperature for 48 hours. After fixation, specimens were decalcified in Kalkitox (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 7 days, embedded in paraffin, and serially sliced in the coronal plane at a thickness of 4 μ m. The slides were stained with hematoxylin and eosin (H&E), safranin O, and picosirius red and toluidine blue (P&T). Digital images of the stained slides were obtained (Olympus System Microscope Model BX53 LED and a cellSens Standard Version 1.17; Olympus Co., Tokyo, Japan). H&E and safranin O staining were evaluated under unpolarized light microscopy, while P&T staining was evaluated under polarized light microscopy. Picosirius red alone was used to stain type 1 and type 3 collagen fibers, but not type 2; therefore, the fibrocartilaginous tissue at the tendon-bone junction was not stained. Because toluidine blue can stain type 2 collagen fiber,

Tendon Graft Thickness for Bone Tunnel Fixation



Tendon Graft Thickness for Anchor Fixation

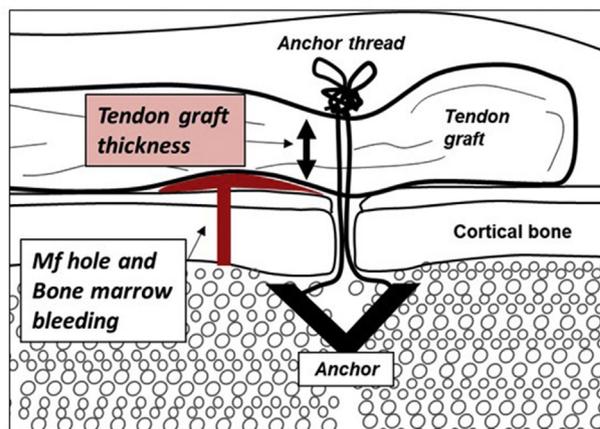


Figure 2 Measurement of the tendon graft thickness at the tendon-bone junction. The thickness of the tendon graft at the tendon-bone junction was measured to investigate the effect of different fixation methods. The tendon graft thickness was measured digitally on the contact site of the tendon graft and edge of the fixed hole (bone tunnel hole or anchor hole). Double-headed black arrow: measurement site of tendon graft thickness.



Figure 3 Biomechanical test setting. (a) A (right) holding device is connected to a digital force measuring instrument. (b) A (left) holding device is grasping a tibial bone, and a (right) holding device is grasping a tendon graft.

double staining with toluidine blue staining was applied to P&T staining to evaluate the continuous formation of type 1 to 3 collagen fibers under polarized light microscopy. Histological analysis was performed to observe the formation of chondrocytes by H&E staining, appearance of type 2 collagen by safranin O, and continuity between the graft tendon and fibrocartilage tissue (containing chondrocytes and type 2 collagen) by P&T double staining.

Modified histological scoring for the tissue engineering of the entheses

Modified histological scoring was performed to comprehensively score and evaluate the cellularity of chondrocytes, type 2 collagen, and tendon-bone junction observed in the formed fibrocartilage tissue (Table 1). We have modified the scale used by Nourissat et al in their histological assessment of entheses reconstruction using rat Achilles tendons.²⁷ They used five assessment points, but one of them, glycosaminoglycan, was not evaluated in our experiment and needed to be modified. Therefore, we evaluated four assessment points consisting of cells at the tendon-bone

junction, type 2 collagen at the tendon-bone junction, collagen organization, and chondrocyte organization. The presence of type 2 collagen was assessed as the area stained red by safranin O. Cells at the tendon-bone junction and chondrocytes were evaluated by observation under the microscope. Collagen organization was assessed under polarized light microscopy for continuity between type 2 collagen and tendon grafts by P&T staining. Scoring was based on four assessment points, each scored from 1 to 4 points, giving a total score of 16 points.

mRNA sample preparation

A total of 72 formalin-fixed paraffin-embedded (FFPE) tissue sections were sliced into 8- μ m tissue sections. Using the H&E specimen as a reference, only the tendon-bone junction of interest was micro-extracted from the unstained slide using a scalpel under a microscope. For mRNA extraction, 10 unstained slides per sample were used. Total mRNA was extracted using a RNeasy FFPE Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The mRNA was quantified using a NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

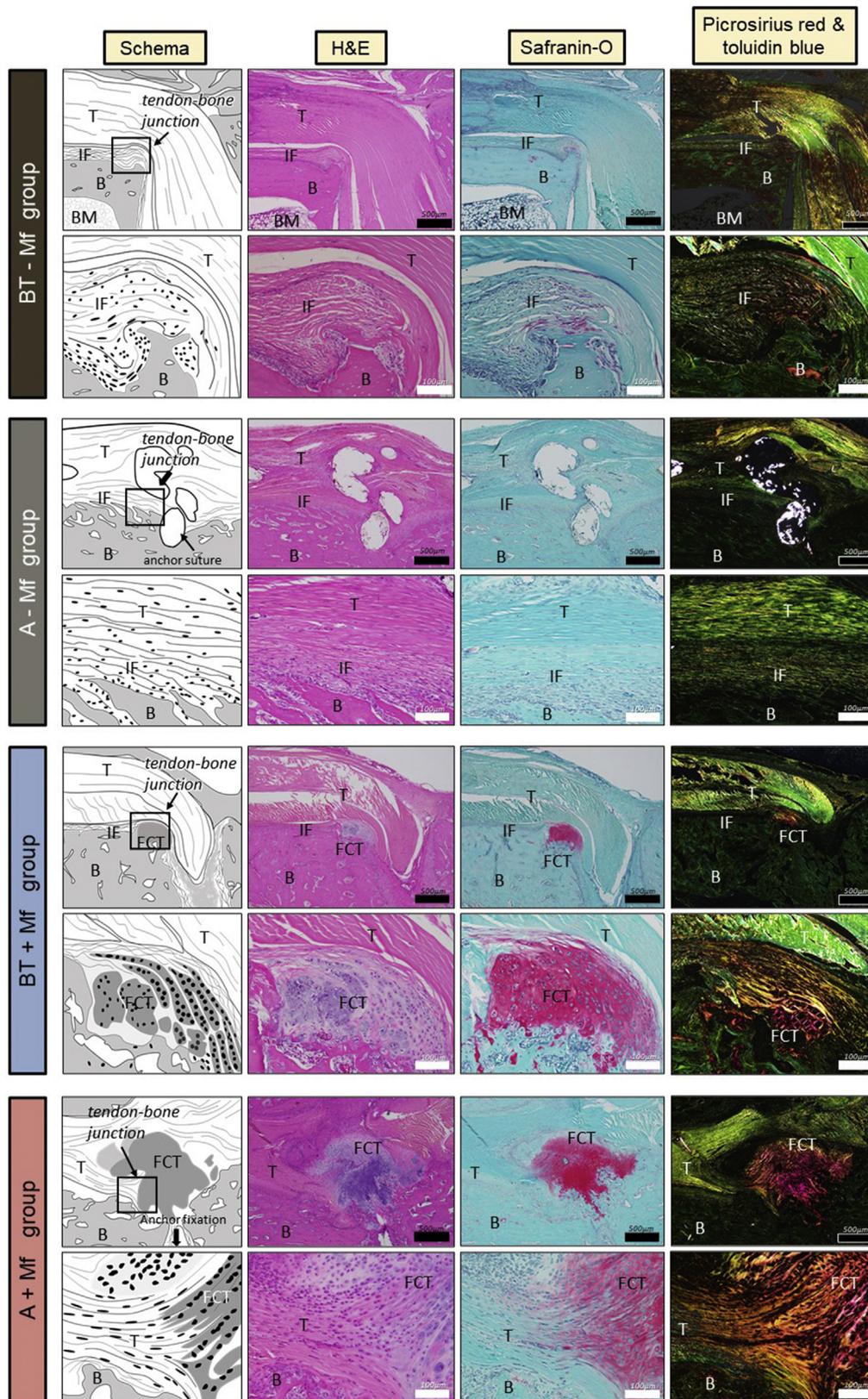


Figure 4 Histological findings. All groups were assessed with three stains: hematoxylin and eosin (H&E), safranin O, and picrosirius red and toluidin blue (P&T). The BT – Mf and A – Mf groups showed no formation of fibrocartilage tissue, and the tendon-bone junction was connected fibrously similar to that in the control group. Conversely, the BT + Mf and A + Mf groups showed fibrocartilage tissue formation at the tendon-bone junction. Fibrocartilage tissue shows columnar alignment and accumulation of chondrocytes in H&E staining; in safranin O staining, the extracellular matrix is stained red; in P&T staining, type 2 collagen is observed in bright red color, and continuity with type 1 and type 3 collagen in the tendon can be observed. P&T staining was observed under a polarized light microscope. The difference in the fixation method also made a histological difference in the formation of fibrocartilage tissue. In the BT + Mf group, fibrocartilage tissue was constructed at the tendon-bone junction (the triangular corner consisting of the fixation hole, cortical bone, and tendon graft), while the A + Mf group was formed under the knot of the anchor thread. T, tendon graft; IF, interface; B, bone; BM, bone marrow; FCT, fibrocartilage tissue. Black scale bars: 500 μm, white scale bars: 100 μm.

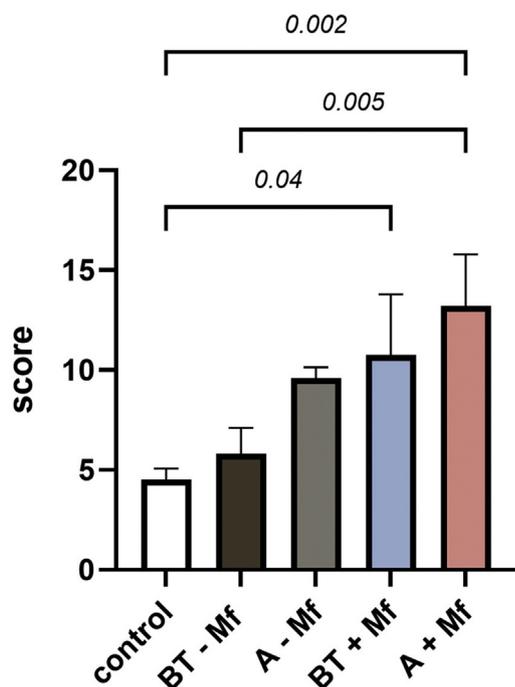


Figure 5 Modified histological score for the tissue engineering of the enthesis. The BT + Mf group showed a significant difference compared to the C group ($P = .04$). The A + Mf group showed a significant difference compared with the C group and BT – Mf group ($P = .002$, $P = .005$). In the comparison of bone tunnel fixation and anchor fixation, the anchor fixation showed higher scores. The bar and error bar in the graphs show the mean and 95% CI (one group, $n = 8$). Statistical analysis comparing multiple groups was conducted with one-way analysis of variance (ANOVA), followed by the Tukey post hoc test. P values $< .05$ were considered statistically significant.

mRNA analysis

To evaluate enthesis reconstruction, tissue from the tendon-bone junction was harvested to examine gene expression of type 2 alpha 1 collagen (Col2a1), bone morphogenetic protein 4 (Bmp4), scleraxis (Scx), and sex-determining region Y-box 9 (Sox9). Moreover, 300 ng of total mRNA was used for cDNA synthesis according to the manufacturer's instructions. cDNA Was synthesized using the PrimeScript RT Master Mix (RR036A; Takara). For qPCR, 15- μ L reactions were set using 2X Agilent Brilliant III Ultra-Fast SYBR (Agilent Technologies, Santa Clara, CA, USA), water, and 10- μ m primers. The qPCR reaction using QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) consisted of 1 cycle at 95°C for 3 minutes; 50 cycles at 95°C for 5 seconds, 55°C for 20 seconds, 95°C for 1 second, 60°C for 20 seconds; and 1 cycle at 95°C for 1 second for melting curve analysis. The quantity of the gene of interest was determined relative to Gapdh cycle threshold (Ct). Primers for Gapdh, Col2a1 (NM_001195671),⁴³ 5'-GACGACA-TAATCTGTGAAGACACC-3' (sense) and 5'-GTTCCTTTCTGCCCTTG-3' (antisense) and Bmp4 (AF042497),¹¹ 5'-TTAACT-CAGCAGCATCC-3' (sense) and 5'-CAGTCTCGTGCCAGTAG-3' (antisense), Scx (NM_198885.376), 5'-CAGCTATGAGCAGGGCCTAT-3' (sense) and 5'-GTCAGTGTCCGGTGCTTAG-3' (antisense), Sox9 (XM_00271949993),²⁵ 5'-TGGAGACTGCTGAACGAGAG-3', and 5'-CTTGATGTCGGGTGGTCTT-3' were purchased. The Ct value was calculated from the value of the C group.

Tendon graft thickness at the tendon-bone junction

Two different fixation methods, BT fixation and anchor fixation, have different effects on the thickness of the tendon at the

tendon-bone junction. In BT fixation, the tendon graft is pulled into the bone hole, whereas, in anchor fixation, the graft is pressed against the bone surface by the anchor thread. Therefore, the thickness of the tendon at the tendon-bone junction was compared in each group. The tendon graft thickness was measured digitally using Olympus System (Olympus System Microscope Model BX53 LED and a cellSens standard version 1.17 [Olympus Co., Tokyo, Japan]) on the contact site, tendon graft, and edge of the fixed hole (BT hole or anchor hole) (Fig. 2). For the C group without fixation holes, measurement was performed at the suture fixation site of the tendon graft.

Biomechanical study

Specimens were kept hydrated with saline solution during preparation and frozen at -80°C until all were mechanically tested simultaneously. After thawing in a 39°C saline solution bath, all muscles attached to the tibia were removed, as were the sutures used for intraoperative fixation of the grafted tendon. The tendon graft was cut at the distal BT and stripped by 1 cm to allow the tendon to be grasped by the device (Fig. 3). The tibia was set in the grip on the pedestal side, and the grafted tendon was gripped by holder with the digital force gage (FGP-5; Nidec-Shimpo Co., Kyoto, Japan). The specimen was manually pulled at a constant speed in a direction of 45° to the long axis of the tibia until the graft and bone connection failed or the tendon ruptured. The strength of the tendon-bone junction at the proximal fixation site was defined as the failure load (N), which was the value of maximum resistance until rupture. Information on resistance values during manipulation was imported from the digital force gage into Excel (Microsoft) in real time and recorded.

Statistical analysis

The statistical differences between two experimental groups were determined by the two-tailed unpaired Student's t-test. Statistical analysis comparing multiple groups was conducted with one-way analysis of variance, followed by the Tukey post hoc test. P values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism v 9.0.1 (GraphPad Software, San Diego, CA, USA).

Results

Histological findings

Figure 4 shows the histological findings. In the no Mf groups, no fibrocartilage tissue bodies were created at the tendon-bone junction. The fibrous tissue on the periosteum in the A – Mf group showed more tight healing than that in the BT – Mf group. In the Mf groups, fibrocartilage tissue was constructed at the tendon-bone junction. Chondrocytes showed clustering on the bone side and columnar alignment toward the fibers on the tendon side in H&E. Under polarized light, P&T staining showed type 2 collagen fibers in continuity between the tendon and bone. However, the difference in the fixation method also showed a histological difference in the area of fibrocartilage tissue formation. In the BT + Mf group, fibrocartilage tissue was constructed at the corner of the BT, while, in the A + Mf group, it was formed under the knot of the anchor thread.

Modified histological scoring

The modified histological scores (mean \pm standard deviation) for each group were as follows: 4.3 ± 0.3 points for the C group,

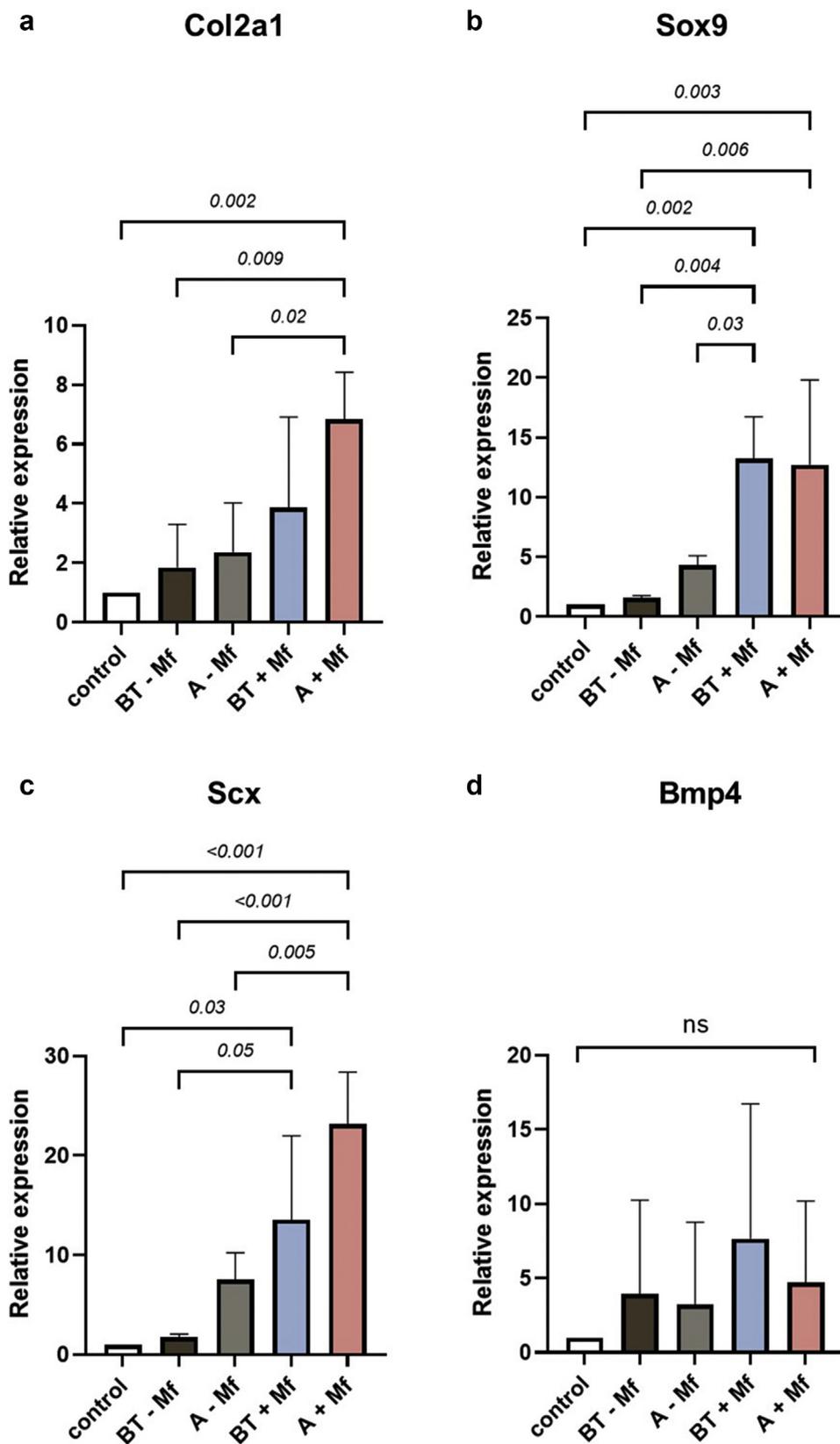


Figure 6 mRNA Analysis. Each gene was normalized by ACTB, and gene expression was calculated relative to the control group. (a) The expression of Col2a1 in the A + Mf group was significantly upregulated to 6.8-fold compared with that in the C group ($P = .002$) and threefold compared with that in the A - Mf group ($P = .02$). (b) For Sox9 expression, the BT + Mf group and A + Mf group showed 13-fold and 12.7-fold upregulation compared with the C group ($P = .002$, $P = .003$). (c) For Scx expression, the BT + Mf group and A + Mf group showed 13.5-fold and 23-fold upregulation compared to the C group ($P = .03$, $P < .001$). (d) Bmp4 was not significantly different between any groups. The bar and error bar in the graphs show the mean and 95% CI (one group, $n = 8$). A statistical analysis comparing multiple groups was conducted with one-way analysis of variance (ANOVA), followed by the Tukey post hoc test. P values $< .05$ were considered statistically significant.

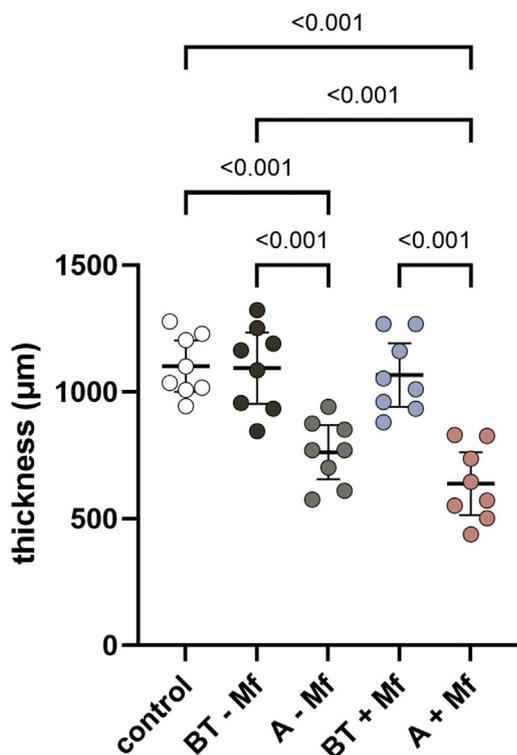


Figure 7 Tendon graft thickness at the tendon-bone junction. There was a significant thickness difference between BT – Mf and A – Mf groups ($P < .001$). There was also a significant difference in thickness between the BT + Mf group and the A + Mf group ($P < .001$). The anchor fixation method also showed a significant difference in thickness for the C group ($P < .001$). The plots in the graph are the values for each group, and the black and error bars show the mean and 95% CI (one group, $n = 8$). A statistical analysis comparing multiple groups was conducted with one-way analysis of variance (ANOVA), followed by the Tukey post hoc test. P values < 0.05 were considered statistically significant.

5.8 ± 0.6 points for the BT – Mf group, 10.8 ± 3.0 points for the BT + Mf group, 9.6 ± 0.2 points for the A – Mf group, and 13.2 ± 1.2 points for the A + Mf group (Fig. 5). There was a significantly higher score in the BT + Mf group and A + Mf group (Mf groups) than in the C group (95% confidence interval [CI], -12.35 to -0.15 ; $P = .043$, and 95% CI, -14.49 to -2.91 ; $P = .002$). Moreover, the A + Mf group had a significantly higher score than the BT – Mf group (95% CI, -12.86 to -1.94 ; $P = .005$). In the BT fixation vs. anchor fixation, the anchor fixation tended to have higher scores. In the Mf groups, anchor fixation showed higher scores. The microfracture procedure showed a score increase of 53.9% in BT fixation (95% CI, -10.74 to 0.83 ; $P = .12$) and 72.7% in anchor fixation (95% CI, -9.05 to 1.85 ; $P = .31$).

mRNA evaluation

In the FFPE sample, β -actin (ACTB) had the highest melting temperature stability. Therefore, we normalized ACTB as a reference gene and calculated as fold change the relative expression of each gene against the control group sample according to the formula $2^{-\Delta\Delta Ct}$ (Fig. 6). The expression of Col2a1 in the A + Mf group was significantly upregulated to 6.8-fold compared with that in the C group (95% CI, -9.77 to -1.9 ; $P = .002$) and threefold compared with that in the A – Mf group (95% CI, -8.41 to -0.54 ; $P = .02$; Fig. 6, A). For Sox9 expression, the BT + Mf group and A + Mf group showed 13- and 12.7-fold upregulation, respectively, compared with the C group (95% CI, -20.68 to -3.81 ; $P = .002$, and 95%

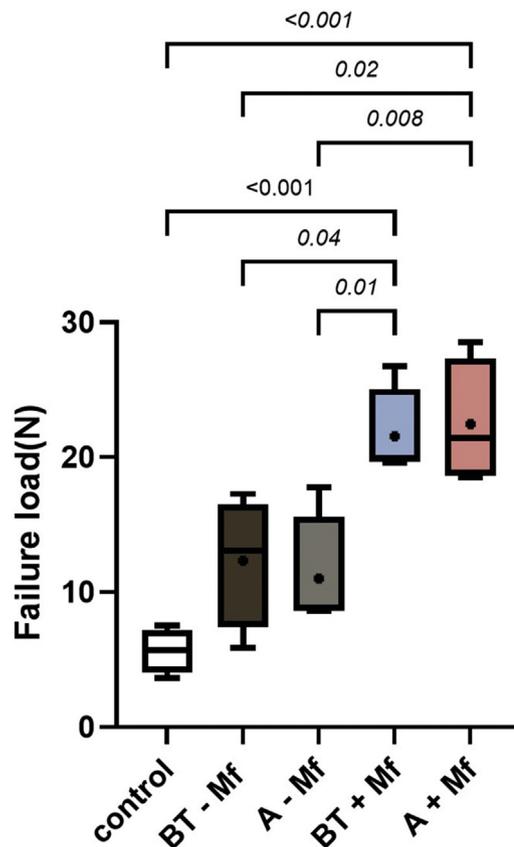


Figure 8 Biomechanical testing. The BT + Mf group showed significant tensile strength differences between C group ($P < .001$) and BT – Mf group ($P = .04$) and A – Mf group ($P = .01$). The A + Mf group also showed a significant difference in tensile strength between C group ($P < .001$) and BT – Mf group ($P = .02$) and A – Mf group ($P = .008$). All comparisons by combination of Mf groups against any of the no Mf groups were significantly different. Thus, the microfracture procedure further significantly enhanced the failure load. The bar and plot in the box show the median and mean (one group, $n = 4$). The whiskers show a 5–95 percentile. A statistical analysis comparing multiple groups was conducted with one-way analysis of variance (ANOVA), followed by the Tukey post hoc test. P values < 0.05 were considered statistically significant.

CI, -20.14 to -3.279 ; $P = .003$; Fig. 6, B). The BT + Mf group also showed a significant upregulation of 8.2-fold compared with the BT – Mf group (95% CI, -20.05 to -3.19 ; $P = .004$). For Scx expression, the BT + Mf group and A + Mf group showed 13.5- and 23-fold upregulation, respectively, compared with the C group (95% CI, -24.21 to -0.89 ; $P = .03$, and 95% CI, -33.81 to -10.5 ; $P < .001$; Fig. 6, C). The BT + Mf group showed 7.8-fold significant upregulation compared with the BT – Mf group (95% CI, -23.48 to -0.16 ; $P = .05$), and the A + Mf group showed threefold significant upregulation compared with the A – Mf group (95% CI, -27.28 to -3.97 ; $P = .005$). Bmp4 level was not significantly different between the groups (Fig. 6, D). The A + Mf group was the only group to show significant gene expression in Col2a1, Sox9, and Scx.

Tendon graft thickness evaluation

The tendon graft thickness at the tendon-bone junction of each group was measured. The measurement results (mean \pm standard deviation) were 1102 ± 113.5 μm for the C group, 1094.2 ± 157.1 μm for the BT – Mf group, 762.3 ± 119.8 μm for the A – Mf group, 1066.7 ± 139.9 μm for the BT + Mf group, and 638.1 ± 138.4 μm for the A + Mf group (Fig. 7). In the no Mf groups, the tendon graft was significantly thinner in the A – Mf group than in the BT – Mf group

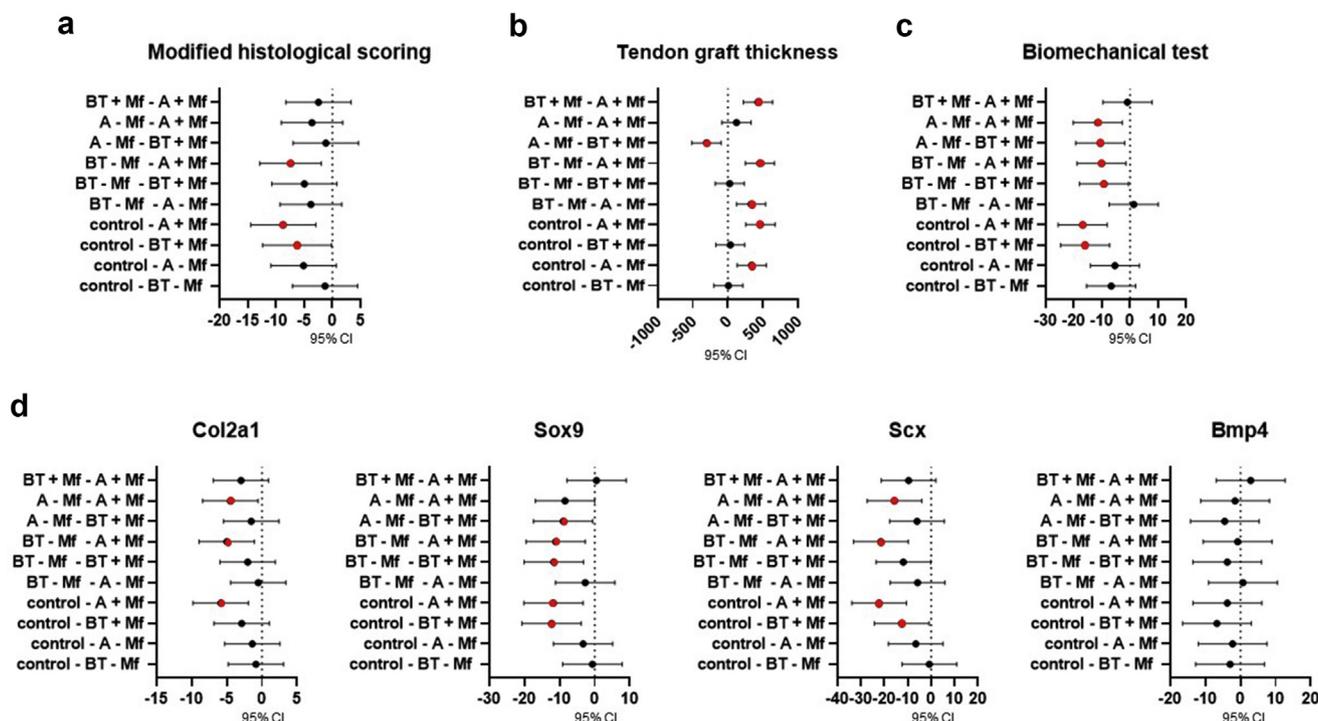


Figure 9 Results of Tukey post hoc analysis for modified histological scoring, tendon graft thickness, biomechanical test, and mRNA analysis. The 95% confidence interval (CI) between each group is shown, where red dots indicate significant differences, and black dots are combinations of groups with no significant differences. (a) Modified histological scoring, (b) tendon graft thickness, (c) biomechanical test, and (d) mRNA analysis. A statistical analysis comparing multiple groups was conducted with one-way analysis of variance (ANOVA), followed by the Tukey post hoc test. P values $< .05$ were considered statistically significant.

(95% CI, 125 to 539; $P < .001$). In the Mf groups, the tendon graft was also significantly thinner in the A + Mf group than that in the BT + Mf group (95% CI, 222 to 636; $P < .001$). The tendon graft thickness with anchor fixation at the tendon-bone junction was significantly thinner than that with BT fixation.

Biomechanical evaluation

The result (mean \pm standard deviation) of the ultimate load to failure at the tendon-bone junction was 5.6 ± 0.8 N in the C group and 12.3 ± 2.4 N and 11.0 ± 2.3 N in the BT – Mf group and A – Mf group, respectively (Fig. 8). Those in the BT + Mf group and A + Mf group were 21.6 ± 1.7 N and 22.5 ± 2.3 N, respectively. There was no significant difference between the C group and no Mf groups. There was no significant difference between the BT – Mf group and the A – Mf group or BT + Mf group and A + Mf group (95% CI, -7.4 to 10.06 ; $P = .99$, 95% CI, -9.62 to 7.83 ; $P > .99$) (Fig. 9). All comparisons by combination of Mf groups against any of the no Mf groups were significantly different. Thus, the microfracture procedure further significantly enhanced the failure load.

Discussion

This study revealed the effects of the microfracture procedure on the tendon-bone junction using two new types of the tendon graft fixation model in laboratory animals. BT and anchor fixation without the microfracture procedure healed by a fibrous connection at the tendon-bone junction. Similarly, BT and anchor fixation under the microfracture procedure led to healing with a fibrocartilage connection in the tendon-bone junction. The difference between BT and anchor fixation under microfracture procedure was shown in the thickness of the tendon and location of fibrocartilage tissue formation at the junction. The augmentation of the

microfracture procedure enhanced histological scores, gene expression levels, and failure load compared with the model without the microfracture procedure. The attachment pattern between the tendon and bone can be histologically divided into two types: indirect entheses and direct entheses.^{4,6,29} Indirect insertions (fibrous entheses), such as the insertion of the tibial side medial collateral ligament and deltoid tendon into the humerus, have no fibrocartilage interface.^{4,5} The tendon and ligament pass obliquely along the bone surface and directly insert into the periosteum at an acute angle.⁶ They are connected by Sharpey's fibers over a broader area of tendon and bone.³⁰ BT and anchor fixation without conducting the microfracture procedure may have led to histological healing as an indirect entheses type. However, direct insertions (fibrocartilage entheses), such as the attachment of anterior cruciate ligament, Achilles tendon, patellar tendon, and rotator cuff, as well as the humeral insertion of the elbow's UCL, are composed of four zones in order of gradual transition: tendon, uncalcified fibrocartilage, calcified fibrocartilage, and bone.²³ The most significant histological difference between indirect and direct entheses is the intervention of an uncalcified fibrocartilage layer containing type 2 collagen fibers, chondrocytes, and aggrecan. BT and anchor fixation under the microfracture procedure created a fibrocartilage tissue at the tendon-bone junction. The formed fibrocartilage tissue contained chondrocytes, and type 2 collagen fibers were observed by toluidine blue staining. Type 2 collagen can be also observed as a color in the red to pink color under a polarized light microscope, and the use of P&T double staining allows for the visualization of gradation of type 1 to 3 collagen fibers. Previous study also indicates that the mineralized interface region exhibits significantly greater compressive mechanical properties than the non-mineralized region.²⁶ However, the results of comparative mechanical studies of fibrocartilage tissue formation at the junction of BT and anchor fixation are unknown. Pierre et al reported that there

was no significant biomechanical or histological difference between BT and anchor fixation in sheep rotator cuff repair models.³⁹ They also revealed that healing between the tendon and bone was mediated by the fibrous interface. In our biomechanical tests, the Mf groups showed significant improvement in failure load compared with the no Mf groups. Therefore, BT and anchor fixation under the microfracture procedure may have led to healing as a direct enthesis type because the fibrocartilage tissue united in the tendon-bone junction.

As tendon development progresses, Scx activates Bmp signaling in tuberosity-forming chondrocytes.^{7,37} Then, Bmp4 increases the expression of Sox9, which stimulates chondrocyte formation and type 2 collagen and aggrecan production to complete the enthesis.^{32,40} Therefore, the expression of Scx, Bmp4, and Sox9 is important in reconstructing fibrocartilage enthesis. In our study, we also evaluated whether these genetic expressions are confirmed into the formed fibrocartilage tissue, focusing on the tendon-bone junction. Gene expression in BT and anchor fixation without the microfracture procedure was low for all genes because a fibrocartilage tissue was not created. Conversely, the gene expression of BT and anchor fixation under the microfracture procedure revealed differences in gene expression of Sox9 and Scx in the BTs and Col2a1 and Scx in the anchors, but there was no consistent trend in expression. In any case, augmentation of the microfracture procedure upregulated gene expression of Col2a1 and Scx and Sox9 although Bmp4 was not significantly different because of its large variance. The difference in results could be related to the inclusion of mesenchymal cells in bone marrow bleeding induced by the augmentation of the microfracture procedure.

We hypothesized that BT fixation would show better histological and mechanical results than anchor fixation. This is because the drill diameter for BT fixation is larger than that for anchor fixation. The outflow of the bone marrow bleeding in the BT fixation will affect the tendon-bone junction more excessively. However, there was no significant difference between the BT – Mf and A – Mf groups or BT + Mf and A + Mf groups in the modified histological scoring, mRNA analysis, and biomechanical test. Thus, there was no association between hole size by fixation method and bleeding. We consider the tendon graft being bent at a near 90° angle at the corner of the fixation hole to be the reason for this result in the BT fixation, and necessary quantities of bone marrow bleeding outflow for fibrocartilage tissue formation did not accumulate at the tendon-bone junction. In the anchor fixation, the tendon graft may have blocked the anchor hole because of the compression caused by the tightening of the anchor thread and prevented the outflow of bone marrow bleeding. Although the compression of the tendon graft at the corner of the junction may have prevented the outflow of bone marrow bleeding, it may also have influenced the healing process. Measurement of tendon graft thickness at the tendon-bone junction suggested that anchor fixation compresses the tendon graft more strongly than BT fixation. However, we consider that compression influences healing in every model, and it is difficult to rule out the effect of compression strictly from healing. Therefore, we focused on whether fibrocartilage tissue was created, as all models in this experiment would have been affected by compression. The role of the microfracture procedure may be to provide a new route to pool bone marrow bleeding into the tendon-bone junction against the outflow of marrow bleeding blocked by the tendon graft fixation methods. Fibrocartilage tissue created by induced bone marrow bleeding showed differences in the location of formation depending on the fixation method. In the BT + Mf group, fibrocartilage tissue was constructed at the tendon-bone junction (the triangular corner consisting of the fixation hole,

cortical bone, and tendon graft), while in the A + Mf group, fibrocartilage tissue was formed under the knot of the anchor thread. Although the reason is unclear, bone marrow bleeding may have penetrated the gap of the distorted tendon fiber alignment by compression of the knot and pooled around the anchor thread. Furthermore, the tendon is connected to the bone through the fibrocartilage tissue, which may be closer to an area with direct healing and results in a stronger failure load. Moreover, the anchor fixation was less invasive than the BT fixation because the drill diameter was smaller, which may have promoted earlier healing. A new finding of this study can enhance the understanding that the anchor fixation method for tendon grafts, when combined with the microfracture procedure, is less invasive and obtains a level of initial fixation similar to that in BT fixation. In clinical practice, this result not only supports the use of anchor fixation for UCL reconstruction but also promotes awareness that the combination of microfracture procedure can enhance the initial fixation.

The limitation of this study is that it was evaluated at 4 weeks postoperatively. In biomechanical tests for a BT model, Rodeo et al reported that the increase in the strength of the interface was greatest in the first 4 weeks after the transplantation to the tendon.³³ Although there was a mild increase in the strength after the fourth week, it was not significant. The first four weeks after surgery, also called the proliferative period, are the most important in tendon-bone healing.¹⁴ Therefore, we focused on the evaluation of fibrocartilage formation in the early stages of healing. However, the long-term postoperative course may show different changes between the BT fixation and anchor fixation with or without the microfracture procedure. We investigated the biomechanical test after 8 weeks in a similar group of models. BT and anchor fixation under the microfracture procedure at 4 weeks and BT and anchor fixation without conducting the microfracture procedure at 8 weeks showed a similar level of failure load ([Supplemental Figure S1](#)). Moreover, BT and anchor fixation under microfracture treatment at 8 weeks showed significantly increased failure load compared with that at 4 weeks. This result suggests that the addition of microfracture procedure may accelerate the initial fixation. For concerns about the number of samples for each analysis, a power analysis could not be performed because there were no previous studies similar to the current experiment and the original standard deviations for each analysis (modified histological score, gene expression, and tendon graft thickness) were unknown. However, biomechanical tests were performed with a similar number of 4 in each group, referring to previous studies using BTs and anchors.³⁹ In mRNA analysis, the control group was used as the negative control. We also examined the native enthesis of the humeral side of the elbow UCL in rabbits as a positive control for gene expression. The results of comparison with native enthesis showed that gene expression was very poor ([Supplementary Figure S2](#)). Our main goal is to examine a more suitable method to reconstruct enthesis and achieve a rigid fixation in the case with UCL reconstruction. However, the model in this study is an original model created to evaluate the attachment of the tendon graft rather than a model mimicking a UCL reconstruction. Therefore, the fixation methods used in this model, such as anchors and BT, are similar to those in clinical cases, but the site of tendon grafting, bone, and the fact that it is a static model are different from UCL reconstruction cases. This is the first study to compare and investigate the effectiveness of BT and anchor fixation with and without the microfracture procedure in a tendon graft model, including gene expression. The results of this study will provide useful information to improve the procedure of ligament reconstruction in terms of initial fixation of the tendon graft.

Conclusion

In two different tendon graft fixation models, the tendon-bone junction of BT and anchor fixation without the microfracture procedure showed fibrous healing, and there was no significant difference in modified histological score, gene expression, and biomechanical test. However, BT and anchor fixation under the microfracture procedure lead to construction of fibrocartilage tissue at the tendon-bone junction and improved the modified histological score, gene expression, and initial fixation strength. The difference between BT and anchor fixation was evident in the thickness of the tendon at the junction and location of the fibrocartilage tissue formation. Therefore, our hypothesis that BT fixation is superior to anchor fixation in histological and mechanical results and that the augmentation of microfracture procedure further improves these results is partially supported.

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Supplementary data

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