An Autograft for Anterior Cruciate Ligament Reconstruction Results in Better Biomechanical Performance and Tendon-Bone Incorporation Than Does a Hybrid Graft in a Rat Model

Hong-De Wang,*†‡§ MD, Tian-Rui Wang,*†‡§‖ MD, Yao Sui,§¶ MD, Juan Wang,*†‡ MD, Wei Chen,*†‡ MD, and Ying-Ze Zhang,*†‡#** MD
Investigation performed at the Third Hospital of Hebei Medical University, Shijiazhuang, China

Background: The biomechanical and tendon-bone incorporation properties of allograft-augmented hybrid grafts for anterior cruciate ligament (ACL) reconstruction compared with traditional autografts are unknown.

Hypothesis: Using an autograft for ACL reconstruction yields better results on biomechanical testing, radiographic analysis, and histological evaluation versus using a hybrid graft.

Study Design: Controlled laboratory study.

Methods: A total of 66 adult male Sprague Dawley rats underwent unilateral ACL reconstruction with an autograft (AT group; n = 33) or a hybrid graft (HB group; n = 33). The grafts used in both groups were harvested from the peroneus longus tendon and were fixed by suturing to the surrounding periosteum. Samples were harvested for biomechanical testing, micro–computed tomography (CT), and histological evaluation at 4, 8, and 12 weeks postoperatively. Bone tunnels on the femoral and tibial sides were divided into 3 subregions: intra-articular (IA), midtunnel (MT), and extra-articular (EA). A cylinder-like volume of interest in the bone tunnel and a tubular-like volume of interest around the bone tunnel were used to evaluate new bone formation and bone remodeling, respectively, via micro-CT.

Results: In the AT group, there were significantly higher failure loads and stiffness at 8 weeks (failure load: 3.04 ± 0.40 vs 2.09 ± 0.54 N, respectively; $P = .006$) (stiffness: 3.43 ± 0.56 vs 1.75 ± 0.52 N/mm, respectively; $P < .001$) and 12 weeks (failure load: 9.10 ± 1.13 vs 7.14 ± 0.94 N, respectively; $P = .008$) (stiffness: 4.45 ± 0.75 vs 3.36 ± 0.29 N/mm, respectively; $P = .008$) than in the HB group. With regard to new bone formation in the bone tunnel, in the AT group, the bone volume/total volume (BV/TV) was significantly higher than in the HB group on the tibial side at 8 weeks (IA: 22.21 ± 4.98 vs 5.16 ± 3.98, respectively; $P < .001$) (EA: 19.66 ± 7.19 vs 10.85 ± 2.16, respectively; $P = .030$) and 12 weeks (IA: 30.50 ± 5.04 vs 17.11 ± 7.31, respectively; $P = .010$) (MT: 21.15 ± 2.58 vs 15.55 ± 4.48, respectively; $P = .041$) (EA: 20.75 ± 3.87 vs 10.64 ± 3.94, respectively; $P = .003$). With regard to bone remodeling around the tunnel, the BV/TV was also significantly higher on the tibial side at 8 weeks (MT: 33.17 ± 8.05 vs 15.21 ± 7.60, respectively; $P = .007$) (EA: 25.19 ± 6.38 vs 13.94 ± 7.10, respectively; $P = .030$) and 12 weeks (IA: 69.46 ± 4.45 vs 47.80 ± 6.16, respectively; $P < .001$) (MT: 33.15 ± 3.88 vs 13.76 ± 4.07, respectively; $P < .001$) in the AT group than in the HB group. Sharpey-like fibers had formed at 8 weeks in the AT group. A large number of fibroblasts withdrew at 12 weeks. In the AT group, the width of the interface was significantly narrower at 4 weeks (85.86 ± 17.49 vs 182.97 ± 14.35 μm, respectively; $P < .001$), 8 weeks (58.86 ± 10.99 vs 90.15 ± 11.53 μm, respectively; $P = .002$), and 12 weeks (42.70 ± 7.96 vs 67.29 ± 6.55 μm, respectively; $P = .001$) than in the HB group.

Conclusion: Using an autograft for ACL reconstruction may result in improved biomechanical properties and tendon-bone incorporation compared with a hybrid graft.

Clinical Relevance: Augmenting small autografts with allograft tissue may result in decreased biomechanical performance and worse tendon-bone incorporation, increasing the risk of graft failure.

Keywords: ACL; autograft; biomechanical performance; hybrid graft; rat model; tendon-bone incorporation

The American Journal of Sports Medicine
1–10
DOI: 10.1177/0363546520967668

A hamstring tendon autograft is commonly used to reconstruct the anterior cruciate ligament (ACL) in patients with an ACL injury.[35,54,56] Compared with the bone–patellar tendon–bone autograft, the hamstring tendon autograft is

reportedly associated with less donor site morbidity, such as anterior knee pain and extension stiffness.[19] Moreover, because of the risk of growth disorders (tibial tuberosity epiphyseal plate fusion and the risk of genu recurvatum induced by transphyseal tunnel bone plugs), ACL reconstruction in growing children should preferably involve a hamstring tendon autograft.[49] However, some patients have a comparatively small hamstring tendon, which may compromise the mechanical properties of an autograft.[45,50] Another tissue commonly used for ACL reconstruction is an allograft.[14,39] The benefits of using an allograft for ACL reconstruction include a predictable graft size and no donor site morbidity.[11,43] Thus, augmenting the autograft with an allograft to create a hybrid graft is theoretically an ideal solution to the problem of an inadequate graft diameter.

Although some authors suggest that using an allograft is a viable means of augmentation when faced with a small autograft during surgery,[18,24] potentially high risks of failure and unknown knee stability associated with hybrid grafting for ACL reconstruction are ongoing concerns.[14,37,52] These potential pitfalls may be caused by differences in graft biomechanical performance[40] and tendon-bone healing capacity between autografts and allografts.[31] Whether the benefits of an allograft for augmentation in ACL reconstruction are equal to those of an autograft with respect to graft biomechanical performance and tendon-bone incorporation remain unclear at least partly because of the difficulty in investigating such comparisons in human patients.

In the current study, autografts were compared with hybrid grafts biomechanically, radiographically, and histologically in a rat ACL reconstruction model. We hypothesized that autografts would yield better results on biomechanical testing, radiographic analysis, and histological evaluation.

METHODS

Study Design

The study was approved by the Institutional Animal Care and Use Committee. A total of 66 adult male Sprague Dawley rats (age, 6-8 weeks; weight, 250-280 g) were randomly allocated to either an autograft (AT) group or a hybrid graft (HB) group (n = 33 each). All rats underwent unilateral ACL resection followed by primary ACL reconstruction, which was conducted by the first 2 authors (H.-D.W and T.-R.W.). The 33 rats in the AT group underwent primary ACL reconstruction with an autograft. The 33 rats in the HB group underwent primary ACL reconstruction with an autograft plus an allograft. Subgroups of rats were euthanized at 4, 8, and 12 weeks postoperatively. At each time point, healing of the graft was assessed via biomechanical testing, micro–computed tomography (CT), and histological evaluation. A summary of the study design is depicted in Figure 1.

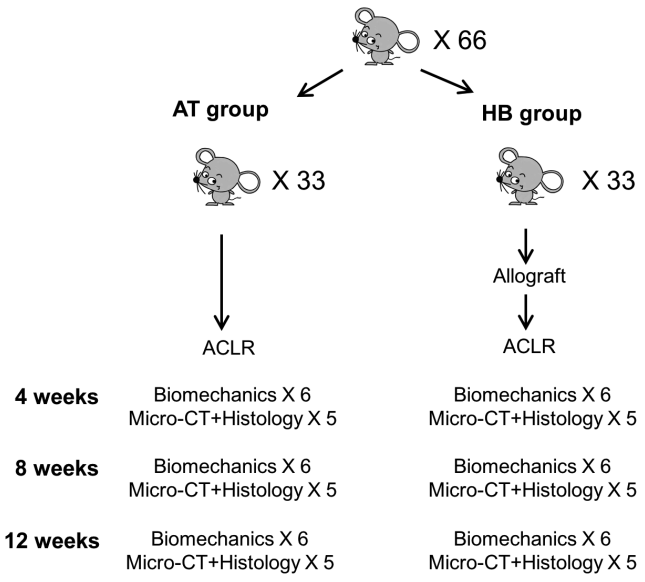

Figure 1. Summary of the experimental design. ACLR, anterior cruciate ligament reconstruction; AT, autograft group; CT, computed tomography; HB, hybrid graft group.

Allograft Preparation

All 33 rats in the HB group were anesthetized with 2% isoflurane in oxygen gas (1.5 L/min) administered via an inhalation mask. The full length of the peroneus longus tendon of the right lower leg was harvested through a percutaneous incision on the lateral aspect of the ankle joint using a stripper generated in house, and then, the wound was closed with sutures. The harvested tendon graft was debrided, washed, and wrapped in saline-soaked sterile gauze immediately. To avoid any tendon graft harvested from a rat from subsequently being used in that same rat

**Address correspondence to Ying-Ze Zhang, MD, Department of Orthopaedic Surgery, Third Hospital of Hebei Medical University, No. 139 Ziqiang Road, Qiaoxi District, Shijiazhuang, Hebei, 050051, China (email: profyzzhang@126.com).
*Department of Orthopaedic Surgery, Third Hospital of Hebei Medical University, Shijiazhuang, China.
†Key Laboratory of Biomechanics of Hebei Province, Shijiazhuang, China.
‡NHC Key Laboratory of Intelligent Orthopaedic Equipment, Third Hospital of Hebei Medical University, Shijiazhuang, China.
§Department of Biochemistry and Molecular Biology, Hebei Medical University, Shijiazhuang, China.
‖Department of Orthopaedic Surgery, Affiliated Hospital of Qingdao University, Qingdao, China.
¶Department of Ophthalmology, Second Hospital of Hebei Medical University, Shijiazhuang, China.
#Chinese Academy of Engineering, Beijing, China.
H.-D.W., T.-R.W., and Y.S. contributed equally to this work.
Submitted January 8, 2020; accepted July 7, 2020.
The authors declared that they have no conflicts of interest in the authorship and publication of this contribution. AOSSM checks author disclosures against the Open Payments Database (OPD). AOSSM has not conducted an independent investigation on the OPD and disclaims any liability or responsibility relating thereto.

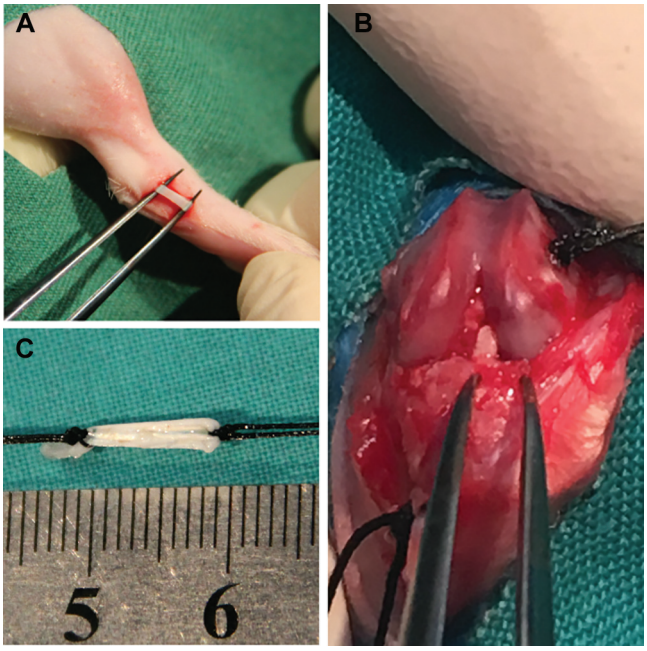

Figure 2. Surgical procedure for anterior cruciate ligament reconstruction. (A) The right peroneus longus tendon was harvested. (B) Both ends of the tendon graft were sutured to the surrounding periosteum at the exit of the tunnel. (C) The final length of the tendon graft.

during the ACL procedure, every tendon graft was placed in a sterile frozen tube and marked with the donor rat number. All tendon grafts were stored at −20°C for at least 1 month before subsequent surgical procedures.[5,44] The frozen tendon grafts were placed in a 4°C refrigerator to thaw 24 hours before surgery.

Surgical Procedure

Rats were anesthetized with 2% isoflurane in oxygen gas (1.5 L/min) administered via an inhalation mask and 4% chloral hydrate solution at a dose of 1 mL per 100 g of body weight. The full length of the peroneus longus tendon of the left lower leg was procured through a percutaneous incision on the lateral aspect of the ankle joint. The harvested autograft was cleaned of muscle on a graft preparation board. In the AT group, the right peroneus longus tendon was also procured for augmentation (Figure 2A). The tendon grafts used in the HB group comprised both an autograft and an allograft. After the tendon grafts were doubled over, the tendon grafts were sutured together with 2-0/T nonabsorbable suture at the top end. To achieve uniform contact between the allograft or autograft and the bone, we staggered each of the 4 half-tendon grafts. The final length of the tendon graft was ≥10 mm (Figure 2B). Standard medial parapatellar arthrotomy was performed to access the left knee. The native ACL was identified and excised. The tibia was then translated anteriorly to confirm that resection of the ACL was complete and to visualize the tibial ACL footprint. With the knee flexed to 90°, a 1.4-mm dental drill was used to create bone tunnels in the proximal tibia and distal femur. The tendon graft was then passed through the tunnels. The knee was next brought to 30° of flexion, at which point the graft was pretensioned to 5 N.[29] Both ends of the tendon graft were sutured to the surrounding periosteum at the exit of the tunnel using 4-0/T nonabsorbable suture[29,47,48] (Figure 2C). The wounds were closed with sutures, and physical activity was not restricted postoperatively. All rats received buprenorphine for pain management, and comfort and recovery were assessed multiple times per day.

Gross Tissue Assessment

After each rat was euthanized, the left lower limb was disarticulated at the hip joint and separated from the ankle joint. The knee joint was carefully dissected under a microscope, and the reconstructed ACL was assessed for synovial coverage.[5,22] Synovial coverage over the grafts was graded as good (coverage >80% around the graft), fair (coverage >50%), or poor (coverage <50%).[21-23] Biomechanical testing and micro-CT of the specimens were performed immediately.

Biomechanical Testing

Specimens were tested using a biomechanical testing machine (ElectroForce 3520-AT; TA Instruments) immediately after harvest at 4, 8, and 12 weeks postoperatively. After removing the fixation construct at the femoral and tibial ends of the graft, the specimen was fixed using a component of the testing apparatus to ensure that the load applied was directed along the longitudinal axis of the graft. Before testing, 10 cycles of longitudinal preloading of 1 N were applied to the specimen for preconditioning.[20] The ultimate failure load was documented at an elongation speed of 0.25 mm/s.[20] Stiffness was calculated from the linear portion of the load-displacement curve using Excel 2016 (Version 15.27; Microsoft).[36] Modes of graft failure were recorded as pullout from the bone tunnel or rupture at the midsubstance. Samples were kept moist with saline solution throughout testing.

Micro-CT

Micro-CT scanning and evaluations of the samples were performed as described in previous reports.[10,27,36] Scanning (SkyScan 1176; Bruker) of specimens was conducted perpendicular to the long axis of the lower limbs at 4, 8, and 12 weeks postoperatively. The parameters used for scanning were the following: source voltage of 65 kV, source current of 385 μA, image rotation of 0.1840, and filter aluminum of 1 mm. After scanning, the sections were 3-dimensionally reconstructed and rotated to align the bone tunnel vertically using NRecon software (Version 1.7.0.4;

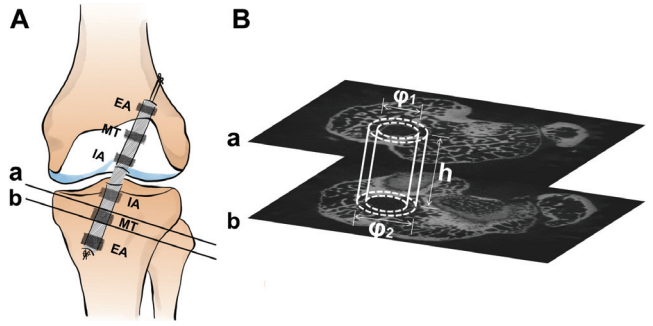

Figure 3. The femoral tunnel and tibial tunnel were divided into 3 subregions of equal length: intra-articular (IA), midtunnel (MT), and extra-articular (EA). The (*a*) and (*b*) planes were defined as the first and the last planes in each subregion. The volume of interest of new bone formation was a cylinder with a diameter of 1.4 mm (φ1) and height (*h*) of 50 sections in each femoral tunnel subregion and 100 sections in each tibial tunnel subregion. The volume of interest of the surrounding bone was a tubular region with an inner diameter of 1.4 mm (φ1) and an outer diameter of 2.0 mm (φ2) as well as a height (*h*) of 50 sections in the femoral tunnel subregion and 100 sections in the tibial tunnel subregion.

Bruker). The image data were analyzed using Analyser software (Version 1.8.1.3; Bruker).

The femoral tunnel and tibial tunnel were divided into intra-articular (IA), midtunnel (MT), and extra-articular (EA) subregions of equal length. The volume of interest of new bone formation was a cylinder with a diameter of 1.4 mm and a height of 50 sections in each femoral tunnel subregion and 100 sections in each tibial tunnel subregion. The volume of interest of the surrounding bone was a tubular volume with an inner diameter of 1.4 mm and an outer diameter of 2.0 mm as well as a height of 50 sections in the femoral tunnel subregion and 100 sections in the tibial tunnel subregion (Figure 3). The bone volume/total volume (BV/TV) ratio was used to evaluate the amount of new bone formation in each subregion and bone changes around each subregion since surgery. The BV was calculated as the total number of thresholded bone voxels within the volume of the cylindrical or tubular volume of interest.

Histological Evaluation

After micro-CT scanning, the proximal tibias of the samples were immediately resected while preserving the entire length of the bone tunnels. The proximal tibia samples were then fixed in paraformaldehyde, decalcified in a solution of EDTA, and embedded in paraffin. The embedded samples were sectioned at a thickness of 5 μm perpendicular to the longitudinal axis of the bone tunnel. Hematoxylin and eosin staining was conducted via a standard protocol.[28] To assess graft-bone integration, the interface between the graft and bone at a depth of 1 mm from the joint surface was assessed via hematoxylin and eosin staining. The interface width was measured as the distance between the edge of the bone tunnel and the outer graft. As noted in previous studies, a thinner interface suggests better integration.[4,7,8,47] The mean interface width of a sample was calculated by averaging the width at every 30° point of the tunnel cross section from 0° to 360°, and 3 sections were measured per sample.[47] The graft-bone integration parameters were analyzed using a previously validated scoring system.[28] This scoring system was based on a scaled maximum of 20 points with a range from 0 to 4 points in each parameter. Parameters assessed were graft degeneration, graft remodeling, percentage of fibrous tissue, collateral connection, and head-to-head connection. For each measure, higher scores indicated better integration. The slides were independently observed using an inverted light microscope (DFC7000T; Leica) by 2 investigators (T.-R.W. and Y.S.) who were blinded to group allocation. Digital images were acquired using Leica Application Suite (Version 4.12.0; Leica). ImageJ software (Java 1.6.0_65; National Institutes of Health) was used for further quantitative analysis performed by an independent investigator (T.-R.W.) who was blinded to group allocation.

Statistical Analysis

Statistical comparisons of biomechanical, radiological, and histological data at each time point were performed using the Student *t* test. Continuous data are presented as the mean ± SD. The Mann-Whitney *U* test was used for a comparison of synovial coverage. All data analyses were performed with SPSS software (Version 21.0.0.0; IBM). Statistical significance was set at $P < .05$.

RESULTS

General and Gross Tissue Findings

Overall, 3 rats died intraoperatively (2 in the AT group and 1 in the HB group), and there were another 2 premature deaths (in the HB group) with no obvious cause. Thus, 5 replacement rats were recruited into the study to return each group to a size of 33 specimens. No synovitis, degenerative changes in articular cartilage, or graft ruptures were apparent. Partial synovial coverage of the graft was observed at 4 weeks in both groups, but better synovial coverage was observed in the AT group at 8 weeks (P = .010) and 12 weeks (P = .027) (Table 1). Moreover, different levels of graft maturity were evident in the HB group at 8 weeks (Figure 4).

Biomechanical Findings

Pullout from the tibial tunnel (graft failure) was evident in 2 rats in the AT group at 4 weeks, 3 rats in the HB group at 4 weeks, and 1 rat in the HB group at 8 weeks. All other grafts in both groups were torn at the midsubstance (Table 2). The mean ultimate failure loads in the AT group were significantly higher than those in the HB group at 8 weeks (3.04 ± 0.40 vs 2.09 ± 0.54 N, respectively; P = .006) and 12 weeks (9.10 ± 1.13 vs 7.14 ± 0.94 N, respectively; P = .008). Failure loads did not differ significantly between the 2 groups at 4

TABLE 1
Results of Synovial Coverage[a]

	4 Weeks			8 Weeks			12 Weeks		
	AT Group	HB Group	*P* Value	AT Group	HB Group	*P* Value	AT Group	HB Group	*P* Value
Synovialization, n			.147			.010			.027
Good	0	0		3	0		7	2	
Fair	2	0		7	5		4	8	
Poor	9	11		1	6		0	1	

[a]AT, autograft; HB, hybrid graft.

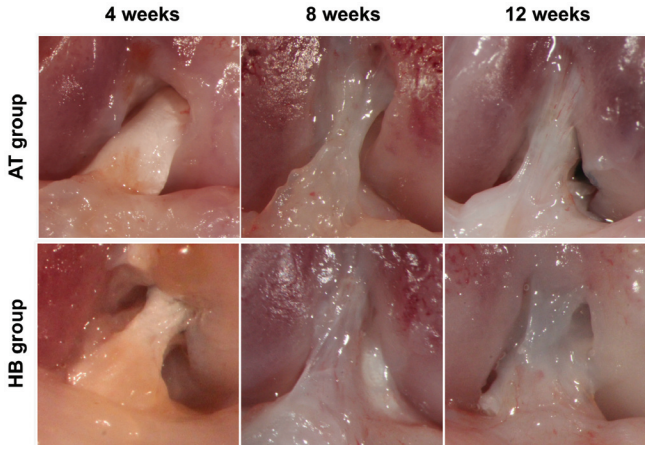

Figure 4. Gross tissue findings at 4, 8, and 12 weeks postoperatively in the autograft (AT) group and the hybrid graft (HB) group.

TABLE 2
Failure Mode During Biomechanical Testing[a]

	Femoral Pullout	Tibial Pullout	Midsubstance Failure
4 weeks			
AT group	0	2	4
HB group	0	3	3
8 weeks			
AT group	0	0	6
HB group	0	1	5
12 weeks			
AT group	0	0	6
HB group	0	0	6

[a]Data are shown as No. For each group, n = 6. AT, autograft; HB, hybrid graft.

weeks (P = .905) (Appendix Table A1 [available in the online version of this article] and Figure 5A). Graft stiffness was significantly higher in the AT group than in the HB group at 8 weeks (3.43 ± 0.56 vs 1.75 ± 0.52 N/mm, respectively; $P <$ .001) and 12 weeks (4.45 ± 0.75 vs 3.36 ± 0.29 N/mm, respectively; P = .008). Graft stiffness did not differ significantly between the 2 groups at 4 weeks (P = .170) (Appendix Table A1 [available online] and Figure 5B).

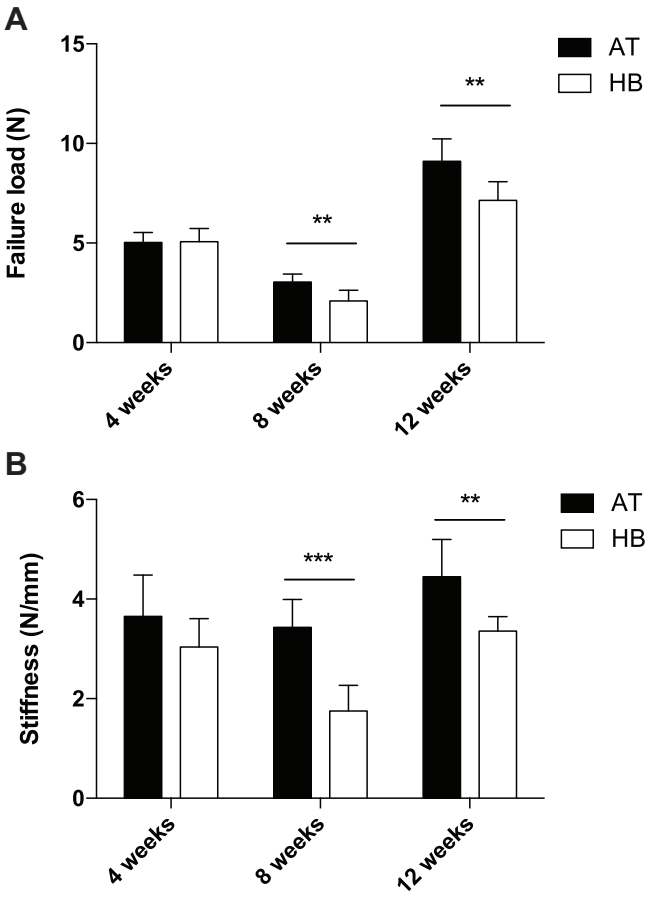

Figure 5. (A) Failure load and (B) stiffness of the grafts after anterior cruciate ligament reconstruction at 4, 8, and 12 weeks postoperatively in the autograft (AT) group and the hybrid graft (HB) group. **$P < .01$. ***$P < .001$.

Micro-CT Findings

With regard to new bone formation, most bone tunnel subregions in both groups exhibited time-dependent increases in the BV/TV from 4 to 12 weeks (Figure 6). On the femoral side, the mean BV/TV was higher in the AT group, although the difference between the 2 groups was not statistically significant at any time point (Appendix Table A2 [available online] and Figure 6, A-C). On the tibial side, at

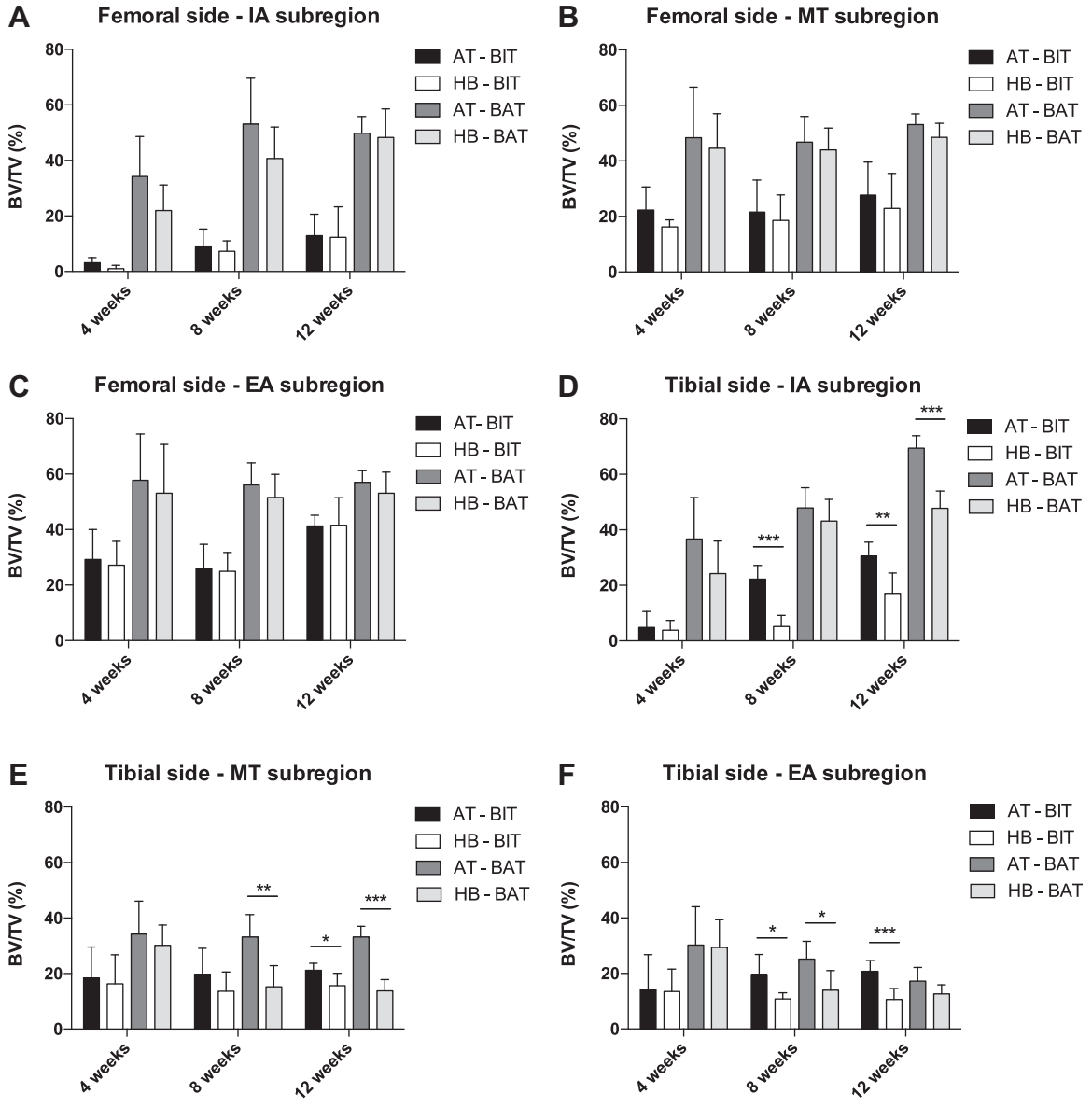

Figure 6. Bone volume/total volume (BV/TV) ratio on (A-C) the femoral side and (D-F) the tibial side after anterior cruciate ligament reconstruction at 4, 8, and 12 weeks postoperatively in the autograft (AT) group and the hybrid graft (HB) group. BAT, bone around the tunnel; BIT, bone in the tunnel; EA, extra-articular; IA, intra-articular; MT, midtunnel. *$P < .05$. **$P < .01$. ***$P < .001$.

8 weeks, the BV/TV was significantly higher in the AT group than in the HB group in the IA subregion (22.21 ± 4.98 vs 5.16 ± 3.98, respectively; $P < .001$) and the EA subregion (19.66 ± 7.19 vs 10.85 ± 2.16, respectively; $P = .030$). At 12 weeks, the BV/TV was significantly higher in the AT group than in the HB group in the IA subregion (30.50 ± 5.04 vs 17.11 ± 7.31, respectively; $P = .010$), MT subregion (21.15 ± 2.58 vs 15.55 ± 4.48, respectively; $P = .041$), and EA subregion (20.75 ± 3.87 vs 10.64 ± 3.94, respectively; $P = .003$) (Figure 6, D-F).

With regard to bone remodeling around the tunnel subregion, the mean BV/TV was higher in the AT group than in the HB group on the femoral side at every time point investigated, but the difference was not statistically significant at any time point (Appendix Table A2 [available online] and Figure 6, A-C). On the tibial side, at 8 weeks, the BV/TV was significantly higher in the AT group than in the HB group in the MT subregion (33.17 ± 8.05 vs 15.21 ± 7.60, respectively; $P = .007$) and EA subregion (25.19 ± 6.38 vs 13.94 ± 7.10, respectively; $P = .030$). At 12 weeks, the BV/TV was significantly higher in the AT group than in the HB group in the IA subregion (69.46 ± 4.45 vs 47.80 ± 6.16, respectively; $P < .001$) and MT subregion (33.15 ± 3.88 vs 13.76 ± 4.07, respectively; $P < .001$) (Figure 6, D-F).

TABLE 3
Results of Histological Evaluation[a]

	4 Weeks			8 Weeks			12 Weeks		
Graft-Bone Integration	AT Group	HB Group	*P* Value	AT Group	HB Group	*P* Value	AT Group	HB Group	*P* Value
Graft degeneration	1.20 ± 0.45	1.60 ± 0.55	.242	2.60 ± 0.55	2.00 ± 0.71	.172	3.40 ± 0.55	3.20 ± 0.45	.545
Graft remodeling	2.20 ± 0.45	1.40 ± 0.55	.035	2.80 ± 0.45	1.80 ± 0.89	.028	2.80 ± 0.45	1.60 ± 0.55	.005
Percentage of fibrous tissue	3.20 ± 0.84	2.80 ± 0.45	.373	3.60 ± 0.55	2.20 ± 0.45	.002	4.40 ± 0.55	3.20 ± 0.45	.005
Collateral connection	2.20 ± 0.84	1.40 ± 0.55	.111	1.20 ± 0.45	1.80 ± 0.84	.195	2.00 ± 0.71	3.20 ± 0.45	.012
Head-to-head connection	1.00 ± 0.71	0.60 ± 0.55	.347	2.80 ± 0.45	1.60 ± 0.55	.005	3.40 ± 0.55	2.20 ± 0.45	.005
Total	9.80 ± 1.10	7.80 ± 1.30	.030	13.00 ± 1.58	9.20 ± 1.64	.006	16.00 ± 1.87	13.40 ± 1.14	.029

[a]Data are shown as mean ± SD. AT, autograft; HB, hybrid graft.

Histological Findings

At 4 weeks postoperatively, a distinct graft-bone interface was evident in both groups. In the AT group, many osteoclasts were identified in the bone around the tunnel. The bone near the interface exhibited intense bone remodeling with osteoblasts and blood vessels. Substantial fibroblasts and some newly formed blood vessels infiltrated the graft. In the HB group, the bone around the tunnel did not exhibit the intense remodeling that was apparent in the AT group, and only a small number of fibroblasts infiltrated the interface.

At 8 weeks postoperatively, in the AT group, Sharpey-like fibers were observed in the interface, connecting the host bone and graft. A large number of fibroblasts had infiltrated the entire graft, and areas of high cellularity were evident at the center of the graft. In the HB group, the graft was surrounded by loose connective tissue with several newly formed blood vessels and a large number of fibroblasts.

At 12 weeks postoperatively, in the AT group, the number of infiltrating fibroblasts had declined sharply. The fibroblasts were uniformly distributed throughout the graft, the graft-bone interface had become thinner, and the fibroblasts had morphed from oval-shaped cells to spindle-shaped cells in the interface. In the HB group, Sharpey-like fibers were evident at the 12-week time point. The number of fibroblasts in the graft was greater than it had been at 8 weeks, especially in the interface. However, the cellular distribution was still inconsistent, and acellular areas were sparse (Figure 7A).

The width of the tendon-bone interface was significantly smaller in the AT group than in the HB group at 4 weeks (85.86 ± 17.49 vs 182.97 ± 14.35 μm, respectively; $P < .001$), 8 weeks (58.86 ± 10.99 vs 90.15 ± 11.53 μm, respectively; $P = .002$), and 12 weeks (42.70 ± 7.96 vs 67.29 ± 6.55 μm, respectively; $P = .001$) (Figure 7B).

The quantitative histological results are reported in Table 3. There was a significant difference in the total score between the AT group and HB group at 4 weeks ($P = .030$), 8 weeks ($P = .006$), and 12 weeks ($P = .029$). At 4 weeks postoperatively, the graft remodeling score was significantly higher in the AT group ($P = .035$). At 8 weeks postoperatively, the graft remodeling score ($P = .028$), percentage of fibrous tissue score ($P = .002$), and head-to-head connection score ($P = .005$) were significantly higher in the AT group. At 12 weeks postoperatively, the graft remodeling score ($P = .005$), percentage of fibrous tissue score ($P = .005$), and head-to-head connection score ($P = .005$) were significantly higher in the AT group. However, the collateral connection score ($P = .012$) was significantly higher in the HB group at 12 weeks postoperatively.

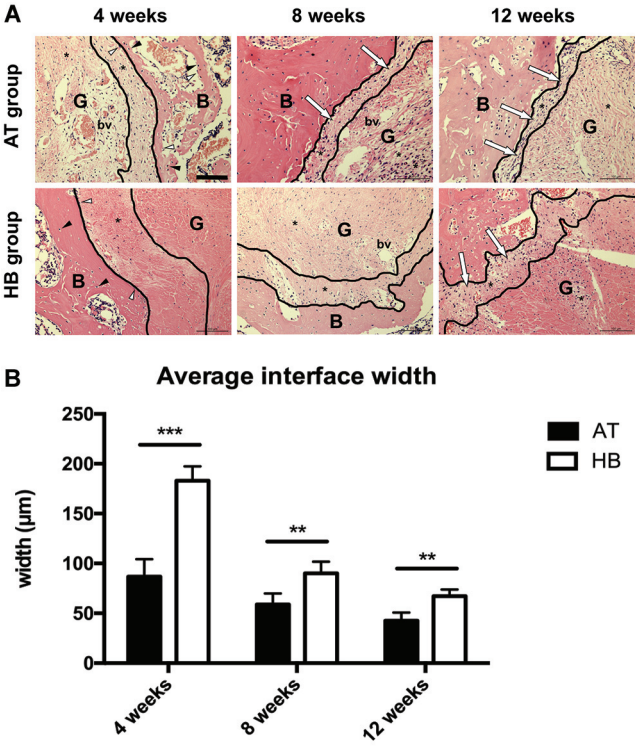

Figure 7. (A) Representative images of hematoxylin and eosin–stained sections of the tendon-bone interface at 4, 8, and 12 weeks postoperatively in the autograft (AT) group and the hybrid graft (HB) group, with (B) statistical comparisons of the mean width of the tendon-bone interface. The tendon-bone interface in (A) is outlined by thick irregular black lines. The white arrows indicate Sharpey-like fibers. Scale bar = 100 μm; magnification for all images is the same. *, fibroblasts; Δ, osteoblasts; ▲, osteoclasts; B, bone; bv, blood vessel; G, graft. $^{**}P < .01$. $^{***}P < .001$.

DISCUSSION

In the current study, the use of gross tissue observation, biomechanical testing, micro-CT, and histological evaluation yielded 2 important findings. One was that after using autografts for ACL reconstruction, the reconstructed ACLs exhibited better biomechanical performance and more mature morphology in the articular cavity than ACLs reconstructed using hybrid grafts. The other was that using an autograft promoted new bone formation in the bone tunnel, bone remodeling around the bone tunnel, and tendon-bone healing at the interface.

Previous studies demonstrated that graft diameter has a strong effect on ACL reconstruction.[9,13] However, using a hamstring tendon autograft less than 8 mm in diameter has been associated with poor clinical outcomes.[12,30] As such, hybrid grafts are recommended in cases of small hamstring tendon autografts because hybrid grafts have the benefit of a hamstring tendon autograft with a customizable graft size. However, to date, few studies have compared the outcomes of ACL reconstruction using a small hamstring tendon graft versus a hybrid graft, and the reported outcomes are varied.[6,16,25,26] Leo et al[25] and Li et al[26] reported that using a hybrid graft for ACL reconstruction resulted in a similar rupture rate and clinical outcomes to reconstruction using a small hamstring tendon autograft, whereas Burrus et al[6] and Darnley et al[14] reported that hybrid grafts may have a higher failure rate and an increased risk for revision. The question of whether there is a difference between hybrid grafts and small hamstring tendon autografts for ACL reconstruction was answered in previous studies.[1,46,53] In a recent meta-analysis, the mean autograft diameter ranged from 6.4 ± 0.2 to 8.8 ± 0.5 mm, and the mean diameter of hybrid grafts ranged from 8.9 ± 1.0 to 9.9 ± 0.8 mm.[53] There was no significant difference in patient-reported outcomes or failure rates between the 2 grafts.[53] This means that using an allograft as augmentation to enlarge a small hamstring tendon autograft for ACL reconstruction did not improve patient-reported outcomes or reduce the failure rate compared with using an autograft. Thus, some authors suggested that in patients with risk factors for small hamstring tendons, grafts should be harvested as bilateral hamstring tendon grafts, and patients should undergo ACL reconstruction with purely a hamstring tendon autograft.[6,52] The purpose of the current study was not to determine whether to augment a small autograft with an allograft but to determine which graft should be used to augment a small autograft for ACL reconstruction, allografts or autografts. Our results suggest that harvesting another autograft for augmentation may be a better option in cases of small hamstring tendon autografts versus using a hybrid graft. Clinicians should be aware that using a hybrid graft for ACL reconstruction will result in poorer biomechanical performance and tendon-bone incorporation, which may lead to worse clinical outcomes compared with an autograft.

Very few clinical studies have examined differences in knee stability outcomes between patients who underwent autografting and those who underwent hybrid grafting.[53] Sufficient biomechanical performance of a reconstructed ACL provides the necessary support for knee stability, but biomechanical testing of reconstructed ACLs cannot be routinely performed on patients. Some authors have recommended not using an allograft as augmentation for ACL reconstruction because this may result in poor knee stability and increase the risk of failure.[6,14,52] We confirmed these potential risks in the current study. The mean failure load and mean stiffness were lower in the HB group from 4 to 12 weeks postoperatively, particularly at 8 and 12 weeks, at which time points there was circuitous fiber orientation and less synovial graft coverage. Potentially slower remodeling with allografts has been confirmed in many studies; however, studies evaluating hybrid graft remodeling are lacking. Shino et al[42] reported that allograft remodeling is delayed in ACL reconstruction and that this results in reduced stability and mechanical function compared with ACL reconstruction with an autograft. Malinin et al[31] examined 9 specimens after ACL reconstruction with an allograft and reported that the central portions of the grafts remained acellular at 2 years postoperatively. In the current study, the allografts in the HB group showed slower remodeling, and different levels of graft maturity with less synovial coverage were observed in the HB group at 8 weeks. In a previous retrospective comparative study, different maturity levels of hybrid grafts were confirmed by second-look evaluations and magnetic resonance imaging conducted 3 years postoperatively.[52] These observations indicate that differences in biomechanical performance may correspond to differences in the remodeling of autografts and allografts within a hybrid graft.

Successful healing between the host bone and a graft can improve mechanical properties, which are vital to knee stability.[27,32] Micro-CT can be used to quantify new bone formation in the bone tunnel and bone remodeling around the tunnel via the BV/TV. In the present study, rats in both groups exhibited an increased BV/TV from 4 to 12 weeks, suggesting a common healing process in the bone tunnel. Notably, however, the samples in the AT group exhibited significantly more bone formation on the tibial side.

Bone remodeling including bone resorption and formation around the tunnel influences graft healing, strength, and function.[2,15,33] In previous studies, different parts of the bone tunnel reportedly changed in different ways during the tendon-bone healing process.[3,10,36,38] Thus, we divided the bone tunnel into 3 portions on the femoral and tibial sides. The maximum diameter of the enlarged part of the bone tunnel was 2.0 mm in all specimens, so we chose a tubular volume of interest with an inner diameter of 1.4 mm and an outer diameter of 2.0 mm. The AT group had a larger mean BV/TV around the bone tunnel on the tibial side at 8 and 12 weeks postoperatively, especially in the IA subregion, and this may explain the histological observation that the bone near the interface exhibited intense remodeling with osteoblasts and blood vessels.

At the tendon-bone interface, fibroblasts play a key role in maintaining homeostasis in normal ACLs and contribute to the formation of strength-bearing Sharpey-like fibers at the interface.[20,55] Contractile fibroblasts express

the alpha–smooth muscle actin isoform, and so-called myofibroblasts participate in ligamentization by producing collagen at an early stage, which can restore in situ tension.[34,41] In the current study, fibroblasts infiltrated the graft earlier in the AT group than in the HB group at 4 weeks, and by 8 weeks, Sharpey-like fibers had formed in the AT group before their formation in the HB group.

In previous studies, myofibroblasts have reportedly receded with graft maturation.[17,51] This was also observed in the present study; at 12 weeks, the number of fibroblasts in the AT group was markedly reduced. A smaller interface width indicated better and more reliable tendon-bone healing.[7] The tendon-bone interface width was significantly narrower in the AT group than in the HB group from 4 to 12 weeks. These differences indicate that using an autograft for augmentation in ACL reconstruction may promote tendon-bone healing.

The current study had some limitations. First, the rat model used may not completely translate to the clinical human patient population. Knee biomechanics, graft blood supply, and fixation methods differ in rats and humans. Despite the limitation inherent in a small animal model, developing a rat model of ACL reconstruction has strong value for further studies of the basic cellular and molecular mechanisms of healing. Second, we did not evaluate the side effects such as anterior knee pain and sensory and strength deficits associated with the additional use of an autograft for augmentation. Whether harvesting more tendon results in more donor site morbidity and increases the risk of infections remains to be investigated in future studies. Finally, all analyses were conducted at an early stage postoperatively. Therefore, it is unknown whether long-term postoperative differences in biomechanical performance and tendon-bone incorporation exist between autografts and hybrid grafts.

CONCLUSION

When faced with a small graft during ACL reconstruction, using an autograft instead of a hybrid graft can result in better graft biomechanical performance and promote new bone formation in the bone tunnel, bone remodeling around the bone tunnel, and tendon-bone healing at the interface.

ACKNOWLEDGMENT

The authors thank Professor Jin-Kun Wen and his team members from the Key Laboratory of Neural and Vascular Biology, Department of Biochemistry and Molecular Biology, Hebei Medical University, for providing experimental technique support.

REFERENCES

1. Abouljoud MM, Everhart JS, Sigman BO, Flanigan DC, Magnussen RA. Risk of retear following anterior cruciate ligament reconstruction using a hybrid graft of autograft augmented with allograft tissue: a systematic review and meta-analysis. *Arthroscopy*. 2018;34(10):2927-2935.
2. Aga C, Wilson KJ, Johansen S, Dornan G, La Prade RF, Engebretsen L. Tunnel widening in single- versus double-bundle anterior cruciate ligament reconstructed knees. *Knee Surg Sports Traumatol Arthrosc*. 2017;25(4):1316-1327.
3. Araki D, Kuroda R, Matsumoto T, et al. Three-dimensional analysis of bone tunnel changes after anatomic double-bundle anterior cruciate ligament reconstruction using multidetector-row computed tomography. *Am J Sports Med*. 2014;42(9):2234-2241.
4. Atesok K, Fu FH, Wolf MR, et al. Augmentation of tendon-to-bone healing. *J Bone Joint Surg Am*. 2014;96(6):513-521.
5. Bhatia S, Bell R, Frank RM, et al. Bony incorporation of soft tissue anterior cruciate ligament grafts in an animal model: autograft versus allograft with low-dose gamma irradiation. *Am J Sports Med*. 2012;40(8):1789-1798.
6. Burrus MT, Werner BC, Crow AJ, et al. Increased failure rates after anterior cruciate ligament reconstruction with soft-tissue autograft-allograft hybrid grafts. *Arthroscopy*. 2015;31(12):2342-2351.
7. Cai J, Wan F, Dong Q, et al. Silk fibroin and hydroxyapatite segmented coating enhances graft ligamentization and osseointegration processes of the polyethylene terephthalate artificial ligament in vitro and in vivo. *J Mater Chem B*. 2018;6(36):5738-5749.
8. Cai J, Wang J, Ye K, et al. Dual-layer aligned-random nanofibrous scaffolds for improving gradient microstructure of tendon-to-bone healing in a rabbit extra-articular model. *Int J Nanomed*. 2018;13:3481-3492.
9. Calvo R, Figueroa D, Figueroa F, et al. Five-strand hamstring autograft versus quadruple hamstring autograft with graft diameters 8.0 millimeters or more in anterior cruciate ligament reconstruction: clinical outcomes with a minimum 2-year follow-up. *Arthroscopy*. 2017;33(5):1007-1013.
10. Camp CL, Lebaschi A, Cong GT, et al. Timing of postoperative mechanical loading affects healing following anterior cruciate ligament reconstruction: analysis in a murine model. *J Bone Joint Surg Am*. 2017;99(16):1382-1391.
11. Carey JL, Dunn WR, Dahm DL, Zeger SL, Spindler KP. A systematic review of anterior cruciate ligament reconstruction with autograft compared with allograft. *J Bone Joint Surg Am*. 2009;91(9):2242-2250.
12. Conte EJ, Hyatt AE, Gatt CJ Jr, Dhawan A. Hamstring autograft size can be predicted and is a potential risk factor for anterior cruciate ligament reconstruction failure. *Arthroscopy*. 2014;30(7):882-890.
13. Cruz AI Jr, Fabricant PD, Seeley MA, Ganley TJ, Lawrence JT. Change in size of hamstring grafts during preparation for ACL reconstruction: effect of tension and circumferential compression on graft diameter. *J Bone Joint Surg Am*. 2016;98(6):484-489.
14. Darnley JE, Leger-St-Jean B, Pedroza AD, Flanigan DC, Kaeding CC, Magnussen RA. Anterior cruciate ligament reconstruction using a combination of autograft and allograft tendon: a MOON cohort study. *Orthop J Sports Med*. 2016;4(7):2325967116662249.
15. DeFroda SF, Karamchedu NP, Owens BD, et al. Tibial tunnel widening following anterior cruciate ligament reconstruction: a retrospective seven-year study evaluating the effects of initial graft tensioning and graft selection. *Knee*. 2018;25(6):1107-1114.
16. Delaloye JR, Murar J, Vieira TD, et al. Combined anterior cruciate ligament repair and anterolateral ligament reconstruction. *Arthrosc Tech*. 2019;8(1):e23-e29.
17. Jackson DW, Grood ES, Goldstein JD, et al. A comparison of patellar tendon autograft and allograft used for anterior cruciate ligament reconstruction in the goat model. *Am J Sports Med*. 1993;21(2):176-185.
18. Jacobs CA, Burnham JM, Makhni E, Malempati CS, Swart E, Johnson DL. Allograft augmentation of hamstring autograft for younger patients undergoing anterior cruciate ligament reconstruction. *Am J Sports Med*. 2017;45(4):892-899.
19. Kartus J, Movin T, Karlsson J. Donor-site morbidity and anterior knee problems after anterior cruciate ligament reconstruction using autografts. *Arthroscopy*. 2001;17(9):971-980.
20. Kawakami Y, Takayama K, Matsumoto T, et al. Anterior cruciate ligament-derived stem cells transduced with bmp2 accelerate graft-bone integration after ACL reconstruction. *Am J Sports Med*. 2017;45(3):584-597.

21. Kim JG, Kang SH, Kim JH, Lim CO, Wang JH. Comparison of clinical results, second-look arthroscopic findings, and MRI findings between the transportal and outside-in techniques for double-bundle anatomic anterior cruciate ligament reconstruction: a prospective, randomized controlled trial with a minimum 2-year follow-up. *Am J Sports Med*. 2018;46(3):544-556.
22. Kim SG, Kim SH, Kim JG, Jang KM, Lim HC, Bae JH. Hamstring autograft maturation is superior to tibialis allograft following anatomic single-bundle anterior cruciate ligament reconstruction. *Knee Surg Sports Traumatol Arthrosc*. 2018;26(4):1281-1287.
23. Kinugasa K, Mae T, Matsumoto N, Nakagawa S, Yoneda M, Shino K. Effect of patient age on morphology of anterior cruciate ligament grafts at second-look arthroscopy. *Arthroscopy*. 2011;27(1):38-45.
24. Kraeutler MJ, Kim SH, Brown CC, et al. Clinical outcomes following primary anterior cruciate ligament reconstruction with hamstring autograft versus planned hybrid graft. *J Knee Surg*. 2018;31(9):827-833.
25. Leo BM, Krill M, Barksdale L, Alvarez-Pinzon AM. Failure rate and clinical outcomes of anterior cruciate ligament reconstruction using autograft hamstring versus a hybrid graft. *Arthroscopy*. 2016;32(11):2357-2363.
26. Li J, Wang J, Li Y, Shao D, You X, Shen Y. A prospective randomized study of anterior cruciate ligament reconstruction with autograft, gamma-irradiated allograft, and hybrid graft. *Arthroscopy*. 2015; 31(7):1296-1302.
27. Liu S, Sun Y, Wan F, Ding Z, Chen S, Chen J. Advantages of an attached semitendinosus tendon graft in anterior cruciate ligament reconstruction in a rabbit model. *Am J Sports Med*. 2018;46(13):3227-3236.
28. Lui PP, Ho G, Lee YW, Ho PY, Lo WN, Lo CK. Validation of a histologic scoring system for the examination of quality of tendon graft to bone tunnel healing in anterior cruciate ligament reconstruction. *Anal Quant Cytol Histol*. 2011;33(1):36-49.
29. Ma R, Schar M, Chen T, et al. Effect of dynamic changes in anterior cruciate ligament in situ graft force on the biological healing response of the graft-tunnel interface. *Am J Sports Med*. 2018;46(4):915-923.
30. Magnussen RA, Lawrence JT, West RL, Toth AP, Taylor DC, Garrett WE. Graft size and patient age are predictors of early revision after anterior cruciate ligament reconstruction with hamstring autograft. *Arthroscopy*. 2012;28(4):526-531.
31. Malinin TI, Levitt RL, Bashore C, Temple HT, Mnaymneh W. A study of retrieved allografts used to replace anterior cruciate ligaments. *Arthroscopy*. 2002;18(2):163-170.
32. Matsumoto T, Kubo S, Sasaki K, et al. Acceleration of tendon-bone healing of anterior cruciate ligament graft using autologous ruptured tissue. *Am J Sports Med*. 2012;40(6):1296-1302.
33. Mermerkaya MU, Atay OA, Kaymaz B, Bekmez S, Karaaslan F, Doral MN. Anterior cruciate ligament reconstruction using a hamstring graft: a retrospective comparison of tunnel widening upon use of two different femoral fixation methods. *Knee Surg Sports Traumatol Arthrosc*. 2015;23(8):2283-2291.
34. Murray MM, Martin SD, Martin TL, Spector M. Histological changes in the human anterior cruciate ligament after rupture. *J Bone Joint Surg Am*. 2000;82(10):1387-1397.
35. Oiestad BE, Holm I, Aune AK, et al. Knee function and prevalence of knee osteoarthritis after anterior cruciate ligament reconstruction: a prospective study with 10 to 15 years of follow-up. *Am J Sports Med*. 2010;38(11):2201-2210.
36. Packer JD, Bedi A, Fox AJ, et al. Effect of immediate and delayed high-strain loading on tendon-to-bone healing after anterior cruciate ligament reconstruction. *J Bone Joint Surg Am*. 2014;96(9):770-777.
37. Pennock AT, Ho B, Parvanta K, et al. Does allograft augmentation of small-diameter hamstring autograft ACL grafts reduce the incidence of graft retear? *Am J Sports Med*. 2017;45(2):334-338.
38. Rodeo SA, Kawamura S, Kim HJ, Dynybil C, Ying L. Tendon healing in a bone tunnel differs at the tunnel entrance versus the tunnel exit: an effect of graft-tunnel motion? *Am J Sports Med*. 2006;34(11):1790-1800.
39. Sanders TL, Maradit Kremers H, Bryan AJ, et al. Incidence of anterior cruciate ligament tears and reconstruction: a 21-year population-based study. *Am J Sports Med*. 2016;44(6):1502-1507.
40. Scheffler SU, Schmidt T, Gangey I, Dustmann M, Unterhauser F, Weiler A. Fresh-frozen free-tendon allografts versus autografts in anterior cruciate ligament reconstruction: delayed remodeling and inferior mechanical function during long-term healing in sheep. *Arthroscopy*. 2008;24(4):448-458.
41. Scheffler SU, Unterhauser FN, Weiler A. Graft remodeling and ligamentization after cruciate ligament reconstruction. *Knee Surg Sports Traumatol Arthrosc*. 2008;16(9):834-842.
42. Shino K, Inoue M, Horibe S, Nagano J, Ono K. Maturation of allograft tendons transplanted into the knee: an arthroscopic and histological study. *J Bone Joint Surg Br*. 1988;70(4):556-560.
43. Sikka RS, Narvy SJ, Vangsness CT Jr. Anterior cruciate ligament allograft surgery: underreporting of graft source, graft processing, and donor age. *Am J Sports Med*. 2011;39(3):649-655.
44. Smith PA, Stannard JP, Bozynski CC, Kuroki K, Cook CR, Cook JL. Patellar bone-tendon-bone autografts versus quadriceps tendon allograft with synthetic augmentation in a canine model. *J Knee Surg*. Published online August 28, 2019. doi: 10.1055/s-0039-1695040
45. Snaebjornsson T, Hamrin Senorski E, Ayeni OR, et al. Graft diameter as a predictor for revision anterior cruciate ligament reconstruction and KOOS and EQ-5D values: a cohort study from the Swedish National Knee Ligament Register based on 2240 patients. *Am J Sports Med*. 2017;45(9):2092-2097.
46. Sochacki KR, McCulloch PC, Lintner DM, Harris JD. Hamstring autograft versus hybrid graft in anterior cruciate ligament reconstruction: a systematic review of comparative studies. *Arthroscopy*. 2019;35(6):1905-1913.
47. Sun Y, Chen W, Hao Y, et al. Stem cell-conditioned medium promotes graft remodeling of midsubstance and intratunnel incorporation after anterior cruciate ligament reconstruction in a rat model. *Am J Sports Med*. 2019;47(10):2327-2337.
48. Takayama K, Kawakami Y, Mifune Y, et al. The effect of blocking angiogenesis on anterior cruciate ligament healing following stem cell transplantation. *Biomaterials*. 2015;60:9-19.
49. Thaunat M, Fayard JM, Sonnery-Cottet B. Hamstring tendons or bone-patellar tendon-bone graft for anterior cruciate ligament reconstruction? *Orthop Traumatol Surg Res*. 2019;105(1S):S89-S94.
50. Tuman JM, Diduch DR, Rubino LJ, Baumfeld JA, Nguyen HS, Hart JM. Predictors for hamstring graft diameter in anterior cruciate ligament reconstruction. *Am J Sports Med*. 2007;35(11):1945-1949.
51. Unterhauser FN, Bosch U, Zeichen J, Weiler A. Alpha-smooth muscle actin containing contractile fibroblastic cells in human knee arthrofibrosis tissue. Winner of the AGA-DonJoy Award 2003. *Arch Orthop Trauma Surg*. 2004;124(9):585-591.
52. Wang HD, Gao SJ, Zhang YZ. Comparison of clinical outcomes after anterior cruciate ligament reconstruction using a hybrid graft versus a hamstring autograft. *Arthroscopy*. 2018;34(5):1508-1516.
53. Wang HD, Gao SJ, Zhang YZ. Hamstring autograft versus hybrid graft for anterior cruciate ligament reconstruction: a systematic review. *Am J Sports Med*. 2020;48(4):1014-1022.
54. Webster KE, Feller JA, Hartnett N, Leigh WB, Richmond AK. Comparison of patellar tendon and hamstring tendon anterior cruciate ligament reconstruction: a 15-year follow-up of a randomized controlled trial. *Am J Sports Med*. 2016;44(1):83-90.
55. Weiler A, Unterhauser FN, Bail HJ, Huning M, Haas NP. Alpha-smooth muscle actin is expressed by fibroblastic cells of the ovine anterior cruciate ligament and its free tendon graft during remodeling. *J Orthop Res*. 2002;20(2):310-317.
56. Zeng C, Gao SG, Li H, et al. Autograft versus allograft in anterior cruciate ligament reconstruction: a meta-analysis of randomized controlled trials and systematic review of overlapping systematic reviews. *Arthroscopy*. 2016;32(1):153-163.e18.