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A Comparative Animal Study of Tendon Grafts Healing After Remnant-Preserving Versus Conventional Anterior Cruciate Ligament Reconstruction

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Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to determine if anterior cruciate ligament (ACL) reconstruction by remnant preservation promotes cell proliferation, vascularization, proprioception recovery, and improved biomechanical properties of the tendon grafts.

Material/Methods: 75 New Zealand rabbits were randomly assigned into the control group (group A), conventional ACL reconstruction group (group B), ACL reconstruction using remnant preservation and graft through remnant sleeve technique group (group C), and ACL reconstruction using remnant preservation and remnant tensioning technique group (group D). The remnant and healing of tendon grafts in groups C and D were observed at 3, 6, and 12 weeks after surgery, and the mRNA expression levels of VEGF, NT-3 and GAP-43 in ACL (group A) or tendon graft samples (groups B, C, and D) were determined by real-time PCR. Tendon graft cell count, microvessel density (MVD), and proprioceptors were determined by H&E staining, CD34, and S-100 immunohistochemical staining. The biomechanical properties of the tendon graft at week 12 in groups B, C, and D were examined by using a tensile strength test.

Results: Remnant and tendon grafts were not healed at 3, 6, and 12 weeks after the operation in groups C and D. VEGF, NT-3, and GAP-43 mRNA expressions in groups B, C, and D were higher than those in group A ($P<0.05$), but no significant difference was observed between groups B, C, and D ($P>0.05$). Furthermore, tendon graft cell count, MVD, proprioception, and biomechanical properties showed no significant differences ($P>0.05$) among groups B, C, and D at various time points.

Conclusions: There was no significant difference in cell proliferation, vascularization, proprioception recovery, or biomechanical properties of the tendon grafts between remnant-preserving and conventional ACL reconstruction methods.

MeSH Keywords: Anterior Cruciate Ligament Reconstruction • Biomechanical Phenomena • Fracture Healing

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Background

Anterior cruciate ligament (ACL) rupture is a common injury in sports medicine. According to an epidemiology survey, the incidence of ACL injury was 1.08 per 1000 competitive playing exposures or 0.7 every 1000 hours of game time in European soccer players [1]. ACL reconstruction is currently the primary treatment for ACL rupture. Although ACL reconstruction has shown good efficacy, failure of the tendon graft after the operation remains a concern [2,3]. Numerous methods have been developed to prevent this failure [4,5], including ACL reconstruction using remnant preservation, which is intended to accelerate the biological conversion of the intra-articular tendon graft. However, it is still debated whether remnant-preserving ACL reconstruction has more biological advantages than traditional surgery.

Since the tibial remnant of the ruptured ACL has been shown to have some capabilities in cellular and vascular regeneration due to inflammatory responses [6], many researchers believe that the remaining cells, blood vessels, and nerves in the remnant can accelerate tendon graft vascularization and promote proprioception recovery in the knee joint [7–9], and therefore have been willing to increase the operation difficulty and surgery time to preserve the ACL remnant. Several remnant preservation techniques have been reported, with the most commonly used methods being "ACL reconstruction using remnant preserving and graft through remnant sleeve technique" [10] and "ACL reconstruction using remnant preserving and remnant tensioning technique" [11]. However, some scholars remain skeptical about the biological advantages of ACL reconstruction by remnant preservation in promoting the functional recovery of the knee joint [12,13]. No recent studies have examined the biological advantages of ACL reconstruction by remnant preservation at the histological and molecular levels. Moreover, whether there is a difference in efficacy between "ACL reconstruction using remnant preserving and graft through remnant sleeve technique" and "ACL reconstruction using remnant preserving and remnant tensioning technique" still needs to be investigated.

To provide a more comprehensive comparison between remnant-preserving and conventional ACL reconstruction methods, we established 3 animal models to compare the healing of tendon grafts in terms of histology, ligament remodeling-related genes and proteins, and the biomechanics of ACL reconstruction using remnant preservation and graft through remnant sleeve technique, remnant preserving and tensioning technique, and conventional methods. We hypothesized that the tendon graft heals better in ACL reconstruction using remnant preservation compared to the conventional method.

Material and Methods

Experimental groups

Seventy-five (75) male New Zealand rabbits, 6–8 months old, weighing 3.0–3.5 kg, were used in this study (rabbits were provided by the Laboratory Animal Center of Xinjiang Medical University, general level, closed group). This study was approved by the Laboratory Animal Center of the First Affiliated Hospital of Xinjiang Medical University. All test rabbits were acclimated for 1 week prior to surgery. The rabbits were randomly assigned to 1 of 4 groups: group A was control (n=9), group B was conventional ACL reconstruction (n=22), group C was ACL reconstruction using remnant preserving and graft through sleeve technique (n=22), and group D was ACL reconstruction using remnant preserving and tensioning technique (n=22). Groups B, C, and D were all subjected to acute-phase ACL reconstruction of both knees. No surgery was performed for group A, and the normal ACL was used as the baseline for RT-PCR detection.

ACL reconstruction

ACL reconstruction using remnant preserving and graft through sleeve technique

Rabbits were intramuscularly injected with 7 mg/kg Zoletil and 0.15 ml/kg Sumianxin II after weighing. When the animals were fully anesthetized, the skin was disinfected using iodine. The middle 1/3 portion of the Achilles tendon (length ~4 cm, diameter ~2 mm) was excised and used as the tendon graft. After cutting through the inner medial patellar ligament (about 3 cm in length), the patella was dislocated outwards which exposed the intra-articular synovium and ACL. The ACL was incised at the femoral junction with retention of the synovium and patellar fat pad. A 2-mm diameter Kirschner wire was used to drill the femoral tunnel at the original femoral attachment site of the ACL. The tibial ACL remnant was carefully isolated to form a cylindrical structure (Figure 1A), and the tibial tunnel was drilled by inserting a fine-needle guide through the center of the remnant mark along the direction of the ACL. After passing through the lateral tibial cortex, a 2.0 mm hollow drill was then passed along the needle guide in the reverse direction (Figure 1B) to ream the tunnel and pass through the articular cartilage. The tendon graft was inserted into the tibial tunnel through the remnant center using a custom-made needle guide. The custom-made needle guide is for a straight needle, and the bottom of custom-made needle guide can fix the tendon graft traction stitch. The needle guide enters the bone tunnel from outside the intra-articular part of the tibial tunnel and is pulled out from the internal intra-articular area, which is in the center of the remnant, then the traction stitch brings the tendon graft into the tibial tunnel and

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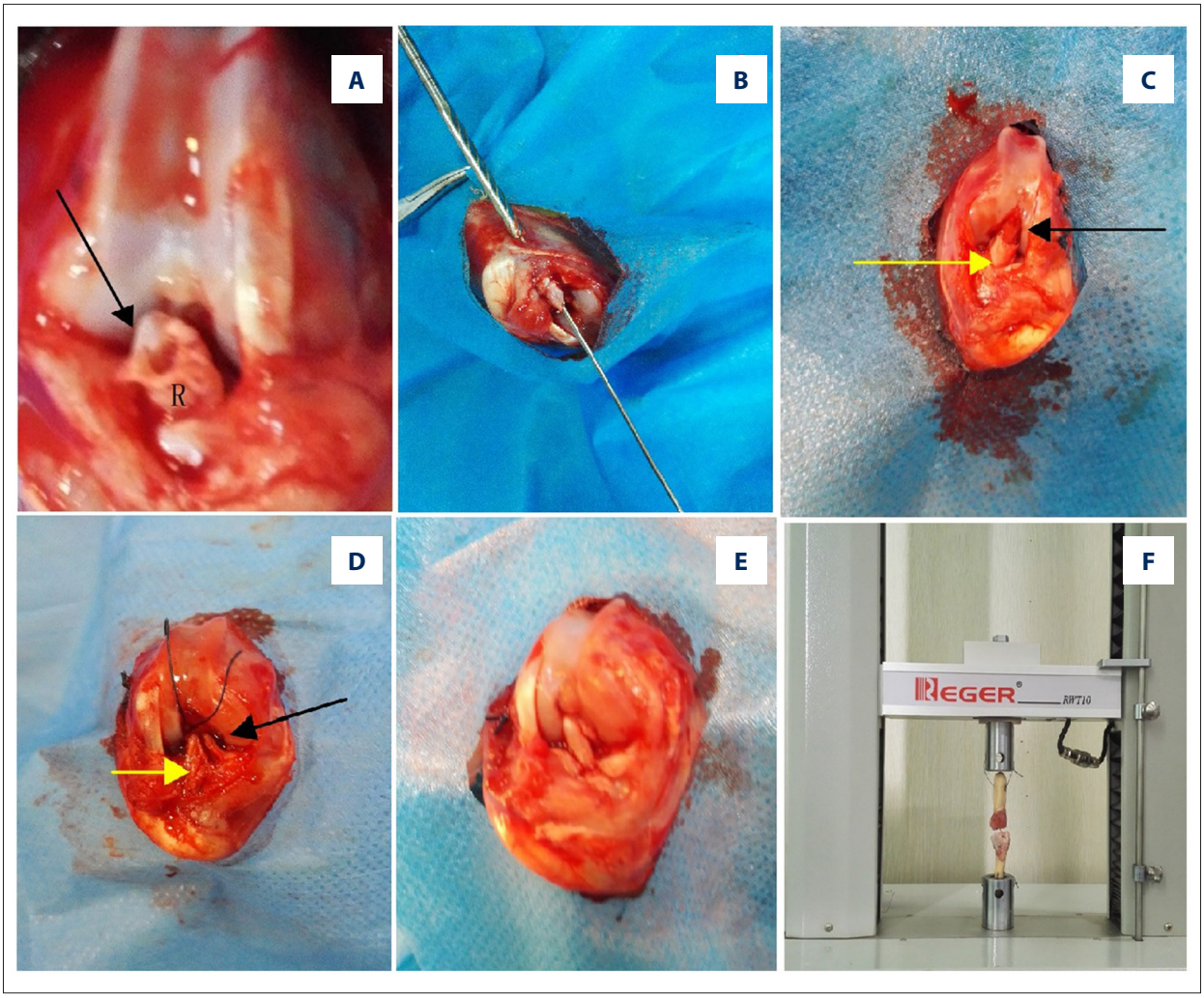

Figure 1. Process of ACL reconstruction. Note: (yellow arrow shows ACL remnant; black arrow shows tendon graft) (**A**) Tibial ACL remnant was formed into a cylindrical structure; (**B**) Needle guide was inserted through the center of the remnant and a 2.0-mm hollow drill was used to drill the tibial tunnel in the direction of the ACL; (**C**) reconstruction using remnant preserving and graft through sleeve technique; (**D**) reconstruction using remnant preserving and tensioning technique; (**E**) conventional reconstruction; (**F**) biomechanical testing.

passes through the center of the remnant, such that the remnant acts as a sleeve that encloses the graft (Figure 1C). The distal femur was sutured to the bone and soft tissues at the opening of the tunnel, and the graft was secured at the tibial end by tightly pulling the graft at a 30° knee flexion. The joint cavity was washed with saline, followed by patella relocation and suturing.

ACL reconstruction using remnant preserving and tensioning technique

After the animals were anesthetized, surgery and femoral tunnel construction were performed as stated above. The tibial tunnel was prepared by making a pull wire by performing a one-stitch suture at the cruciate ligament remnant, and was used to pull the ligament remnant during the construction of the tibial tunnel to avoid damage from the drilling of the tibial tunnel. The medial opening of the tibial tunnel was located at approximately 1 mm posterior to the end of the ACL [14], and a Kirschner wire was used to drill the tunnel. The tendon graft was inserted through the tibial joint from the exterior and secured at the proximal tibia. The tendon graft was stretched accordingly, sutured to the original ACL remnant at extension, and secured at the femoral end. The remnant was pulled into the femoral tunnel as much as possible by passing the pull wire through the tunnel to create some tension in the remnant (Figure 1D).

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Conventional ACL reconstruction

Anesthetic and surgical procedures were similar to those previously mentioned, and the ACL was completely removed after exposing the ligament. A tibial tunnel was drilled from the center of the ACL remnant mark left on the tibia after its removal. Preparation of the femoral tunnel and securing the tendon graft were performed the same as in the remnant-preserving reconstruction group (Figure 1E). Both legs of the rabbits from all groups were allowed to move freely post-surgery. The surgical incision site was not wrapped, and 400 000 U/kg penicillin was injected intramuscularly for 5 days. The wound was disinfected daily and stitches were removed after 10 days.

Sample preparation

Normal bilateral ACL were obtained from both knees of 3 rabbits (6 knees) from group A following euthanasia (anesthesia overdose) at weeks 3, 6, and 12. From the reconstruction groups (B, C, and D), 6 rabbits (12 knees) were euthanized at weeks 3 and 6, and 10 rabbits were sacrificed at week 12 post-surgery (4 of the rabbits at week 12 [8 knees] were used for biomechanical testing). Grafts were carefully incised from the joint cavities of the rabbits in the reconstruction groups. The graft from the left knee was fixed in 10% paraformaldehyde, then dehydrated, cleared, embedded in paraffin wax, and cut into 5-μm sections for H&E staining, and CD34 and S-100 IHC staining (H&E, CD34, and S-100 were only compared among groups B, C, and D). The graft from the right knee was snap frozen in liquid nitrogen, stored at –80°C, and used for real-time quantitative PCR.

Sample examination

Gross observations

The tension, integrity, and healing of the remnant and tendon graft after reconstruction was observed.

Histological observations

The number of fibroblasts, healing of remnant and tendon graft, and number of proprioceptors were observed in each reconstruction group by H&E staining.

Immunohistochemistry staining

Graft microvessel density (MVD) was determined by concentrated mouse anti-rabbit CD34 monoclonal antibody (ZSGB-Bio, China), and graft proprioceptors were detected by concentrated mouse anti-rabbit S-100 monoclonal antibody. Samples were visualized using the En Vision two-step system consisting of DAB colorization, hematoxylin staining, dehydration, clearing, and mounting. MVD value was calculated in a blinded fashion by the same pathologist based on Weidner's method [15]. The site of highest MVD (the hotspot) was first determined under low-power magnification (10×10), then the number of vessels was counted from 5 fields of view under high-power magnification (40×10), and the average value was recorded as the MVD.

Table 1. Primer sequence of various target genes.

Name	Primer sequence
β-action-F	TCACCATGGATGATGATATCGC
β-action-R	CGTGCTCGATGGGGTACTTCA
VEGF-F	CGAGGAGTTCAACGTCACCA
VEGF-R	CCTTGCCCTTTCCTCGAACT
GAP-43-F	AAAATTCAGGCGAGCTTCCG
GAP-43-R	TTCTTCTCCACCCCATCAGC
NT-3-F	ACGAGATGCAAAGAGGCCAG
NT-3-R	CTATCCGTATCCACCGCCAG

Biomechanical testing

About 4 cm of bone structure was reserved from the tibia and femur after obtaining the knee joint sample, and the ends were embedded in methyl methacrylate. After all soft tissues (joint capsule, meniscus, and posterior cruciate ligament) apart from the graft were removed, the bones were wrapped with saline-soaked gauze in a sealed Ziploc bag stored at –20°C and were thawed at 4°C overnight prior to the tensile strength test. An electronic digital universal testing machine (Reger Instrument Co., Ltd., Shenzhen, China) was used for the test, which was conducted at room temperature with a humidity of 40% and a loading speed of 2 mm/min. The maximum tensile load (N) and stretching distance (mm) were recorded by computer (Figure 1F).

VEGF, GAP-43, and NT-3 mRNA expression levels were detected using RT-PCR. Graft tissue was homogenized and total RNA was extracted using Trizol. Reverse transcriptase was used for cDNA synthesis according to the protocol, and the primers were synthesized by Sangon Biotech, Shanghai (Table 1). The PCR conditions consisted of 94°C×5 min, 56°C×40 s, and 72°C×30 s for a total of 40 cycles, and 5 μL of the PCR product was verified by agarose gel electrophoresis. Using β-actin as the control gene, the relative mRNA expression of various genes was calculated by $2^{-\Delta\Delta Ct}$. Expression levels of each gene were normalized to the expression level of the same gene in the control group.

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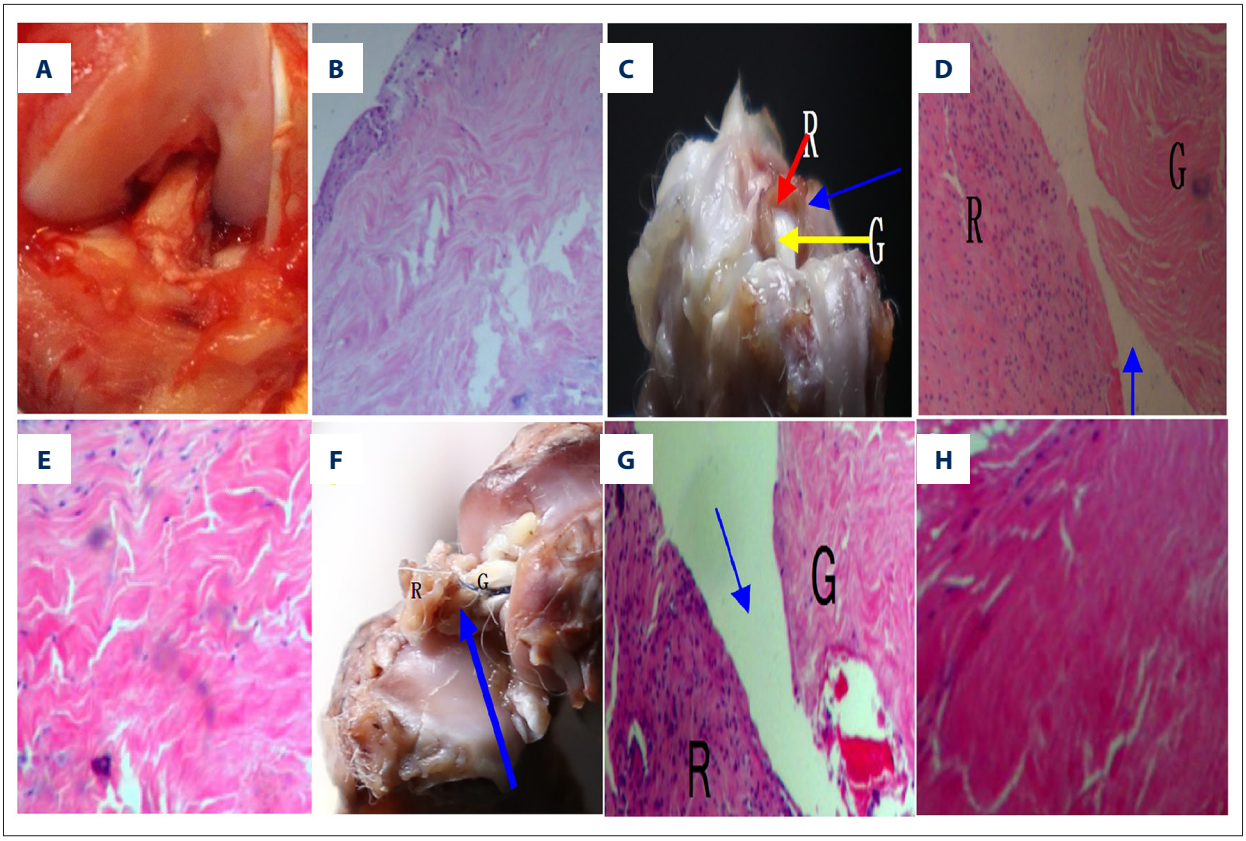

Figure 2. Gross observation and histology of tendon graft in various reconstruction groups at week 3. Note: G – tendon graft, R – remnant, gap between tendon graft and remnant (blue arrow). (**A**) gross observation of conventional reconstruction group; (**B**) tendon graft histology of conventional reconstruction group (HE×40); (**C**) gross observation of reconstruction with remnant-preserving and graft through sleeve technique group; (**D**) tendon graft histology of reconstruction with remnant-preserving and graft through sleeve technique group showing clear gap between remnant and tendon graft (HE×40); (**E**) cell growth in the tendon graft of reconstruction with remnant-preserving and graft through sleeve technique group (HE×40); (**F**) gross observation of reconstruction with remnant-preserving and tensioning technique group; (**G**) tendon graft histology of reconstruction with remnant-preserving and tensioning technique group showing a clear gap between remnant and tendon graft (HE×40); (**H**) cell growth in tendon graft of reconstruction with remnant-preserving and tensioning technique group (HE×40).

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software, and all data are presented as mean ±SD ($\bar{x}\pm s$). One-way ANOVA was used to compare groups and the Bonferroni test was used for pairwise comparison. $P<0.05$ was considered to be statistically significant.

Results

Gross observation

The integrity and tension of the tendon grafts in all reconstruction groups at 3, 6, and 12 weeks post-surgery were satisfactory. Further observation of the healing between the remnant and the tendon graft after remnant-preserving reconstruction showed a significant gap between the remnant and the tendon graft, indicating that the tissues did not merge.

Histological observation

At 3 weeks post-surgery, tendon grafts in group B showed collagen disintegration, cell necrosis, and very low cell counts. Tendon grafts were surrounded by a very thin layer of synovial tissue and a low level of fibroblast proliferation was seen at the junction between the graft and the synovium (Figure 2A, 2B). Although the group C remnant showed significant cell growth, the remnant and the tendon graft remained poorly merged (Figure 2C, 2D). We also observed collagen disintegration, cell necrosis, and very low cell counts, with little cell proliferation, especially in the periphery of the tendon graft (Figure 2E). No merging was observed between the tendon graft and remnant in group D (Figure 2F, 2G), and the tendon graft histology was similar to those of groups B and C (Figure 2H).

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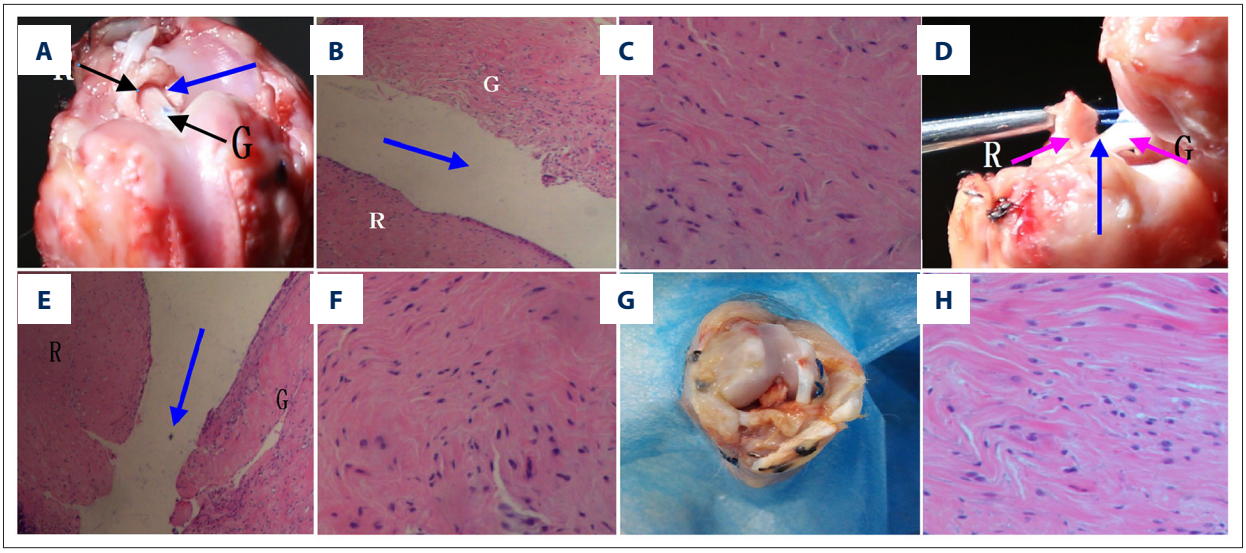

Figure 3. Gross observation and histology of tendon graft in various reconstruction groups at week 6. Note: G – tendon graft, R – remnant, gap between tendon graft and remnant (blue arrow). (**A**) gross observation of reconstruction with remnant-preserving and graft through sleeve technique group showing an obvious gap between remnant and tendon graft; (**B**) histology of reconstruction with remnant-preserving and graft through sleeve technique group showing a lack of merging between remnant and tendon graft (HE×40); (**C**) cell growth in the tendon graft of reconstruction with remnant-preserving and graft through sleeve technique group(HE×100); (**D**) gross observation of reconstruction with remnant-preserving and tensioning technique group showing clear gap between remnant and tendon graft; (**E**) histology of reconstruction with remnant-preserving and tensioning technique group showing a lack of merging between remnant and tendon graft (HE×40); (**F**) cell growth in reconstruction with remnant-preserving and tensioning technique group (HE×100); (**G**) gross observation of conventional reconstruction group at week 6; (**H**) cell growth in conventional reconstruction group (HE×100).

At 6 weeks post-surgery, more fibroblasts were found with an uneven distribution in the tendon grafts of group B, and parts of the graft still showed necrotic disintegration (Figure 3G, 3H). Complete integration between the remnant and the tendon graft in groups C and D was still absent (Figure 3A 3B, 3D, 3E), and there was no significant difference in cell numbers compared to that of group B (Figure 3C, 3F, 3H).

At 12 weeks post-surgery, fibroblasts in the periphery and center of tendon graft in group B were significantly more numerous compared to week 6, with disorganized cell arrangement and irregular collagen (Figure 4G, 4H). In addition to the clear gap between the remnant and tendon grafts in groups C and D (Figure 4A, 4B, 4D, 4E), increased fibroblast growth was also observed in the periphery and center of the graft (Figure 4C, 4F), and there was little difference in the histological observations between remnant-preserving and conventional reconstruction techniques.

Vascular regeneration of tendon graft

VEGF mRNA expression in tendon graft

mRNA expression in all reconstruction groups at weeks 3, 6, and 12 were higher than in the control group ($p<0.05$), with no significant difference between group B and groups C and D ($p>0.05$), or between groups C and D ($p>0.05$). VEGF mRNA expression was increased in all reconstruction groups at weeks 3 and 6, with expression levels peaking at week 6 and reduced at week 12 (Table 2).

CD34-labeled MVD in tendon graft

There was no significant difference between group B and groups C and D ($p>0.05$) or between groups C and D ($p>0.05$) at weeks 3, 6, and 12. MVD was similar between groups and increased over time. Week 12 showed the highest MVD (Table 3, Figure 5).

Nerve growth factor and proprioceptor regeneration in tendon graft

Proprioceptor

Pacinian red blood cells and free nerve endings were observed under the microscope (Figure 6). No proprioceptor was found in the tendon grafts of groups B, C, or D at 3 weeks post-surgery. In contrast, proprioceptors were observed in the treatment groups at 6 and 12 weeks post-surgery, with no significant difference between the groups ($p>0.05$) (Table 4).

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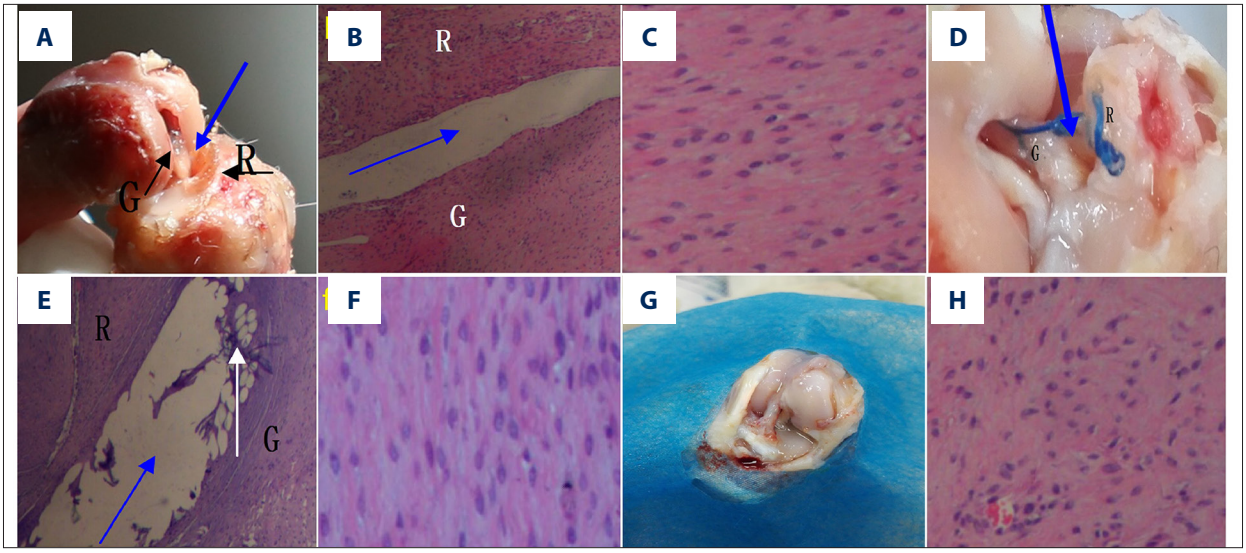

Figure 4. Gross observation and histology of tendon graft in various reconstruction groups at week 12. Note: G – tendon graft, R – remnant, gap between tendon graft and remnant (blue arrow). (**A**) gross observation of reconstruction with remnant-preserving and graft through sleeve technique group showing presence of gap between the remnant and tendon graft (blue arrow); (**B**) histology of reconstruction with remnant-preserving and graft through sleeve technique group showing the presence of a gap between the remnant and tendon graft (HE×40); (**C**) cell growth in the tendon graft of reconstruction with remnant-preserving and graft through sleeve technique group(HE×200); (**D**) gross observation of reconstruction with remnant-preserving and tensioning technique group showing the presence of a gap between remnant and tendon graft with stitches clearly shown; (**E**) histology of reconstruction with remnant-preserving and tensioning technique group showing a gap between remnant and tendon graft (HE×40) and the presence of the stitches (white arrow); (**F**) cell growth in reconstruction with remnant-preserving and tensioning technique group (HE×200); (**G**) gross observation of conventional reconstruction group at week 12; (**H**) cell growth in conventional reconstruction group (HE×200).

Table 2. RT-PCR detection of relative VEGF mRNA expression in various reconstruction groups at different time points post-surgery (n=6, $\bar{\chi}$ ±s).

Groups	Postoperative time (weeks)		
	3	6	12
A group	1.00[a]	1.00[a]	1.00[a]
B group	1.98±0.48[ab]	4.35±0.45[ab]	1.14±0.62[ab]
C group	2.06±0.14[abc]	4.18±0.63[abc]	1.19±0.35[abc]
D group	2.21±0.73[abc]	4.07±0.24[abc]	1.23±0.06[abc]
F value	409.48	149.8	4.41
P value	0.000	0.000	0.026

[a] P<0.05 when compared to group A; [b] p>0.05 when group B was compared to groups C and D; [c] p>0.05 when group C was compared to group D.

NT-3 mRNA expression

NT-3 mRNA expression in various reconstruction groups was higher than that of the controls at weeks 3, 6, and 12 (p<0.05). There was no significant difference in expression between group B and groups C and D (p>0.05), or between groups C and D (p>0.05). Over time, NT-3 mRNA expression gradually increased in the 3 reconstruction groups, reaching peak expression at week 12 (Table 5).

GAP-43 mRNA expression

The relative GAP-43 mRNA expression level in groups B, C, and D was significantly higher than in group A at 3, 6, and 12 weeks post-surgery (p<0.05). Further analysis showed that there was no significant difference in expression between group B and groups C and D (p>0.05), or between groups C and D (p>0.05). The GAP-43 mRNA expression in various reconstruction groups

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Table 3. CD34-labeled MVD in various reconstruction groups at different time points post-surgery (n=6, $\bar{\chi}$ ±s).

Groups	Postoperative time (weeks)		
	3	6	12
B group	7.95±2.06[a]	13.14±3.72[a]	16.06±3.15[a]
C group	8.04±2.78[ab]	11.92±3.37[ab]	17.10±4.16[ab]
D group	8.67±1.81[ab]	12. 83±2.96[ab]	16.98±4.78[ab]
F value	2.34	3.45	1.43
P value	0.13	0.06	0.26

[a] p>0.05 when group B was compared to groups C and D; [b] p>0.05 when group C was compared to group D.

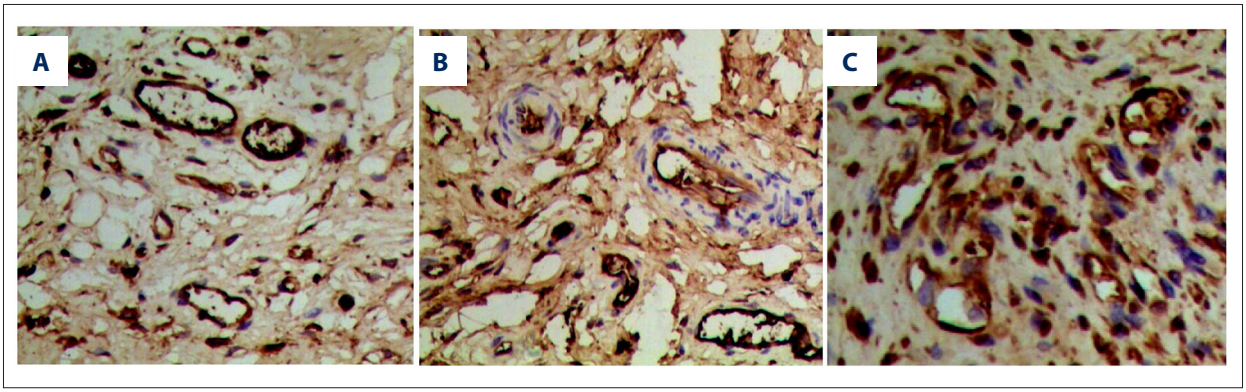

Figure 5. MVD in various groups at week 12 post-surgery. Note: MVD (microvessel density). (**A**) MVD of the control group (CD34×200); (**B**) MVD of reconstruction with remnant-preserving and tensioning technique group (CD34×200); (**C**) MVD of reconstruction with remnant-preserving and graft through sleeve technique group (CD34×200).

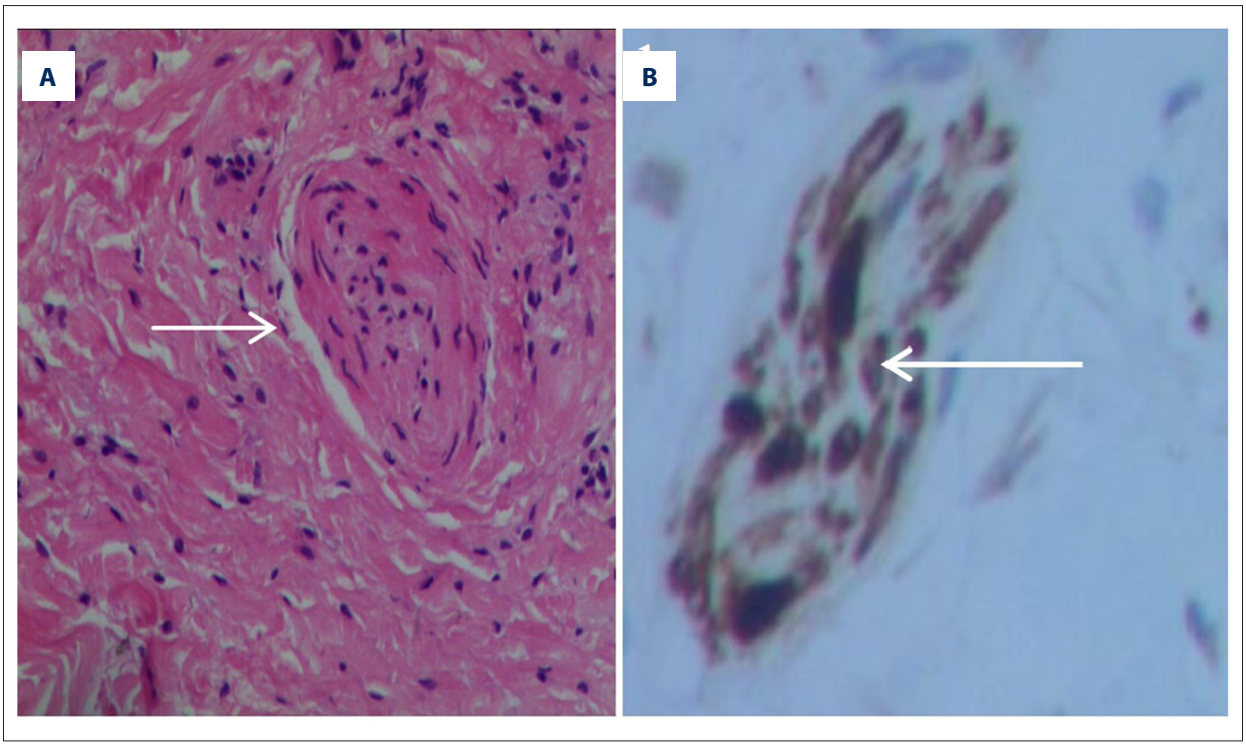

Figure 6. Proprioceptor. (**A**) Pacinian corpuscles (HE ×100); (**B**) free nerve endings (S-100 ×400).

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Table 4. Number of proprioceptors in various reconstruction groups at different time points.

Groups	Postoperative time (weeks)		
	3	6	12
B group	0	2.67±0.81[a]	4.9±1.14[a]
C group	0	3.00±1.09[ab]	5.8±0.44[ab]
D group	0	3.17±1.16[ab]	5.4±1.51[ab]
F value		0.36	1.63
P value		0.70	0.23

[a] p>0.05 when group B was compared to groups C and D; [b] p>0.05 when group C was compared to group D.

Table 5. RT-PCR detection of the relative NT-3 mRNA expression in various reconstruction groups at different time points (n=6, $\bar{\chi}$ ±s).

Groups	Postoperative time (weeks)		
	3	6	12
A group	1.00[a]	1.00[a]	1.00[a]
B group	1.88±0.12[ab]	4.79±0.46[ab]	7.81±1.02[ab]
C group	1.89±0.41[abc]	4.68±0.71[abc]	7.68±0.87[abc]
D group	2.03±0.23[abc]	4.71±0.84[abc]	7.79±0.46[abc]
F value	179.1	503.78	9809.0
P value	0.000	0.000	0.000

[a] P<0.05 when compared to group A; [b] p>0.05 when group B was compared to groups C and D; [c] p>0.05 when group C was compared to group D.

Table 6. RT-PCR detection of the relative GAP-43 mRNA expression in various reconstruction groups at different time points post-surgery

Groups	Postoperative time (weeks)		
	3	6	12
A group	1.00[a]	1.00[a]	1.00[a]
B group	1.73±0.61[ab]	5.86±1.07[ab]	2.94±0.69[ab]
C group	1.66±0.23[abc]	5. 76±0.74[abc]	3.13±0.95[abc]
D group	1.58±0.12[abc]	6.01±0.43[abc]	3.10±0.34[abc]
F value	33.41	452.09	180.1
P value	0.000	0.000	0.00

[a] P<0.05 when compared to group A; [b] p>0.05 when group B was compared to groups C and D; [c] p>0.05 when group C was compared to group D.

peaked at 6 weeks post-surgery, and was reduced to the lowest levels at week 12 (Table 6).

Biomechanical testing

All graft samples were ruptured at the intra-articular ligament during the tensile test at 12 weeks post-surgery, without any prolapse of the graft from the bone tunnel. There was no significant difference in maximum tensile load and stretching distance between group B and groups C and D (P>0.05), or between groups C and D (P>0.05) (Table 7).

Discussion

This study is the first to establish a model for ACL reconstruction using remnant preserving and graft through remnant sleeve technique, and also the first to examine the healing

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Table 7. Biomechanics of tendon graft in various reconstruction groups at week 12 post-surgery.

Groups	The maximum tensile load (N)	The maximum stretching distance (mm)
B group	38.9±1.97[a]	6.08±0.34[a]
C group	40.8±1.28[ab]	5.92±0.13[ab]
D group	39.7±1.49[ab]	5.73±0.48[ab]
F value	2.366	2.282
P value	0.118	0.127

[a] p>0.05 when group B was compared to groups C and D; [b] p>0.05 when group C was compared to group D.

of tendon grafts after different remnant-preserving and conventional ACL reconstruction methods from histological, molecular, and biomechanical perspectives. Our results showed that there were no advantages in tendon graft cell proliferation, vascularization, proprioception recovery, or biomechanical properties after ACL reconstruction with remnant preservation compared to the conventional method. These results contradicted our hypothesis.

Fibroblast count in the tendon graft is an important indicator for assessing graft healing. Our results showed that the number of cells in various groups increased with time, indicating that the tendon grafts in every group passed through the healing process of ischemic necrosis, cell disappearance, and cell growth. There was no significant difference in the number of fibroblasts in the tendon grafts between the remnant-preserving and conventional reconstruction groups at various time points post-surgery, which suggests that remnant preservation does not enhance cell growth in the tendon graft. Since the number of cells and blood vessels increased in the ruptured ACL due to inflammation, some researchers speculated that the remnant could enhance cell proliferation and vessel regeneration in the tendon graft. However, the ability of the remnant to promote the healing of the tendon graft is dependent on the integration of the remnant and the tendon graft, so that the cells and blood vessels from the remnants can migrate into the tendon graft. Murray et al. [16,17] found that despite fine-suturing of the torn ACL, healing of the ruptured ends was very poor, with a gap of about 50–100 μm wide; any gap between ruptured ends that is greater than 50 μm can affect cell ingrowth [18]. ACL reconstruction using the graft through use of the remnant sleeve technique involves passing the tendon graft through the center of the remnant, and does not require the suturing of the 2 tissues. ACL reconstruction using the tensioning technique involves imply securing the remnant and tendon graft with a few stitches, which not only cannot eliminate the gap between the 2 tissues, but also increases the difficulty of healing because the remnant is suspended in the synovial fluid over a long period of time. Our study further demonstrated that ACL reconstruction using both graft through remnant sleeve and tensioning techniques cannot promote a tight merging between the remnant and the tendon graft, so that cells from the remnant were unable to migrate into the tendon graft; therefore, remnant preservation has a very limited role in promoting cell growth in the tendon graft.

Angiogenesis is a key part of tendon graft remodeling. Using MRI to assess the ligamentization of the tendon graft following remnant-preserving and conventional ACL reconstruction, Gohil [19] demonstrated that retaining the remnant can promote tendon graft vascularization. Because the use of imaging to evaluate ligament vascularization can be very subjective, we examined the mRNA expression of VEGF and determined the MVD by CD34 labeling instead to clearly demonstrate the growth of blood vessels in the tendon graft. In this study, no significant difference was found in VEGF mRNA expression between various reconstruction groups at different time points. The reasons for this could be: 1) VEGFmRNA is mainly produced by fibroblasts and macrophages, but the number of cells was not increased by remnant preservation compared to the conventional technique; and 2) although inflammation following ACL rupture can increase VEGF secretion [20], the lack of a tight integration between the remnant and the tendon graft prevents paracrine VEGF signaling from the remnant to the tendon graft, and thus the difference in VEGF between remnant-preserving and conventional reconstruction groups becomes insignificant. CD34 is selectively expressed on hematopoietic stem cells and vascular endothelial cells in human and other mammals [21]. Mineo [22] believes that CD34 is the most effective marker for labeling microvessels. Results from our study indicate that the MVD of various reconstruction groups was increased over time, reaching a maximum density at week 12, but the difference in the post-surgery MVD between each reconstruction group was not significant. This result may be due to: 1) VEGF can promote the proliferation and differentiation of $CD34^+$ cells, and is closely associated with the MVD [23], thus lack of a significant difference in VEGF mRNA expression levels between various reconstruction groups led to insignificant differences in post-surgery MVD between these groups; and 2) peripheral synovial tissue is the main blood supply for the tendon graft following ACL reconstruction, so we retained the knee synovium to the best of our abilities during

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the surgery regardless of the reconstruction method; therefore, the difference in tendon graft vascularization in various reconstruction groups was not significant.

ACL is not only a major stabilizer of the knee joint, but also an important proprioceptive organ. Previous studies have shown proprioceptors can be found within the ACL [24], and proprioceptors can be regenerated following ACL reconstruction [25]. In contrast with the current study, Mifune [26] found that remnant-preserving ACL reconstruction (partial cruciate ligament rupture) can promote the regeneration of proprioceptors in rats. However, a study by Song et al. [14] comparing conventional and ACL reconstruction using remnant preservation and tensioning in rabbits indicated no difference in the number of proprioceptors post-surgery between the 2 reconstruction methods. While our results were similar to those reported by Song, they were different than those from Mifune's study. This may be due to the fact that the volume of blood supply is different between partial and complete cruciate ligament rupture, and since proprioceptors are mainly distributed around the blood vessels, the generation of proprioceptors is largely dependent on the blood supply. In our study we found that the tendon graft vascularization was similar between the 3 ACL reconstruction groups; therefore, no difference was observed in the number of proprioceptors between the groups.

GAP-43 is a key nerve growth and regenerative factor [27]. Studies have shown that GAP-43 mRNA expression can be detected in tendon grafts following ACL reconstruction, and this was considered to be a sign of nerve regeneration [28]. Our results demonstrated that the relative GAP-43 mRNA expression of all reconstruction groups was higher than that of the controls, and the expression was the highest at week 6 and reduced at week 12, indicating the presence of nerve regeneration and remodeling. On the other hand, no significant difference was observed in the amount of GAP-43 mRNA in the tendon grafts between group B and groups C and D after surgery, and this result may have been due to the close association between the nerve and blood vessel regeneration processes. The proliferation and activation of vascular endothelial cells are coupled in terms of time and space with neurogenesis: VEGF both regulates the regeneration of new blood vessels and promotes the regeneration of the peripheral nerves [29]. Therefore, the difference in nerve regeneration among the 3 reconstruction groups was insignificant because the VEGF mRNA and MVD were similar in the tendon grafts post-surgery.

Tissue NT-3 is synthesized by the target cells and is transported retrogradely through the nerve to the neurons, creating a series of physiological effects. Not only does NT-3 play an important role in maintaining proprioception [30], it is also vital in the repair of proprioception and motor neuron damage [31,32]. A study by Xie et al. demonstrated that the levels of NT-3 mRNA in the tendon graft were higher in the remnant-preserving group compared to in the conventional group at 2 and 12 weeks after reconstruction [33]. The large discrepancy between Xie's findings and ours is likely because the detection of NT-3 in Xie's study was performed using both the remnant and the tendon graft, without considering the healing between graft and remnant. In contrast, we did not include the remnant tissue for NT-3 mRNA detection in order to more objectively assess the expression of the gene in the tendon graft after observing an absence of integration between the 2 tissues. Moreover, NT-3 mRNA expression in the graft of various reconstruction groups increased over time, suggesting that proprioception recovery following ACL reconstruction may be a long-term process.

Biomechanics is an important parameter for evaluating tendon graft healing after ACL reconstruction. Whether remnant-preserving ACL reconstruction has better biomechanical properties is still a matter of debate. We have found that the maximum tensile load and stretching distance among groups B, C, and D were not significantly different, and this could be because: 1) the biomechanical properties of the tendon graft are dependent on its remodeling, and since no difference was observed in ligamentization and vascularization of the grafts between the various reconstruction groups, the biomechanical properties of these group were also not different; or 2) only the tendon graft, but not the remnant, provides mechanical stability after surgery as a result of the poor healing between the 2 tissues in the remnant-preserving reconstruction groups; therefore, the difference in biomechanical properties was not significant among the groups.

Conclusions

In summary, our study has demonstrated that the ACL remnant was unable to integrate with the tendon graft after ACL reconstruction using the graft through remnant sleeve and tensioning techniques in New Zealand rabbits during the acute phase. There were no significant differences in tendon graft cell proliferation, vascularization, nerve regeneration, proprioception recovery, and biomechanical properties between the 2 remnant-preserving and conventional ACL reconstruction methods. Therefore, ACL reconstruction using remnant preservation has no biological advantages in animal studies.

Declaration of interest

None.

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References:

1. Waldén M, Hägglund M, Magnusson H, Ekstrand J: Anterior cruciate ligament injury in elite football: a prospective three-cohort study. Knee Surg Sports Traumatol Arthrosc, 2011; 19(1): 11–19
2. van Eck CF, Schkrohowsky JG, Working ZM et al: Prospective analysis of failure rate and predictors of failure after anatomic anterior cruciate ligament reconstruction with allograft. Am J Sports Med, 2012; 40: 800–7
3. Whitehead TS: Failure of anterior cruciate ligament reconstruction. Clin Sports Med, 2013; 32: 177–204
4. Vogrin M, Rupreht M, Dinevski D et al: Effects of a platelet gel on early graft revascularization after anterior cruciate ligament reconstruction: A prospective, randomized, double-blind, clinical trial. Eur Surg Res, 2010; 45: 77–85
5. Sánchez M, Anitua E, Azofra J et al: Ligamentization of tendon grafts treated with an endogenous preparation rich in growth factors: Gross morphology and histology. Arthroscopy, 2010; 26: 470–80
6. Nguyen DT, Ramwadhdoebe TH, van der Hart CP et al: Intrinsic healing response of the human anterior cruciate ligament: An histological study of reattached ACL remnants. J Orthop Res, 2014; 32: 296–301
7. Sun L, Wu B, Tian M et al: Comparison of graft healing in anterior cruciate ligament reconstruction with and without a preserved remnant in rabbits. Knee, 2013; 20: 537–44
8. Li H, Chen J, Chen S: Remnant repair-enhanced polyethylene terepthalate artificial ligament graft ligamentization. Int J Sports Med, 2015; 36: 1015–20
9. Lee BI, Kwon SW, Choi HS et al: Anatomic single-bundle anterior cruciate ligament reconstruction with remnant preservation using outside-in technique. Arthrosc Tech, 2015; 4: e331–34
10. Lee BI, Min KD, Choi HS et al: Arthroscopic anterior cruciate ligament reconstruction with the tibial-remnant preserving technique using a hamstring graft. Arthroscopy, 2006; 22: 340.e1–7
11. Ahn JH, Wang JH, Lee YS et al: Anterior cruciate ligament reconstruction using remnant preservation and a femoral tensioning technique: clinical and magnetic resonance imaging results. Arthroscopy, 2011; 27: 1079–89
12. Kondo E, Yasuda K, Onodera J et al: Effects of Remnant Tissue Preservation On Clinical And Arthroscopic Results After Anatomic Double-Bundle Anterior Cruciate Ligament Reconstruction. Am J Sports Med, 2015; 43: 1882–92
13. Hong L, Li X, Zhang H et al: Anterior cruciate ligament reconstruction with remnant preservation: a prospective, randomized controlled study. Am J Sports Med, 2012; 40: 2747–55
14. Song GY, Zhang J, Li X et al: Biomechanical and biological findings between acute anterior cruciate ligament reconstruction with and without an augmented remnant repair: A comparative *in vivo* animal study. Arthroscopy, 2016; 32: 307–19
15. Foote RL, Weidner N, Harris J et al: Evaluation of tumor angiogenesis measured with microvessel density (MVD) as a prognostic indicator in nasopharyngeal carcinoma: Results of RTOG 9505. Int J Radiat Oncol Biol Phys, 2005; 61: 745–53
16. 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-A: 1387–97
17. Murray MM, Spindler KP, Ballard P et al: Enhanced histologic repair in a central wound in the anterior cruciate ligament with a collagen-platelet-rich plasma scaffold. J Orthop Res, 2007; 25: 1007–17
18. Murray MM, Martin SD, Spector M: Migration of cells from human anterior cruciate ligament explants into collagen-glycosaminoglycan scaffolds. J Orthop Res, 2000; 18: 557–564
19. Gohil S, Annear PO, Breidahl W: Anterior cruciate ligament reconstruction using autologous double hamstrings: A comparison of standard versus minimal debridement techniques using MRI to assess revascularisation. A randomised prospective study with a one-year follow-up. J Bone Joint Surg Br, 2007; 89: 1165–71
20. Cheng P, Han P, Zhao C et al: High-purity magnesium interference screws promote fibrocartilaginous entheses regeneration in the anterior cruciate ligament reconstruction rabbit model via accumulation of BMP-2 and VEGF. Biomaterials, 2016; 81: 14–26
21. Gangenahalli GU, Singh VK, Verma YK et al: Hematopoietic stem cell antigen CD34: Role in adhesion or homing. Stem Cells Dev, 2006; 15: 305–13
22. Mineo TC, Ambrogi V, Baldi A et al: Prognostic impact of VEGF, CD31, CD34 and CD105 expression and tumor vessel invasion after radical surgery for IB-II A non-small cell lung cancer. J Clin Pathol, 2004; 57: 591–97
23. Lai T, Li M, Zheng L et al: Over-expression of VEGF in marrow stromal cells promotes angiogenesis in rats with cerebral infarction via the synergistic effects of VEGF and Ang-2. J Huazhong Univ Sci Technolog Med Sci, 2012; 32: 724–31
24. Bali K, Dhillon MS, Vasistha RK et al: Efficacy of immunohistological methods in detecting functionally viable mechanoreceptors in the remnant stumps of injured anterior cruciate ligaments and its clinical importance. Knee Surg Sports Traumatol Arthrosc, 2012; 20: 75–80
25. Shimizu T, Takahashi T, Wada Y et al: Regeneration process of mechanoreceptors in the reconstructed anterior cruciate ligament. Arch Orthop Trauma Surg, 1999; 119: 405–9
26. Mifune Y, Ota S, Takayama K et al: Therapeutic advantage in selective ligament augmentation for partial tears of the anterior cruciate ligament: results in an animal model. Am J Sports Med, 2013; 41: 365–73
27. Liu F, Liao F, Li W et al: Progesterone alters Nogo-A, GFAP and GAP-43 expression in a rat model of traumatic brain injury. Mol Med Rep, 2014; 9: 1225–31
28. Aune AK, Hukkanen M, Madsen JE et al: Nerve regeneration during patellar tendon autograft remodelling after anterior cruciate ligament reconstruction: an experimental and clinical study. J Orthop Res 1996; 14: 193–99
29. Schänzer A, Wachs FP, Wilhelm D et al: Direct stimulation of adult neural stem cells *in vitro* and neurogenesis *in vivo* by vascular endothelial growth factor. Brain Pathol, 2004; 14: 237–48
30. Sheard PW, Bewick GS, Woolley AG et al: Investigation of neuromuscular abnormalities in neurotrophin-3-deficient mice. Eur J Neurosci, 2010; 31: 29–41
31. Genç B, Ozdinler PH, Mendoza AE, Erzurumlu RS: A chemoattractant role for NT-3 in proprioceptive axon guidance. PLoS Biol, 2004; 2: e403
32. Johnson PJ, Parker SR, Sakiyama-Elbert SE: Controlled release of neurotrophin-3 from fibrin-based tissue engineering scaffolds enhances neural fiber sprouting following subacute spinal cord injury. Biotechnol Bioeng, 2009; 104: 1207–14
33. Xie GM, Huang Fu XQ, Zhao JZ: The effect of remnant preservation on patterns of gene expression in a rabbit model of anterior cruciate ligament reconstruction. J Surg Res, 2012; 176: 510–16

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