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The histological analysis of the anterior cruciate ligament of canine after radiofrequency shrinkage

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Abstract. – OBJECTIVE: Radiofrequency (RF) shrinkage has been widely conducted in clinical practice and the anterior cruciate ligament (ACL) laxity is regarded as one of the indications. However, basic researches regarding the postoperative histological changes were still insufficient. The study aimed to investigate postoperative histological changes of different areas of ACL for further identifying the optimal area for RF shrinkage.

MATERIALS AND METHODS: A total of 29 healthy canine (16.5 ± 2.2 kg, 4.1 ± 0.7 years) were recruited, 24 of which were randomly divided into group A and group B. The epiphyseal arrest was confirmed by X-ray examination in all animals. On one canine, an ACL's vascular perfusion model was established by the ink-perfusion method to observe the blood supply of the ACL. The mid-portion of ACL was conducted by RF in group A while the amph-portions of ACL were conducted in group B. Two legs of each canine were sub-divided into fixation group (group A_1 and B_1) and non-fixation group (group A_2 and B_2). 8 ACLs were separated from the rest 4 canine. 2 ACLs were sent for the histological examination after RF shrinkage and the rest 6 ACLs were served as blank controls. Masson staining and hematoxylin-eosin (H-E) staining were applied to observe the features of inner fibrous changes of ACL, cell count and vascular density.

RESULTS: According to the Masson staining, collagenous tissues were observed in area after RF shrinkage, which was more evident among group B_1 than the others. The cellar density in both group A and B was found lower at 12 weeks postoperatively than that at 6 weeks postoperatively ($p < 0.05$). In addition, the cellar density in B_1 group was found higher than that in A_1 group at both 6 and 12 weeks postoperatively ($p < 0.05$). The density of subsynovial vessel in B_1 group was found higher than that in A_1 group at 6 weeks postoperatively ($p < 0.05$) and the density of subsynovial vessel in both A_1 and B_1 groups was found lower at 12 postoperatively weeks than that at 6 weeks postoperatively ($p < 0.05$). In both A_2 and B_2 groups, all ACLs were found ruptured at 12 weeks postoperatively.

CONCLUSIONS: The postoperative revascularization pattern of RF-treated ACL was permeating from the synovium to the RF-treated areas, and the best area for the RF shrinkage treatment was the amph-portions of the ACL. Moreover, the application of postoperative external fixation to restrict the movement of injured limb was necessary.

Key Words:
Anterior cruciate ligament, Radiofrequency, Histology.

Introduction

The radiofrequency (RF) technology has been widely conducted in clinical practice and the anterior cruciate ligament (ACL) laxity is regarded as one of its indications. After the RF energy was firstly applied in the treatment of ACL laxity by Thabbit[1] in 1998, many clinical reports using RF technology to treat the ACL laxity have been published, but the therapeutic efficacy is still inconclusive. Khan et al[2] applied the monopolar-RF to 38 patients with ACL laxity and the outcome of 17 weeks follow-up demonstrated a definite therapeutic efficacy (only one patient received ligament reconstruction operation). Indelli et al[3] showed successful outcome in up to 96% of 28 ACL laxity patients with RF shrinkage at a 2-year follow-up. A random controlled trial (38 patients recruited) conducted by Wei et al[4] confirmed that the RF shrinkage treatment could significantly improve patient's symptom. Perry and Higgins[5] observed the rupture

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of the ACL and the posterior cruciate ligament after the RF shrinkage treatment, but they did not use the external fixation or other protective instruments to restrict the movement of injured limbs. Carter et al[6] showed failing outcomes in up to 61% of 18 patients with RF shrinkage for ACL laxity at the 20.5-month follow-up. Nevertheless, Halbrect[7] reported a better early efficacy of the monopolar RF shrinkage treatment, but the long-term efficacy decreased, which was similar to the outcomes of the recent animal experiments[8].

The reason for the inconsistent findings from previous studies may be due to the different areas in which the RF shrinkage was conducted. Although the RF treatment was performed following the same principles, the operation procedure has not yet been unified. In addition, the effect of RF energy on the structure and histology of ACL was not clear, and basic studies on the postoperative histological change of ACL were still scarce. In order to investigate the postoperative histological change of ACL and provide more details on the shrunken pattern of RF treatment, RF treatment was conducted at the different areas of canine's ACL and the features of postoperative revascularization were recorded in the present study. Furthermore, we also compared the postoperative efficacy among different RF-treated areas.

Materials and Methods

Grouping

A total of 29 healthy canine (average weight: 16.5±2.2 kg, average age: 4.1±0.7 years) were recruited, which were supplied by the Experimental Animal Center of Southern Medical University. The epiphyseal arrest had been confirmed by the X-ray examination in all animals. If any pathology was found on the knee-joint cyst, accessory ligament, cruciate ligament, meniscus and cartilage, the experimental subjects were excluded from the study. In order to observe the blood supply of the ACL, one dog was chosen to build an ACL's vascular perfusion model by the ink-perfusion method. 24 dogs were randomly divided into group A and group B for observing the postoperative histological changes. The mid-portion of ACL in group A was conducted by RF while in group B, the amph-portions of ACL was conducted. Group A and B were further sub-divided into A1 and B1 (the hind limb with postoperative external fixation), A2 and B2 (the hind limb without any external fixation). Moreover, a total of 8 ACLs were separated from the rest 4 dogs. 2 ACLs were sent for the histological examination just after RF shrinkage treatment and the rest 6 ACLs served as blank controls. The animal experiment in our study met the requirement of Guidance suggestion of caring laboratory animals (statuted by The Ministry of Science and Technology of the People's Republic of China in 2006)[9].

The Vascular Model of the ACL of Canine

A total of 150 g gelatin (Fluka Ltd., Gillingham, UK) was heated and dissolved in 1000 mL distilled water, and then Zhonghua ink (200 ml) was added into the solution and mixed until the solution was equable. Then, a 10-layers gauze was used to filter the impurity and the smaller carbon particles were reserved. Experimental animal was fixed on the operation table after anesthesia, and then its femoral artery and vein were precisely separated. The proximal end of femoral artery was ligatured and the distal end was intubated. After the Krebs equilibrium solution (including Sodium Nitroprusside 10 g/L, Heparin Sodium 5000 U/L, 37°C, from Hyclone Ltd., Cramlington, UK) was used to perfuse the artery at a flow rate of 50 mL/(min·kg) for 15 minutes, the proximal end of the femoral vein of the experimental animal was ligatured and the distal end was intubated. A 50 mL syringe, which was full of ink-perfusion solution, was connected with the tube on the distal end of femoral vein and the ink-perfusion would be performed repeatedly until the color of the hind limbs skin turned into black. The skin and joint capsule were cut opened and the coloring pattern of the ACL was observed. Furthermore, the vascular distribution of the ACL on the pre-patella and fossa intercondyloidea were also recorded.

RF Shrinkage

ACL and synovial plica of knee-joint were exposed through a patellar anterolateral incision, and then RF shrinkage (output power 25W, operating temperature 60-70°C, Linvatec Ltd., Watchfield, UK) was conducted in group A (on the middle 1/3 parts of ACL) and B (on the rest parts of ACL), respectively. The RF probe was moved from one side to the other side at a speed of 2.5 mm/s, and each part of the ACL had been treated without redundancy.

Postoperative Treatment

After the operation, all animals were immediately returned to their cages and one of their hind

limbs was randomly chosen to be fixed (the area of fixation ranged from 5 cm below inguinal to 3 cm above ankle) by gypsum at 30° of flexion, while the other ones were not fixed. A metal cable was placed into the gypsum, and hung on the back of canine. Six animals from each group would be randomly chosen at the 6 and 12 weeks postoperatively for histological examination. For analyzing the cell count and the distribution and orientation of fibers in ACL, the ligament from each sample was cut into two parts via the gap between anterior and posterior bundle, and one part was used for preparing the longitudinal slices following the fibrous direction. Meanwhile, the other part was used for preparing the lateral slices to analyze the revascularization of ligament.

After the conventional fixation, dehydration, transparency, wax, sectioning, Masson and H-E staining, the histological observation, cell count and comparison of vascular density could be conducted.

H-E Staining for Cell Count

10 hotspots (distributed uniformly on the whole slice between the front and back side of the ligament) of the same size were chosen under the low-power field. Then, the hotspot contained the largest number of cells was observed under the high-power field (×200 times) for cell count. The total number of cells was standardized according to the area (mm^2) of the hot spot.

Microvessel Count

Each specimen was divided into two cross-sections for observation and counting. The center of each cross-section was marked and the line connected the center of cross-section with the margin of synovial ligament was, then, divided into three regions: subsynovial, intermediate and central region. At first, hotspot was sought in the RF-treated area under the low-power field (×40 times), and then the number of vessels in the hotspot area stained in brown using the antibody of VIII factor, was counted under the high-power field (×200 times). The stained endothelial cells, which connected with adjacent microvessels, histocytes and connective tissues, were regarded as countable microvessels. The cell counts in subsynovial and intermediate regions were conducted under five different high-power fields (×200 times), and three different high-power fields (×200 times) were counted in the central region. The outcome of cell count in the high-power field of each region was averaged, and then normalized according to the area (mm^2) for evaluating the microvascular density on the cross-section of the ligament.

Evaluation and Parameters

Parameters were the features of inner fibrous changes of ACL, cell count and vascular density. Our experiment was designed and blind-evaluated by the first and third author, respectively. The other authors were responsible for performing the operation and collecting information.

Statistical Analysis

Statistical analysis was conducted with SPSS 11.0 software package (SPSS Inc., Chicago, IL, USA). The factorial analysis was applied to analyze the data at 6 and 12 weeks postoperatively and test the influence of postoperative fixation and different RF-treated areas. Furthermore, the interactive effect was also tested. The independent and paired sample *t*-test was used to compare the differences of cellular and vascular density between different groups. A statistical difference was achieved for a p-value ≤ 0.05.

Results

Observation of Vascular Ink-perfusion ACL Model

The vessels distributed around the ACL as the branches, and the distribution of vessels on the amph-portions of ACL (included the tibial and femoral insertion site) was higher than that on the mid-portion of ACL. The vasoganglion on the amph-portions connected with the offside via the communicating branches, which were located on the mid-portion (Figure 1). The blood supply of

Figure 1. The subsynovial vascularity in the amph-portions was obviously higher than in the midportion.

synovium and ligament were depended on the vasoganglion, which was distributing on the surface of ACL.

General Observation of ACL

The experimental animals' hind limbs were opened via the former operative path and their ACLs were exposed for observation at the 6 and 12 weeks postoperatively. Then, the comparison of the general observation (synovial coverage of ACL, effusion quantity of knee-joint, the continuity of ligament, etc.) was performed (Table I).

MASSON Staining

After the Masson staining was performed, the repaired tissues on the RF-treated areas in each group were observed (Figure 2).

All repaired tissues on the RF-treated areas were confirmed as collagenous tissues. In both group A1 and B1, the RF-treated areas on the ACL were found compact at 6 weeks post-treatment, and the features of striped alignment of the collagen fibrils were still unclear. The collagen fibrils distributed on most of the areas had merged with each other and the surrounding cells were aligned within the direction of the collagen fibrils. The distribution of collagen fibrils in the mid-portion group was sparser than that in the amph-portions group. The striped alignment of collagen fibrils in both group A1 and B1 at 12 weeks postoperatively, was more evident than those at 6 weeks postoperatively. The cells were more equably distributed between the collagen fibrils at 6 weeks postoperatively, but there were still some insufficiencies compared with the normal orientation of collagen fibrils, which could be found more significant in group A. The collagen fibrils in the repaired tissues of ACL were less organized in both group A2 and B2, and the interruption began to show at 6 weeks postoperatively and the rupture of ACL was also found in both groups at the 6th weeks.

H-E Staining and Cell Count

In the normal ACL, the striped alignment of collagen fibrils should be observed and there should be lots of cells distributed between the collagen fibrils or under the synovium. The cellar density of normal ACL was (920±81)/mm^2. Because the intervention factors in the amph-portions group were identical, significant differences in cell count were not found. The cell count was conducted in four RF-treated groups at 6 and 12 weeks after the operation, and the result in the amph-portions group was presented as the mean value (Table II).

According to the factorial analysis, two major efficacies (external fixation and RF treated on

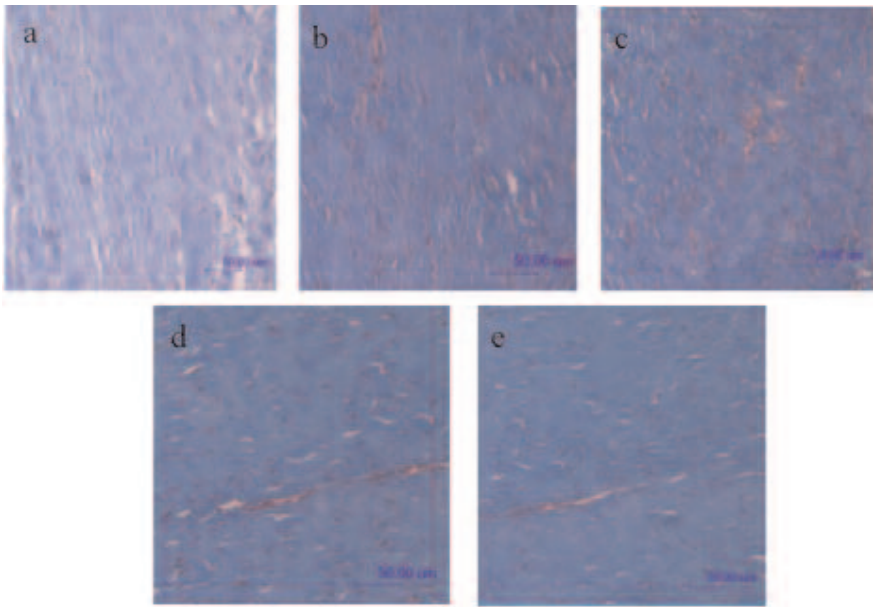

Figure 2. Features of repaired tissues in RF-treated areas of each group (MASSON staining, ×200). ***A*** Normal ACL; ***B,*** A1 Midportion group at week 6; ***C,*** B1 Amph-portions group at week 6; ***D,*** A1 Midportion group at week 12; ***E*** B1 Amph-portions group at week 12.

Table I. General observation of ACL.

Item	Synovial coverage	Effusion of knee-joint	Continuity of ligament
Normal ligament	The synovium was thin, the vessel was less but transparent, bright and shining	Transparent or light yellow	The ligament was full bright, and the contour of bundle and texture was clear.
Group A_1 6 weeks	The ACL was covered by thin synovium, and the adipose tissues hadn't been found around the ligament but majorly on the RF-treated areas	Light yellow	A little floating sensation could be found on the RF-treated areas of the synovium. The contour of bundle was unclear and the orientation of collagen fibrils was a little flatness
Group B_1 6 weeks	The ACL was covered by thin and coarse synovium and the adipose tissues hadn't been found around the ligament but majorly on the RF-treated areas	Light yellow	The coverage of thin synovium was clear on the RF-treated areas. The contour of bundle was unclear and the orientation of collagen fibrils was a little flatness.
Group A_2 Group B_2 6 weeks	The peripheral synovium was yellow, thicker (may result from inflammation) and dispersedly distributed. The ligament was wrapped by prepatellar fat plica with the form of chordae tendineae	Dull yellow, muddy and ropiness	Patent floating sensation could be found on the RF-treated areas. The ligament wasn't interrupted, the contour of bundle was unclear and the orientation of collagen fibrils was a little flatness.
Group A_1 12 weeks	Lacking the coverage of synovium on the RF-treated areas	Light yellow	Ligament wasn't interrupted, the RF-treated areas weren't enough plump, the contour of bundle was unclear and the orientation of collagen fibrils was a little flatness
Group B_1 12 weeks	The blood supply of the ligament was abundant and the coverage of the synovium was well but the thickness had decreased	Light yellow	Ligament was plump and not interrupted, no defect was found on the RF-treated areas. The contour of bundle was clear and the orientation of collagen fibrils was a little flatness
Group A_2 Group B_2 12 weeks	The ligament was pale, lacking the coverage of synovial and the distribution of vessels, the prepatellar plica was obviously swelling.	Dull yellow, muddy and ropiness	Ligament was ruptured and shrunk to be blunt.

Table II. The cellular density in each group by H-E staining (mean ±s, n=6, /mm^2).

Time	Area	Fixation	Non-fixation
6 weeks	midportion	2431 ± 282 (Group A_1)	1359 ± 148 (Group A_2)
	amph-portions	2887 ± 266 (Group B_1)	1517 ± 167 (Group B_2)
12 weeks	midportion	1580 ± 128 (Group A_1)	1175 ± 172 (Group A_2)
	amph-portions	1807 ± 184 (Group B_1)	1224 ± 75 (Group B_2)

amph-portions) were found to have significant differences (F = 178.2, p = 0.00 < 0.05; F = 11.279, p = 0.003, respectively, but the differences of the synergistic effect between them were not found (F = 2.661, p = 0.118 > 0.05) at 6 weeks postoperatively. At 12 weeks postoperatively, the significant differences of the two major efficacies still existed (F = 68.7, p = 0.00 < 0.05; F = 5.336, p = 0.032, respectively) while the synergistic effect between them was still insignificant (F = 2.225 = p = 0.151 > 0.05). Based on these observations, we speculated that either the external fixation or the RF treated on amph-portions could promote the cellularization on the RF-treated areas of the ACL, which was only the outcome of additive effects.

The Comparison of Vascular Density in Each Group

The ACL was covered by a layer of synovium, in which was full of vessels on the mid-portion of ACL. The vascular density was recorded as followed: subsynovial region 66.0±17.0/mm^2, intermediate region 40.8±29.3/mm^2, central region 25.6±11.2/mm^2. The vascular density of the tibital and femoral insertion site of ACL was averaged as followed: subsynovial region 78.7±17.2/mm^2; intermediate region 48.8±33.3/mm^2; central region 25.6±11.2/mm^2. The number of vessels of the ACL at each period was estimated by the means of the number of subsynovial vessels on the cross-section of the ACL (Table III).

After the operation, the RF-treated areas on the ACL were the regions that the number of vessels increased the most while the vascular density on the rear of untreated ACL did not show significant change (Table III). At the 6th and 12th weeks, the blood supply of amph-portions of ACL was represented by the average value of vascular density on the tibial and femoral insertion sites of ACL in group B1. According to the factorial analysis, both external fixation and RF treatment could promote the revascularization on the ACL, but only the additive effects were observed (F = 31.098, p = 0.000; F = 4.485, p = 0.047, respectively). The vascular density on the mid-portion of the ACL in group B1 at 12 weeks postoperatively had significantly increased than that in 6 weeks ago (p = 0.024), and this was not found in group A (p > 0.05). In addition, the change of the vascular density on the central region of ACL was also insignificant (p > 0.05). Then the vascular density on each region of both A1 and B1 groups was compared at 6 and 12 weeks postoperatively, and the result demonstrated that the vascular density on the subsynovial region was significantly higher than that on the intermediate region at both 6 and 12 weeks (A1, p = 0.015; B1, p = 0.027). However, the differences had decreased at 12 weeks postoperatively.

Discussion

In our study, the ink-perfusion technology was employed in demonstrating the vascular distribution of the ACL and the outcome showed that vessels were majorly distributed on the amph-

Table III. Comparison of cell density at week 6 and 12 following RF reefing between the groups (m±s, n=6, /mm^2)

Time	Vascular density (/mm^2)	A_1	A_2	B_1	B_2
6W	Subsynovial	181.8 ± 42.8	97.0 ± 40.4	242.3 ± 59.1	116.5 ± 40.0
	Intermediate	46.7 ± 15.0	44.8 ± 18.5	54.5 ± 20.1	39.2 ± 19.0
	Central	19.7 ± 11.2	20.4 ± 13.9	26.6 ± 18.5	23.4 ± 15.2
12W	Subsynovial	121.2 ± 29.9	46.7 ± 22.0	156.2 ± 40.2	51.0 ± 24.3
	Intermediate	71.7 ± 28.6	29.5 ± 18.3	99.0 ± 35.9	34.6 ± 29.7
	Central	30.9 ± 15.1	16.4 ± 10.3	26.8 ± 11.7	14.5 ± 9.2

portions of the ACL, which was similar to that in human[10]. As a result, the canine could be an ideal experimental animal in simulating the revascularization of the RF-treated ACL of human.

The goal of RF shrinkage treatment was to make the typical triple-helical structure of collagen fibrils into the irregular crimp pattern, which would further shorten the ligament. Although the adequate molecular mechanism of the thermal heating related collagenous degeneration had not yet been confirmed, 60-80°C was estimated to be the critical temperature for the thermal shrinkage[11-13].

Vangsness et al[14] applied the laser energy (70C°) in shrinking the patellar ligament of human in the 37°C saline solution and found that the ligament could be shortened to 70% of its resting length. In our study, the output power of the RF probe was 25 W, and the thermal energy increased the temperature on the surface of the ACL to 60-70°C with almost 2.0-3.0 mm of the penetrative depth of RF energy, which was related to the time of contact and the distance between the probe and tissue. This was similar to the result of Hecht et al[15], in which the penetrative depth of RF was thought to be 1.0-5.0 mm.

The mechanical properties of the ACL had decreased (sometimes might be ruptured if there was not any protection) after the RF shrinkage treatment. Lopez et al[16] applied the RF treatment on the ACL of dog's one of the hind limbs without any postoperative protection, and the rupture of ACL was observed at the 55th days. In this study, two hind limbs of dog were subjected for either fixation or non-fixation. At the 60th days after the treatment, the sign of the ACL rupture was observed in the non-fixation group while animals in the fixation group were normal, which suggested that the gypsum could restrict the extension or flexion of the joint and promote the recovery of the RF treated ACL. The postoperative weight bearing should not be regarded as the contraindication of RF shrinkage treatment, but restricting the movement and maintaining the stability of the joint were more pivotal.

The good repair and recovery of tissues relied on a good blood supply. The revascularization of injured ligament was important for the generation of collagen fibrils. Both the fat pad and synovium were thought to be essential in the repair of the injured ligament. As once the patellar fat pad was removed, the revascularization of the ligament would be delayed[17]. Unterhauser et al[18] showed that the revascularization started with the growth of the synovium; then, vessels in the synovium would grow into the ligament while the growth of vessels on the central portion of the ligament was the last step. Furthermore, in their study, the synovial fluid was not considered to be crucial during the revascularization. Scheffler et al[19] observed the histological changes on the RF-treated ACL, and yet did not found the accomplishment of revascularization. The new vessels majorly distributed on the subsynovium and intermediate regions. However, the specimens in their study were observed only at 24 weeks postoperatively, which might neglect the revascularization of ACL in the early stage. In addition, their specimens were obtained from the mid-portion of the ACL and the amph-portions in which had a richness of blood supply were neglected. The vasoganglion and synovium supplied nutrients to the ligament, nevertheless, Ma et al[20] discovered that both of them could be destroyed during the RF shrinkage treatment. After the RF treatment, the growth of the collagen fibrils and new vessels would begin anew. As the factor VIII antibodies could specifically combine with the factor VIII that was distributed on the endothelial surface cells of blood vessels, the microvessels (even if the microvessels of the vascular smooth muscle) could be recognized easily. In this experiment, the revascularization on the RF-treated areas was observed at the 6th and 12th weeks. At the 6th week, microvessels were majorly distributed on the subsynovium region of the ACL and at the 12th week, the density of microvessels on the mid-portion of the ACL had increased with the decrease on the subsynovium region. The vascular density in group B1 was higher than that in group A1 at 6 weeks postoperatively, with significant differences ($p = 0.008$). However, the vascular density in both groups had obviously decreased at 12 weeks postoperatively without significant difference ($p > 0.05$). The blood supply of the posterior and central regions of the ACL remained intact during the RF shrinkage treatment. During the process of revascularization, the vascular density on these regions did not changed, which suggested that the revascularization on the RF-treated areas started on the synovium (a few weeks after the treatment), though the previous synovium had been destroyed. The revascularization on the amph-portions of the ACL was prior to that on the mid-portion.

The postoperative efficacy of the tissular recovery was also associated with the revascularization. In this study, the result of Masson staining revealed that all the repaired tissues were col-

lagenous tissues. The collagenous density in ACL was higher at 6 weeks postoperatively, the orientation of the collagen fibrils was littery and the outcome of cell count in the amph-portions group was significantly higher than that in the mid-portion group. In addition, the repaired tissues of ACL in group B1 was better than that in group A1. At the 12th week after the operation, the rudiment of the bundle alignment of the fibrils in ACL could be observed in both group A1 and B1, the cells were uniformly distributed between the collagen fibrils and the repair of the ligament in group B1 was particularly obvious.

Conclusions

Our findings suggest that the pattern of the postoperative revascularization was from the synovium to the RF-treated areas via permeating. The recovery on the amph-portions of the ligament was better than that on the mid-portion, and the application of external fixation to restrict the movement of injured limb was necessary.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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