Effects of tendon-bone healing of anterior cruciate ligament reconstruction by osteoprotegerin combined with deproteinized bovine bone

Guoyao Zou
Enhong Song
Bing Wei

Department of Spinal and Joint Surgery, Affiliated Hospital of Guilin Medical University, Guilin, China

Corresponding author:

Guoyao Zou
Department of Spinal and Joint Surgery,
Affiliated Hospital of Guilin Medical University
No.15, Lequn Road, Xiufeng District
541001 Guilin, China
E-mail: guoyao1891@163.com

Summary

Background: The healing of a tendon graft in a bone tunnel depends on bone ingrowth into the interface between tendon and bone, or that can enhance tendon-bone healing, which is important to reduce the failure rate after ACL reconstruction.

Methods: Sixty skeletally mature, New Zealand white rabbits underwent left ACL reconstruction. OPG/DBB compound (concentration ratio of 30%, 60%, 100%) was delivered to the tendon-bone interface with use of a DBB carrier, and nothing as control group. Twenty animals were killed at 4, 8 and 12 weeks after surgery. I-IV levels of semi-quantitative and Sharpey fibers at the healing tendon-bone interface were evaluated, and the biomechanical properties were tested.

Results: A significantly greater amount of Sharpey fibers at the healing tendon-bone interface in the concentration ratio of 100% OPG/DBB-treated group was found compared with the others at all time-points ($P < 0.05$), and it is the same to the Grade Scores at 12 weeks ($P < 0.05$). The femur-ACL-tibia complex of the concentration ratio of 100% OPG/DBB-treated group has significantly increased stiffness compared with the others at 12 weeks ($P < 0.05$).

Conclusion: The concentration ratio of 100% OPG/DBB compound significantly improve bone formation around the grafted tendon and improve the stiffness at the healing tendon-bone junction in a rabbit model.

KEY WORDS: anterior cruciate ligament, biomechanical, deproteinized bovine bone, grade score, osteoprotegerin, sharpey fibers.

Background

Anterior Cruciate Ligament (ACL) rupture is a common injury in young and active patients. ACL reconstruction using tendon grafts is one of the most common procedure performed in sport traumatology worldwide, and the number of patients undergoing ACL reconstruction seems to be increasing annually¹. It is thought that the mechanical strength of an inserted graft is largely dependent on the process of tendon-bone healing. However, the slow rate of healing and the weakness of the tendon-bone interface limit rehabilitation and delay return to activity². Interventions that can accelerate and improve tendon-bone healing are attractive, because they can potentially limit graft fixation failures and allow for early aggressive rehabilitation³. Therefore, how to effectively enhance tendon-bone healing has increasingly attracted strong attention and more research.

It has been found that the application of growth factors, like BMP, BFGF, TGF- β , improve tendon-bone healing^{4, 5}. Osteoprotegerin (OPG) is a newly discovered growth factor which belongs to a member of the tumor necrosis factor receptor superfamily, inhibiting osteoclast maturation and bone resorption, thereby increasing bone density⁶⁻⁸. Rodeo et al.⁹ compared OPG with RANKL, which stimulates osteoclastic activity, for ACL reconstruction in a rabbit model. The Authors found that OPG significantly improved bone formation around the grafted tendon and improved the stiffness at the healing tendon-bone junction.

Deproteinized Bovine Bone (DBB) is a bone substitute material that has conductivity, low immunogenicity and induced characteristics, etc.¹⁰ It has a special three-dimensional porous structure that can provide a sustained release space for OPG¹¹. The literature supports that the application of slow release carrier containing growth factor is better than a single application for tendon-bone healing^{12, 13}. Zhai et al.¹⁴ compared PRG/DPB compound with PRG, which reduced the chance of bone tunnel enlargement and improved the tensile strength of the healing interface. The Authors found that the application of DPB carrier improved the stiffness at the healing tendon-bone junction.

The aim of the study is that different concentrations ratios of OPG/DBB compound may improve the stiff-

ness at the healing tendon-bone junction in a rabbit model.

Materials and methods

Study Design

The study was performed on sixty healthy, 5~6 months of age, male or female, New Zealand White rabbits (provided from Experimental Animal Center of Guilin Medical University) weighing 2.3 to 3.1 kg. According to the ratio of OPG to DBB, all rabbits were randomly divided into four groups ($n = 15$), control group (Group A), 30% (Group B), 60% (Group C) and 100% (Group D) OPG/DBB-treated group. Animal experimentation was carried out under the rules of the Animal Care and Use Committee. This article submits to the ethical standards of the Journal¹⁵.

Preparation of different concentrations of OPG/DBB compound

Femur epiphyseal of bovine were harvested, with the soft tissue and bone cortex removed, processed into bone particles of a size of about 3 mm × 3 mm × 3 mm, and then malaxated and douched repeatedly by warm water. They were soaked into 30% H₂O₂ for 72 hours after drying out, and then were put into methanol/chloroform solution to skim for 4 hours. Washed with water, ethylene oxide sterilization, spared. The OPG (Bioss, Beijing, China) was dissolved in sterile distilled water and formulated into three different concentrations of solution, they were marked as 30 µg/mL (Group B), 60 µg/mL (Group C), 100 µg/mL (Group D). According to the proportion of two grains of DBB soaked in a solution of 2 ml OPG¹⁶ and three concentrations ratios of OPG/DBB compound were obtained, they were marked as 30% (Group B), 60% (Group C), 100% (Group D) of OPG/DBB compound. Then they all were stored at 4°C for 6 hours. At last, they were aliquot and spare using EP tubes at -20°C.

Surgical Procedure

Each animal was anesthetized with pentobarbital sodium (30 mg/kg) in the form of ear vein injection, and left knee anesthetized paracentesis with lidocaine (2%, 2 ml). The left hind limb was shaved, scrubbed with betadine, and draped in a sterile fashion. The achilles tendon was identified and harvested. Both ends of the achilles tendon were secured with a 3-0 mersilk suture (Ethicon). A parapatellar incision was used to expose the knee joint. The soft tissue of medial patella was step detached and the patella was dislocated laterally. The native ACL was identified and transected. Remnant ACL stumps were debrided and the footprints were identified. The tibial and femoral tunnels were made at the anatomic footprints using a 2-mm drill in an inside-out manner. One inner diameter of 1.0-mm bone bridge was obtained at the 10mm above the outside of the femoral tunnel and another bone bridge was obtained at the 10 mm at

the lower part of the tibial tunnel. The two bone bridges were paralleled to the coronal plane and used to fix both ends of the tendon graft.

The tendon graft was passed through the tunnels. The different concentrations ratios of OPG/DBB compound were implanted in the femoral and tibial bone tunnel of groups B, C, D. Then, the tendon graft was fastened to the bone bridge at both ends of tendon. Intra-articular residual bone were carried out. The joint capsule, the subcutaneous tissues, and the skin were closed in a standard manner. The wounds were dressed, and the left limbs were bandaged. The legs were not immobilized postoperatively. Intramuscular injections of penicillin (400,000 U/d) within 3 days.

Histological Evaluation

The rabbits of each group were killed at 4 weeks (five rabbits), 8 weeks (five rabbits), 12 weeks (five rabbits) after the operative procedures. At the time of dissection, the appearance of the synovial tissues was observed. The proximal part of the tibia and the distal part of the femur were resected, with preservation of the tunnel holes. All of the soft tissues (except for the ACL graft) were removed. The specimens were fixed in a 10% neutral-buffered formalin solution immediately after harvesting for 48 hours. After the specimens had been decalcified for 1 week, they were cut vertical to the longitudinal axis of the bone tunnels. Five-micrometer-thick sections were cut after they were embedded in paraffin blocks. The sections were stained with hematoxylin and eosin and Masson trichrome and then were examined with light microscopy (Carl Zeiss, Germany).

Histological evidence of the healing process at the tendon-bone interface was assessed according to Demirag score¹⁷. An ocular micrometer was used to measure the percentage of a 10-mm length of the tendon-bone interface that was occupied by Sharpey collagen fibers. Grade I denoted an interface with scant and unorganized fibrovascular tissue; Grade II denoted organized fibrovascular tissue with few collagen fibers, occupying <30% of the interface length; Grade III denoted mature fibrovascular tissue with decreased cellularity and vascularity, occupying 30% to 60% of the interface length, and Grade IV denoted fibrovascular tissue organized to dense connective tissue, with >60% of the length of the interface containing Sharpey fibers.

Biomechanical Testing

Each specimen were analyzed for biomechanical testing. The suture that had been used for operative fixation of the graft was removed. With use of specially designed clamps, the distal part of the femur and the proximal part of the tibia were loaded on a micro-computer control electronic universal testing machine (Shenzhen Sans Material Test Instrument Company, China). The displacement rate was 25 mm/min. A tensile load was applied along the longitudinal axis of the graft, and the ultimate load to failure was recorded on a personal computer.

Figure 1 a-d. Tissue morphology at 4 weeks after operation (hematoxylin and eosin, $\times 100$). Tendon-bone healing is an indirect insertion which characteristic with Sharpey fibers (marked with an arrow). a) Group A; b) Group B; c) Group C; d) Group D. Abbreviation: T, tendon; B, bone.

Statistical Analysis

The SPSS 18.0 software was used for statistical analysis. Significance of the differences between groups was determined using one-way ANOVA and the post-hoc multiple comparison Bonferroni test. To identify differences in the Grade score, Kruskal-Wallis Test was used. Differences were regarded as statistically significant at values of $P < 0.05$.

Results

Histological Findings at Four Weeks

Both group A and group B, the interface between tendon graft and bone was filled with poorly fibrovascular tissue with few or no irregular collagen fibers, tendon-bone connection is not tight. Group C, a small amount of collagen fibers arranged irregular and more fibrovascular tissue formation in tendon-bone interface. Irregular collagen fibers consistent with Sharpey fibers were observed between the bone and the tendon graft. Group D, part of the interface between tendon graft and bone exhibited a small amount of Sharpey fibers, tendon-bone binded tightly than others (Fig.1).

Histological Findings at Eight Weeks

The interface between tendon graft and bone was filled with fibrovascular tissue with few or no Sharpey fibers in Group A. There were few irregular Sharpey fibers and uncalcified fibrocartilage in the interface between tendon graft and bone in Group B. Abundant perpendicular Sharpey fibers and uncalcified fibrocartilage were present between tendon graft and bone in Group C. In the specimens of Group D, there was little calcified fibrocartilage in the interface between bone and uncalcified fibrocartilage, and the tendon had few new bone tissue ingrowth. Quite a few perpendicular Sharpey fibers and uncalcified fibrocartilage were filled in the interface between tendon and bone (Fig. 2).

Histological Findings at Twelve Weeks

In the specimens of Group A, the bone had few new calcified fibrocartilage ingrowth. In Group B, quite a few Sharpey fibers filled the interface between tendon and bone, uncalcified fibrocartilage connected with calcified fibrocartilage were observed also. An irregular and blur "tidal line" could be seen in part of the specimens. In both the Group C and D specimens, there were more areas of uncalcified fibrocartilage

Figure 2 a-d. Tissue morphology at 8 weeks after operation (hematoxylin and eosin, $\times 100$). Tendon-bone healing is an indirect insertion which characteristic with Sharpey fibers (marked with an arrow). a) Group A; b) Group B; c) Group C; d) Group D. Abbreviation: T, tendon; B, bone; UFC, uncalcified fibrocartilage; CFC, calcified fibrocartilage.

and calcified fibrocartilage formed. Compared with Group C, there's been a noticeable irregular and blur "tidal line" in Group D (Fig. 3).

Results of Grading of the Fibrous Tissue

With the help of hematoxylin and eosin and Masson trichrome, there was significant difference between Group D and others in the Grade scores (Tabs. I, II).

Biomechanical Testing

At 4 weeks, the ultimate load to failure was significantly greater in the Group D specimens than in the group A specimens and Group B specimens, with the significant difference ($P < 0.05$). At 8 weeks, the transplant tensile strength of the Group C specimens and D specimens were higher than that of the Group A specimens, with the significant difference ($P < 0.05$).

Table I. Results of grading of the fibrous tissue at the tendon-bone interface.

grade	weeks 4 (n=5)				weeks 8 (n=5)				weeks 12 (n=5)			
	A	B	C	D	A	B	C	D	A	B	C	D
I	4	4	3	1	3	3	1	-	2	-	-	-
II	1	1	1	3	2	2	2	1	2	3	1	-
III	-	-	1	1	-	-	1	2	1	1	2	1
IV	-	-	-	-	-	-	1	2	-	1	2	4

Figure 3 a-d. Tissue morphology at 12 weeks after operation (hematoxylin and eosin, $\times 100$). a) Group A; b) Group B; c) Group C; d) Group D. T, tendon; B, bone; UFC, uncalcified fibrocartilage; CFC, calcified fibrocartilage; TM, Tide mark.

Table II. Grade Scores in each group within 12 weeks. The bone tunnel had more areas of denser connective-tissue ingrowth in Group D compared with others, the difference was significant ($P < 0.05$).

Grade Score	Group A	Group B	Group C	Group D*
Grade I	9	7	4	1
Grade II	5	6	4	4
Grade III	1	1	4	4
Grade IV	0	1	3	6
Total	15	15	15	15

Until to 12 weeks, the transplant tensile strength of the group D specimens was higher than others, with the significant difference ($P < 0.05$) (Fig. 4).

Discussion

The ability of a tendon to heal firmly to its osseous attachment site is vital to musculoskeletal function.

However, the healing of achilles tendon to bone is not yet clearly understood despite many experimental studies.

Tendon-bone healing in a bone tunnel usually occurs with formation of fibrovascular interface tissue between the tendon and bone. There are two types of entheses at the tendon-bone junction: direct entheses (fibrocartilage entheses) and indirect entheses (fibrous entheses)^{18, 19}. Direct entheses contain 4 mor-

Figure 4. Histogram of ultimate tensile load between groups at different time points.

phologically distinct phases in the transition from tendon to bone: tendon, uncalcified fibrocartilage, calcified fibrocartilage and bone. In addition, there is an irregular and blur "tidal line" between the uncalcified fibrocartilage and calcified fibrocartilage. Indirect entheses consist primarily of superficial fibers, called Sharpey fibers, which insert into the periosteum at acute angles²⁰. Likewise, the fibers that insert into calcified cartilage were also called Sharpey fibers.

The tendon-bone interface tissue goes through a maturation process that continues until its matrix is composed of collagen fibers²¹. Then, they were formed of the typical 4 layers structure of direct entheses that included uncalcified fibrocartilage, calcified fibrocartilage. The normal entheses of tendon-bone healing is the fibrocartilage entheses that were considered to compensate for the various elastic moduli of the ligament and bone. The normal ACL entheses shows fibrocartilage entheses through formation of a fibrocartilage zone²². Therefore, tendon-bone healing through fibrocartilage interface tissue, could show more effective elastic modulus compensation rather than fibrous healing. In addition, previous animal studies already showed that tendon-bone healing through fibrocartilage entheses interface tissue was biomechanically stronger. The direct entheses buffers the stress mainly through its typical 4 layers structure of fibrocartilage entheses. However, the indirect entheses buffers the stress mainly through the large area of fibrous entheses. Therefore formation of this fibrocartilage zone as a transition structure is thought to be more functional than indirect healing through Sharpey fibers²³.

Our results showed that few of irregular Sharpey collagen fibers were observed at 4 weeks after operation in each group and trend to arrange regularly. Until to 12 weeks, the compound groups were found to have a typical 4 layers structure of direct entheses, with the clear evidence in the Group D. In the all compound

groups, the growth of new bone tissue was obvious, which was closely related to the tendon graft, rather than control group. In addition, The Grade scores of collagen fibers in Group D were significantly higher than those in others. Histologic results in Group C and D showed broad fibrocartilage entheses healing between the tendon graft and bone resembling the normal ACL direct entheses. The evidence indicated that OPG/DBB compound could contribute to the formation of direct entheses and promote the healing process at the tendon-bone junction.

Importantly, histologic improvements were associated with biomechanical improvement, indicating that the stresses at the interface of tendon-bone were related to composed types of entheses. Thus, biomechanical changes can directly represent the degree of tendon-bone healing²⁴. The greater the tensile load of the tendon graft shows the higher degree of tendon-bone healing. The results of this study showed that the ultimate tendon graft load of Group D was maximum at 12 weeks, and the ultimate tendon graft load of Group D was higher than that of Group A and B at 4 or 8 weeks. These demonstrated that the concentrations ratio of 100% OPG/DBB compound could enhance tendon-bone healing and increase the stresses at the interface of tendon-bone.

Since DBB is a three-dimensional porous structure which can store and control the release of OPG. Our study adopted the DBB as the carrier, took full advantage of osteogenesis of OPG and the release characteristics of the carrier which reduced the loss of OPG, so that to ensure effective concentration of OPG. Therefore, the grafted tendon was exposed to the bone, sufficient and lasting. Further, they promoted the formation of fibrocartilage entheses in the interface of tendon-bone. It is precise because of the slow release characteristics of OPG with the DBB carrier, the results were analyzed at three time points and compared with the three concentrations of OPG.

The important finding of our study was that OPG/DBB compound could contribute to enhancing tendon-bone healing through the interface area with fibrocartilage entheses. It can significantly improve the stiffness at the healing tendon-bone junction when the concentration of OPG is 100%.

There are some limitations in this study. Owing to fewer samples observed in each group and the shorter observation time, the duration of the compound was still need to be further researched.

The conflict of interest

The Authors declare no conflicts of interest.

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