The underlying mechanism of partial anterior cruciate ligament injuries to the meniscus of knee joint in rabbit models

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Abstract

Background The diagnosis, treatment and efficacy evaluation of anterior cruciate ligament (ACL) remains controversial. This research aims to investigate the underlying mechanism of partial ACL injuries to the meniscus degeneration in rabbit knee. **Methods** Sixty New Zealand, white rabbits were randomly divided into three groups: the anteromedial bundle (AMB) splitting, the posterolateral bundle (PLB) splitting and a control group. Finally, eight rabbits were sampled randomly on the second, fourth and eighth weeks respectively. We observed the typical form of the meniscus through HE staining. Expressions of inflammatory factors including interleukin-1 β (IL-1 β) and IL-17 in the knee joint fluid were determined by means of an ELISA. Analysis of the mRNA expressions of MMP-13 was performed to evaluate the inflammatory mediators in the pathogenesis of the meniscus. **Results** HE staining results showed that the surface was rough and the tissues were loose displaying collagen fibers of varying thickness. Both IL-1 β and IL-17 in the synovial fluid, and the positive rate of MMP-13 in addition to MMP-13 mRNA showed a demonstrable increase trends from the 2nd to the 8th week. The significant difference was found ($P < 0.05$) compared to the control group. **Conclusion** Our findings illustrated that the elevated levels of IL-1 β and IL-17, along with increased MMP13 expression, resulting in meniscus degradation in the rabbit knee joint model with partial ACL injury. When the partial ACL injury on the different bundles occurred, the reconstruction of the reserved AMB or PLB must be operated on time due to uncertainty of conservative treatment.

Background

With the development of mass sports and the increase in traffic accidents, anterior cruciate ligament (ACL) splitting has become an extremely common injury, which accounts for about half of all knee ligament injuries by increasing year by year incidence in China [1]. Clinically, the severity of ACL injury is divided into three types: mild, moderate and severe injuries. In the cases of moderate and severe injuries, ACL reconstruction surgery is recommended. Injury to one-quarter of a single bundle of the ACL are commonly referred to as a mild injury or termed partial ACL injury. In partial ACL injury, it is difficult to assess its functional retention with arthroscopy because of physical examination and imaging performance [2, 3].

The diagnosis, treatment and efficacy evaluation of the partial ACL injury remain challenging with conflicting reports in the literature [4, 5]. When the ACL injury develops, poor knee stability results in a greatly increased probability of joint injury. Therefore, treatment of ACL after different functional bundle injuries is related to aspects of the patient's knee joint, such as articular cartilage, meniscus and other structures of acute or long-term damage [6]. In this aspect, establishing an animal model of partial ACL injury is helpful in developing research to gain better understanding of the basic mechanism and selective treatments of ACL injury.

Numerous factors influence knee trauma and osteoarthritis (OA) progression. It has been suggested that the elevated levels of inflammatory cytokines within the joint environment were associated with knee

trauma in addition to OA [7]. Additionally, matrix metalloproteinases (MMPs) are a class of enzymes widely present in the connective tissue of the articular cartilage extracellular matrix (ECM). Physiological and pathological degradation of ECM plays an important role in the protease super-family, which can be divided into many sub-types according to dissimilar substrates. MMP-13 was chosen as a bio-marker of ligament damage as it belongs to the collagenase sub-type of MMPs, which can directly degrade type 2 collagen in the cartilage matrix. MMP-13 can reflect the metabolic changes in cartilage matrix [8]. The pathogenesis of ACL injury leading to OA has not been fully elucidated. Studying the mechanism can help physicians choose appropriate treatments for patients including surgery, drugs and tissue engineering to reduce the incidence of OA.

This study was designed to establish a model of discrete functional bundle injuries in the knee joint of rabbits, and to observe whether the differences in the morphology and histology of the meniscus are impacted by different functional bundle injuries on the knee meniscus. In addition, we aimed to determine the clinical significance of inflammatory factors in the articular fluid of meniscus degeneration. We also presented these findings as a theoretical basis for the selection of clinical treatment methods.

Methods

Animals and Groups

This experiment was approved by the Animal Experiments Ethics Committee of the Associated Hospital of Beihua University (Ethical approval number: Protocol Number 2017-08-16). Animal care was in accordance with the Animal Research: Reporting in Vivo Experiments guidelines. The study was conducted on 60 mature New Zealand rabbits weighing between 2.5-3.0 kg. Animals were obtained from the Experimental Animal Breeding and Research Centre, Bethune Medical College Animal Experimental Center, Jilin University. A total of 60 rabbits were randomly assigned into three groups: the anteromedial bundle (AMB) fracture group, the posterolateral bundle (PLB) fracture group and the control group without fractures. Fifteen rabbits were randomly assigned to each group.

Plan-operative procedure

The experimental knee model was created by cutting the partial ACL of the rabbits under the arthroscopy (Andover, MA, USA; 72200616) as previously described [9]. The right legs were utilized to build AMB and PLB splitting models. In brief, anesthesia was administered intramuscularly at dosages of ketamine (100 mg/kg). An adequate opening was obtained through the medial incision of the knee into the joint cavity. One quarter of the ACL was transversely cut off under a clear arthroscopic field of vision. The anterior drawer experiment confirmed that the model of ACL splitting had been successfully done while the incision was then sutured with 3/0 absorbable sutures. The additional 10 rabbits were invoked as a sham operation control, the same procedure as the medial incision of the knee into the joint cavity was exposed without cutting off the joint cavity directly. Four days after surgery, all postoperative rabbits were intramuscularly injected with 400,000 U penicillin to prevent infection. The legs of rabbits did not make

any fabrication. All rabbits were housed with sub-cage feeding. The cage size was 60 cm×60 cm×40 cm with a temperature of 23-25 °C and a relative humidity of 55%.

General observations of animal status and meniscus

Eight rabbits were randomly sacrificed in the experimental group at the 2nd, 8th and 10th weeks after the operation. The knee was experimentally dissected and the typical meniscus shapes were observed along with other properties including fractures, color, elasticity and surface smoothness.

Histopathological and immunohistochemistry evaluation

The posterior angles of the medial meniscus in the three groups were cut separately and the surrounding tissue was thoroughly removed. 0.9% sodium chloride brine was rinsed and fixed for 24 hours. The histological section of the operational side of the meniscus was stained with haematoxylin-eosin. Histological changes in the meniscus tissue were measured according to the method as previously described [10].

Immunohistochemical staining using antibodies directly against MMP-13 was adopted with the Histostain-SP kit (Zymed, San Francisco, CA). The tissue sections were incubated with a specific antibody against MMP-13 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:500). Normal goat IgG was used as a negative control. The expression of MMP-13 was detected by a biotin-streptavidin-peroxidase system using diaminobenzidine as a chromogen. Counterstaining was carried out with hematoxylin.

Cytological measurement of knee joint fluid samples

Primary outcomes included levels of IL-1 β and IL-17 in the knee joint fluid. For each operative animal, a minimum of 1 ml of synovial fluid was aspirated from the knee joint prior to orthopedic surgery via direct needle aspiration. Samples were placed in a BD Vacutainer test tube and centrifuged at 3,000 rpm for 10 minutes. The supernatant was stored at 20 °C until the assay. The levels of IL-1 β and IL-17 were measured by means of an enzyme-linked immunosorbent assay (ELISA) with the Quantikine® HS Human IL-1 β and IL-17 Immunoassay kit (R&D Systems, MN, USA). The limit of detection was lower than 2 pg/mL for IL-1 β , IL-17, and the intra-batch CV and inter-batch CV were < 9 % and <15 % respectively.

RNA isolation and analysis of the mRNA expressions of MMP-13

0.1g of fresh meniscus tissue was cut into a foam with scissors. The pellets were collected and total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized with a cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The primer sequences are as follows: MMP-13 forward TGACCACTCCAAGGACCCAG; reverse GAGGATGCAGACGCCAGAAGA. G3PDH forward CCACTTTGTGAAGCTCATTTCT; reverse TCGTCCTCCTCTGGTGCTCT.

qRT-PCR analysis of the mRNA was performed using a standard kit (Invitrogen, Carlsbad, CA, USA). The relative transcript levels of the target genes were normalized to that of G3PDH using the $2^{-\Delta\Delta C_t}$ assay. Relative levels of gene expression are presented as the mean $\pm$ standard deviation of three independent experiments.

Statistical analysis

Statistical analysis was performed with SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA) and data are expressed as the mean $\pm$ standard deviation. Normality of data distribution was assessed by the Jarque-Bera test. When the cytokine concentrations were non-Gaussian distribution, both *t*-test and Mann-Whitney-Wilcoxon (MWW) tests were utilized. Analysis of variance (one-way ANOVA) and post hoc Bonferroni multiple comparisons test was utilized to compare differences between groups. A value of $P < 0.05$ was considered statistically significant.

Results

Characteristics of animal status and meniscus in the ALB and PLB groups

At two weeks post-surgery, the incisions in all groups had recovered without any signs of infection or delayed healing and the control group remained healthy. Operated limbs could be gradually moved and no abnormalities were found. In the ALB and PLB control groups, the structure of the meniscus was complete and the surface was bright white, very smooth, had good elasticity and showed no tears each week. In ALB group after 2 weeks, the meniscus was smooth, dark yellow in color and had complete general structure, showed good toughness with no obvious tears on the body and the free margin. In week 4, the general meniscus structure was still complete. The surface was rough and pale yellow with poor elasticity, relaxation and no tears. In week 8, the free margin on the meniscus appeared to be worn out, had a rough surface, was dark yellow, had poor toughness and the degree of tension significantly decreased. The meniscus had an intact structure and poor surface flatness, was pale yellow, had poor elasticity and no tears at the second week. At the fourth week, the inner edge of the meniscus showed wear and tear, the surface was rough, yellowish, with poor elasticity and obvious signs of relaxation. The meniscus body was damaged, the surface flatness had significantly deteriorated, it was deep yellow, the toughness had noticeably deteriorated, and the degree of tension was significantly reduced after eight weeks.

Pathological morphology of meniscus on the ALB and PLB groups

In the ALB control groups, the meniscus HE staining characteristics showed that the surface was dense and smooth. Chondrocyte cells and collagen fibers were arranged neatly and were well defined. Non-clustered chondrocytes and inflammatory cells were found (Fig. 1 a). In the ALB groups, staining was consistent and the surface structure was complete. The collagen fibers were arranged in a compact area, and only the surface cells were enlarged (Fig. 1 b) for the 2nd week. The staining depth changed and the surface was uneven. Also, the tissue was loose and the cells were irregularly arranged (Fig. 1 c) at the 4th

week. At the 8th week, the smoothness of the meniscus was significantly reduced showing that the local slag formation and collagen fiber tissue sequence had changed dramatically and a small amount of inflammatory cell infiltration was observed (Fig. 1 d). In addition, the meniscus HE staining exhibited the same characteristics in PLB groups as the AMB groups (Fig. 2 a). After two weeks, the meniscus was structurally intact, the surface was poorly formed, it was pale yellow, the elasticity was worse and there was relaxation. The surface had poor flatness, the tissue was loose and showed irregular arrangement of cells (Fig. 2 b). After four weeks, the flatness of the meniscus was week, and local slag formation was observed. The sequence of the collagen tissue had changed obviously, and a small amount of inflammatory cells had infiltrated (Fig. 2 c). After eight weeks, the smoothness of the meniscus was worse, the tissue was loose, the sequence of the collagen tissue was altered and the thickness was uneven. A large number of inflammatory cells had also infiltrated (Fig. 2 d).

Expressions of inflammatory factors in knee joint fluid of different groups

Both IL-1 β and IL-17 in the synovial fluid showed a demonstrable increase trends from the 2nd to the 8th week. Compared with the control group, there was a difference between ALB and PLB groups at three time phases ($P < 0.05$) as showed in Table 1 and Table 2.

Levels of MMP-13 and MMP-13 mRNA in the meniscus of different groups

Immunohistochemical staining for MMP-13 showed the same characteristics in the experimental groups. MMP-13 showed weak positive expression, both the cytoplasm and matrix were not expressed in the control groups. The surface, matrix and cytoplasm showed weak positive staining, the cells were rounded, full, and exhibited some cytoplasmic expressions for the 2nd week. The expressions of the surface and interstitial inside the specimen showed strong positive staining, partial degradation of the matrix, relaxation of collagen fibrous tissue and change in cell morphology. Staining degree of meniscus decreased from 4 weeks, especially cytoplasm, matrix degradation, partial collagen fiber fracture, cell size and shape were different.

MMP-13 positive cells were counted in 100 images and compared in the three groups. The positive rate of MMP-13 expression in the AMB and PLB groups showed an increase trends from the 2nd to the 4th week. Compared with the control group, a significant difference was observed among 2, 4 and 8 weeks ($P < 0.05$). However, the positive rate of MMP-13 expression in the AMB and PLB groups showed a decrease after eight weeks (Table 1, Table 2).

The expressions of MMP-13 mRNA in the meniscus of the three groups were identified by RT-PCR. The findings demonstrated that the expression of MMP-13 mRNA increased from the 2nd week to peak at the 4th week and the difference was significant ($P < 0.05$) compared to the control group on the AMB group and the PLB group. The level of MMP-13 mRNA decreased in the eighth week in both groups, although a significant difference was found ($P > 0.05$) compared to the control group (Fig. 3).

Discussion

The ACL is an important stable structure in the knee joint. Its principal role is tantamount to limit the tibial advance and to adjust the stability of the knee joint rotation function [11]. However, the ACL is also the most easily damaged structure in the knee joint. Anatomically, the ACL can be subdivided into the AMB and the PMB [12]. Each function bundle affects the tibial tuberosity advancement and rotation in different tension modes over the entire flexion and extension of the knee joint. If any of these band, it affects the stress distribution for all parts of the knee and can lead to damaging [13, 14]. As the anatomical structure and histochemistry of rabbit knee joints are similar to humans, rabbits were served as experimental models. Knee modeling was utilized to study the pathological process, histopathological features and cartilage biochemical metabolism of articular cartilage after partial ACL injury [15, 16].

In this study, we successfully established a partial ACL injury model in rabbit knee joint and systematically observed effects on the meniscus at the second, fourth and eighth weeks. When the ACL injury on the discrete bundles occurred, the part of meniscus slowly silted, the meniscus surface becomes gradually rough and yellow, and the elasticity of meniscus deteriorated and become relaxed. The control side of the meniscus structure was complete, the surface was bright white, and very smooth, had good elasticity. In a viewpoint of HE staining findings, the injuries of different functional bundles of the ACL were clearly found to cause damage to the meniscus. More severe meniscus damages were associated with prolongation of the ACL injury. Immunohistochemistry results showed that MMP-13 was involved in the pathogenesis of meniscus degeneration. Due to the massive damage and death of chondrocytes, the amount of MMP-13 decreased accordingly. It is worth noting that the low expression of MMP13 did not necessarily represent the end of injury and chondrocyte apoptosis.

The meniscus lies in the articular space of the knee joint and also is a key structure of the knee joint. The meniscus acts transfer load and absorbs shock to maintain joint stability which results from vital functions of collagen which is composed of fibrous tissue [17]. The medial collateral ligament is contacted to the medial meniscus, and the fiber is connected with the diaphragm muscle [18]. The meniscus maintains stability and flexibility of the normal physiological function. Some studies have revealed that the knee joint can change the mechanical properties of the medial meniscus after injury of the knee at different angles [19]. When the meniscus angle is considerably higher than the anterior horn, the knee joint tomography suggests that the meniscus of the posterior horn is vulnerable to damage. This can lead to secondary knee medial meniscus damage which requires ACL reconstruction as soon as possible [20].

In the course of keen OA after ACL injury, inflammatory mediators play an important role at the beginning of the process and mechanical factors accelerate progression of the OA [21]. The pathogenesis of ACL injury leading to OA has not been fully elucidated. Studying the mechanism can help physicians choose appropriate treatments for patients including surgery, drugs and tissue engineering to reduce the incidence of OA [22, 23].

In the present study, we measured the levels of IL-1 β and IL-17 in synovial fluid concerning the role of inflammatory mediators in the process of chronic degeneration in the knee meniscus during eight weeks.

Our findings indicated that the elevated levels of IL-1 β and IL-17 suggested that inflammatory factors were involved in the degeneration of the meniscus. In previous studies, several studies have confirmed that IL-1 β and IL-17 within synovial inflammation were associated with meniscus or cartilage degeneration as primary inflammatory mediators in humans or animals. Our study was in accord with these reporters [24, 25]. High levels of IL-1 β and IL-17 in knee traumatic areas demonstrated the level of meniscus or cartilage damage and the presence of a local inflammatory response that triggers early knee OA [26]. IL-17 is also a pro-inflammatory cytokine, which has been declared to increase synovitis and joint destruction following intra articular injection. IL-17 is mainly secreted by Th17 and can promote the proliferation and activation of T cells. Activated T cells develop a large number of cytokines such as IL-1 β to accelerate meniscus injury. IL-1 β can inhibit the proliferation of chondrocytes by interfering with the metabolism of meniscus chondrocytes, and activate the MMPs signaling pathway. Studies indicated that a variety of inflammatory mediators play critical roles in the pathogenesis of knee trauma and OA progression. Expressions of various matrix MMPs are significantly increased, including MMP-1, MMP-2, MMP-3 and MMP-7 in addition to MMP-13 [27]. The increase in MMPs triggers a series of biological reactions that increase collagen degradation in the cartilage matrix and destroy meniscus integrity.

Current surgical treatment of the part ACL injury is different from bundle injury diagnosis, remains controversial [28, 29]. The part ACL injury is a common ACL injury model, however, the treatment standards of patients with ACL injury are inconsistent [30, 31]. An orthopedic expert reported that patients with part ACL injury who had been followed up for 9 to 15 years have developed a complete rupture, but only 32% of patients recovered to pre-injury levels. 48% of patients had a poor prognosis and 86% of patients had persistent symptoms [32]. The complete breakdown of the ACL single bundle injury may depend on the number of damaged fibers, the type of injury and the presence of secondary injury. These factors may be particularly important after posterolateral bundle injury and impact the stability of the knee joint [33].

Conclusions

We successfully established models with distinctive damage to functional bundle on ACL in the rabbit knee. Our findings illustrated that the elevated levels of IL-1 β and IL-17 increased MMP13 expression in the posterior horn of the medial meniscus in the rabbit knee joint model with partial ACL injury, resulting in meniscus degradation and knee OA occurrence. When the partial ACL injury on the different bundles occurred, the reconstruction of the reserved AMB or PLB must be operated on time due to uncertainty of conservative treatment.

Declarations

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Disclosure statement

The authors declare that they have no competing interests. The authors alone are responsible for the content and writing of the paper.

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Tables

Table 1 Comparison of MMP-13, IL-1β and IL-17 on AMB between the control and experimental groups ($\bar{X} \pm s, n=8$)

Weeks	Experimental group				Control group		P value
	MMP-13 (%)	IL-1β (ng/ml)	IL-17(ng/ml)	MMP-13(%)	IL-1β (ng/ml)	IL-17(ng/ml)	
2	1.56 ± 0.21	17.54 ± 4.60	20.31 ± 8.45	1.28 ± 0.17	1.52 ± 1.60	1.31 ± 0.55	0.05*
4	12.24 ± 0.13	21.44 ± 6.70	24.41 ± 7.35	1.47 ± 0.21	1.54 ± 1.70	1.33 ± 0.45	0.05*
8	10.53 ± 0.25	25.53 ± 6.30	38.31 ± 6.53	1.17 ± 0.16	1.53 ± 1.50	1.32 ± 0.55	0.05*

AMB: Anteromedial bundle

MMP-13: Matrix metallo proteinase-13

Data are presented as mean±standard deviation of three independent experiments, each in triplicates.

***P*<0.05 compared with the control group determined by ANOVA and post hoc Bonferroni multiple comparisons test.

Table 2 Comparison of MMP-13, IL-1β and IL-17 on PLB between the control and experimental groups ($\bar{X} \pm s, n=8$)

Weeks	Experimental group			Control group			P value
	MMP-13 (%)	IL-1 β (ng/ml)	IL-17(ng/ml)	MMP-13(%)	IL-1 β (ng/ml)	IL-17(ng/ml)	
2	1.76 $\pm$ 0.17	17.54 $\pm$ 4.60	20.31 $\pm$ 8.45	1.15 $\pm$ 0.20	1.52 $\pm$ 1.60	1.31 $\pm$ 0.55	$\neq$ 0.05*
4	12.46 $\pm$ 0.23	21.44 $\pm$ 6.70	24.41 $\pm$ 7.35	1.26 $\pm$ 0.16	1.54 $\pm$ 1.70	1.33 $\pm$ 0.45	$\neq$ 0.05**
8	10.79 $\pm$ 0.21	25.53 $\pm$ 6.30	38.31 $\pm$ 6.53	1.13 $\pm$ 0.25	1.53 $\pm$ 1.50	1.32 $\pm$ 0.55	$\neq$ 0.05**

PLB : Posterolateral bundle

MMP-13: Matrix metallo proteinase-13

Data are presented as mean $\pm$ standard deviation of three independent experiments, each in triplicates.

** P <0.05 compared with the control group determined by ANOVA and post hoc Bonferroni multiple comparisons test.

Figures

Figure 1

Morphologies of the knee meniscus and HE staining on the rabbits at the 2nd, 4th and 8th week after the operation in the experimental and control groups amongst the AMB groups (HE staining $\times 400$). a. Structures of the knee meniscus and HE staining on the rabbits sacrificed after two weeks were imaged in the control sides of the AMB groups. b. Structures of the knee meniscus and HE staining on the rabbits sacrificed after two weeks were imaged in the experiment in the AMB groups. c. Structures of the knee meniscus and HE staining on the rabbits sacrificed after four weeks were imaged in the experiment in the AMB groups. d. Structures of the knee meniscus and HE staining on the rabbits sacrificed after eight weeks were imaged in the experiment in the AMB groups. AMB: Anteromedial bundle

Figure 2

Morphologies of the knee meniscus and HE staining on the rabbits at the 2nd, 4th and 8th weeks after the operation in the experimental and control groups amongst the PLB groups (HE staining, $\times 400$). a.

Structures of the knee meniscus and HE staining on the rabbits sacrificed after two weeks were imaged in the control sides of the PLB groups b. Structures of the knee meniscus and HE staining on the rabbits sacrificed after two weeks were imaged in the experiment in the PLB groups. c. Structures of the knee meniscus and HE staining on the rabbits sacrificed after four weeks were imaged in the experiment in the PLB groups. d. Structures of the knee meniscus and HE staining on the rabbits sacrificed after eight weeks were imaged in the experiment in the PLB groups. PLB : Posterolateral bundle

Figure 3

Levels of MMP-13 mRNA in the meniscus of different experimental groups. The expression of MMP-13 mRNA increased from the 2nd week to the peak on the 4th week which was significantly different ($P < 0.05$) compared with the control group on the AMB group and the PLB groups. Whilst the level of MMP-13 mRNA decreased at the 8th week in both groups, the difference was not statistically significant ($P > 0.05$) compared with the control group. AMB: Anteromedial bundle; MMP-13: Matrix metalloproteinase-13 M: Marker;