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Original Article

Promotion of adipose stem cell transplantation on the healing of rabbit anterior cruciate ligament injury and its mechanism

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Abstract: Objective: To observe the ligament healing and mechanism in rabbits with anterior cruciate ligament (ACL) injury. Methods: 60 New Zealand white (NZw) rabbits were randomly assigned to control group (CG) (n = 20), model group (MG) (n = 20) and study group (SG) (n = 20). In CG, the rabbits were given longitudinal incision. In MG, 70% of ACL was cut off. In SG, 10 μL of third-generation adipose stem cell suspension was injected into the injured site on the basis of MG. The rabbits were anesthetized and executed 4 weeks after surgery. The samples of anterior ligament were taken. The relative TGF-β (transforming growth factor-β) and type I collagen expressions of the three groups were detected, and Western blotting was performed for testing the protein expressions of TGF-β and type I collagen. Results: The relative expressions of TGF-β mRNA and protein of MG and SG were significantly higher than those of CG ($P < 0.05$), but the relative expression of type I collagen mRNA and protein of CG were significantly higher than those of MG and SG groups ($P < 0.05$). But CG exhibited the highest relative type I collagen and protein expressions, and SG at second ($P < 0.05$). The relative TGF-β mRNA and protein expressions in SG were lower than those in MG ($P < 0.05$). SG, relative to other two groups displayed a lower postoperative impairment rate ($P < 0.05$). The postoperative ligament stress ability of MG was significantly lower than that of CG and SG ($P < 0.05$). Conclusions: Adipose stem cell transplantation can reduce the damage of rabbit anterior ligament, increase the degree of ligament stress and healing strength by reducing the expression of TGF-β and increasing the expression of type I collagen.

Keywords: Adipose stem cell transplantation, rabbit anterior cruciate ligament injury, TGF-β, type I collagen, healing, mechanism

Introduction

Anterior cruciate ligament (ACL) rupture is a prevalent impairment [1], which is easy to cause instability of knee joint. As a result, articular cartilage or meniscus is injured [2]. ACL injury is induced by many factors, including biomechanical abnormality, neuromuscular abnormality, collagen gene mutation and knee joint main structure. ACL cannot heal spontaneously after injury. The incidence of ACL injury is increasing year by year, and the surgical reconstruction is required [3].

Adipose stem cells (ASCs) are separated from adipose tissues, with self-renewal and multi-direction differentiation abilities, and can be differentiated into cells with the features of hepatocytes, endothelial cells or neurons [4]. Due to its unique properties, it can also enhance the ability of nerve regeneration and restore the repair functions of tissue cells [5]. Adipose stem cells have been widely used in adipogenesis, osteogenesis and chondrogenesis [6-8]. Transforming growth factor-β (TGF-β) plays an important role in embryogenesis and homeostasis of adult tissues by regulating cell differentiation, proliferation, migration and death [9]. A study has shown [10] that the up-regulation of TGF-β expression in New Zealand white rabbits can facilitate tendon bone recovery following ACL reestablishment. Type I collagen is mainly

distributed in tendons, skins, etc. It is reshaped and degraded in tissues and inflammation sites. Therefore, it can chemotactically stimulate the fiber cells *in vivo* and attract these cells to repair damaged tissues [11]. Another study has shown that the expression of type I collagen reflects the healing of ligament injury during ligament injury and healing [12].

Rabbits are the commonly used experimental animals in the study of cruciate ligament injury [13]. This study investigated the therapeutic effect and mechanism of adipose stem cell transplantation on ACL in rabbits. Meanwhile, the effect of TGF-β and type I collagen expressions in ligament tissues was studied.

Materials and methods

Animal data

Materials: 61 New Zealand white rabbits were purchased from the Experimental Animal Center of Zhejiang Academy of Medical Sciences (Hangzhou, China). The weight was 3.0-4.5 kg in average. The rabbits were raised in a clean environment and fed with rabbit food for the SPF experiment. The rabbit food was provided by Jiangsu Xietong Pharmaceutical Bioengineering Co., Ltd. The rabbits were allowed to freely eat and drink. This study has been approved by the Hospital Ethics Committee. The study process obeyed the *Guidelines for the Protection and Use of Experimental Animals* [14].

Separation and identification of rabbit adipose stem cells

(1) Separation of rabbit adipose stem cells: A rabbit was randomly selected to prepare the adipose stem cell suspension required by the experiment. First, intramuscular injection anesthesia was performed with 0.3 ml/kg of Sumianxin II. Then, it was fixed on the anatomical scaffold. Under aseptic conditions, the infrainguinal adipose tissues were taken and placed under the stereo microscope. Then the extraneous tissue surrounding the adipose tissue was removed carefully and washed with PBS for 3-5 times to remove red blood cells (Shanghai Xinfan Biotechnology Co., Ltd., Art. No.: XF-P2999). Then, tissues were cut with scissors (Shanghai Qiansheng Biotechnology Co., Ltd., Art. No.: QS-0064). 3 mL of 0.15% type I collagenase (Shanghai Yanjin Biotechnology Co., Ltd., Art. No.: Js10053-500 mg) was added for mixing and then placed in a carbon dioxide incubator for 1 h of incubation. The suspension was filtered with a 200-mesh screen. The centrifugation was performed at the speed of 1500×*g* for 10 min, and then the supernatant was removed. Resuspension was performed with DMEM medium (Wuhan Chundu Biotechnology Co., Ltd., Art. No.: CD-100042GM). Inoculation was conducted in a culture dish. The morphological features of cells were observed with a microscope.

(2) Identification of adipose stem cells: Cells were observed under microscopes for morphology. When the cell growth reached 80% to 95% confluence, cell pass-generation test was performed for three generations. By the third generation, cell climbing, fixation and PBS rinsing were performed. Primary antibody diluent (Shanghai Hengfei Biotechnology Co., Ltd., Art. No.: A1810) was added. The resulting substance was placed in a wet box at 4°C overnight and then rinsed with PBS before addition of secondary antibody (Shanghai Xitang Biotechnology Co., Ltd., Art. No.: c000) for incubation at 37°C, after which, the mixture was rinsed with PBS and sealed. The test was performed strictly in accordance with the instructions of CD43 immunofluorescence kit (Shanghai Kemin Biotechnology Co., Ltd., Art. No.: IQP-133F). Under fluorescence microscope (Guangzhou Keshite Scientific Instrument Co., Ltd., Art. No.: UMC 800TFL), the result was judged as positive if CD43 immunofluorescence staining was green. After 2 days of culture, cells were subcultured continuously, and the positive CD43 staining was observed to indicate successful culture.

Establishment method and adipose stem cell transplant in the healing model

Sixty New Zealand white rabbits were classified into control group (CG) (n = 20), model group (MG) (n = 20) and study group (SG) (n = 20) according to the random number table. After anesthesia, all the animals were fixed and performed with skin preparation and routine sterilization. After sterilization, the knee joint and ligaments were exposed. In CG, only the ACL was exposed. The incision was sutured then. In MG, 70% of ligament was resected, and the ligament and incision were sutured. In SG, 10 μL of adipose stem cell suspension (cell density: 1×10^6 cells/μL) was injected on the basis of

Table 1. TGF-β, type I collagen and β-actin primer sequence

	Upstream Primer	Downstream Primer
TGF-β	5'-TGCTTCAGCTCCACAGAGAA-3'	5'-TGGTTGTAGAGGGCAAGGAC-3'
Type I collagen	5'-GCGGTGGTTACGACTTTGGTT-3'	5'-AGTGAGGAGGGTCTCAATCTG-3'
β-actin	5'-AGGGAAATCGTGCGTGACAT-3'	5'-GAACCGCTCATTGCCGATAG-3'

Table 2. General conditions of New Zealand white rabbits [n (%)] ($\bar{x}$ ± sd)

Category	Mock Surgical Group (n = 20)	Model Group (n = 20)	Transplant Group (n = 20)	F/χ^2	*P*
Gender				1.616	0.446
Male (%)	11 (55.00)	13 (65.00)	9 (45.00)		
Female (%)	9 (45.00)	7 (35.00)	11 (55.00)		
Week-age (Weeks)	11.78±0.27	11.93±0.25	11.87±0.28	1.600	0.211
Length (cm)	35.11±1.76	35.26±1.79	34.98±1.77	0.125	0.883
Indoor temperature (°C)	24.05±1.24	23.78±1.08	24.25±0.93	0.935	0.398
Indoor humidity (%)	51.52±2.63	50.74±1.93	50.71±1.35	1.015	0.368
Body mass before modeling (g)	221.54±12.35	227.12±14.24	218.36±9.87	2.606	0.082
Body mass after modeling (g)	209.63±7.57	208.53±7.41	206.47±7.82	0.891	0.416

70% of ligament resection, and then the incision was sutured. The success rates of modeling in the three groups were 100%.

Outcome measures

(1) The postoperative ligament injury and post-recovery ligament stress degree in each group were observed and recorded. Rabbits of all the three groups were anesthetized and sacrificed at the 4th week after surgery, and the ligament tissue was removed. The repair of ligament injury was observed visually and stored at -80°C.

(2) Expressions of TGF-β protein and type I collagen in rabbit ACL: After the ACL tissue was taken out at constant temperature, western blot was used for detection. The test was performed in strict accordance with the instructions of TGF-β protein and type I collagen test kits. After determining the protein content, the gel imaging software was applied to analyze the gray value of the protein band, and the gray value of the target protein/internal reference protein was used to indicate the relative protein expression.

(3) Expressions of TGF-β mRNA and type I collagen mRNA: The ligament tissue was removed in advance, and the expression was measured by PCR after grinding. First, TRIzol was used to collect total RNA from the tissue (purchased from Shanghai Xinfan Biotechnology Co., Ltd., Art. No.: XFR1030). Second, reverse transcription kit was used for reverse transcription of the total RNA (Beijing Huada Protein Research and Development Center Co., Ltd., Art. No.: BPI01030). The reaction system: 10 μL. The reaction conditions: at 37°C for 20 min and at 80°C for 5 s. β-actin was used as the internal reference. The reaction system: 25 μL. The reaction conditions: at 95°C, predegeneration for 3 min, degeneration at 95°C for 35 s, annealing at 58°C for 35 s, extending at 73°C for 45 s, annealing and extending for 40 cycles, and extending at 73°C for 5 min. $2^{-\Delta\Delta Ct}$ method was adopted to calculate the relative expression of TGF-β and type I collagen mRNA (**Table 1**).

Statistical method

The statistical analysis was implemented by SPSS19.0 (Yiyun (Shanghai) Information Technology Co., Ltd.). The measurement data were represented by mean ± standard deviation ($\bar{x}$ ± SD). Chi-square test was applied to analyze the enumeration data among the groups. One-way analysis of variance (one-way ANOVA) was adopted to compare the mean values among groups. $P < 0.05$ was considered as significant difference.

Results

General information

There was no difference in gender, week age, length, indoor temperature, indoor humidity,

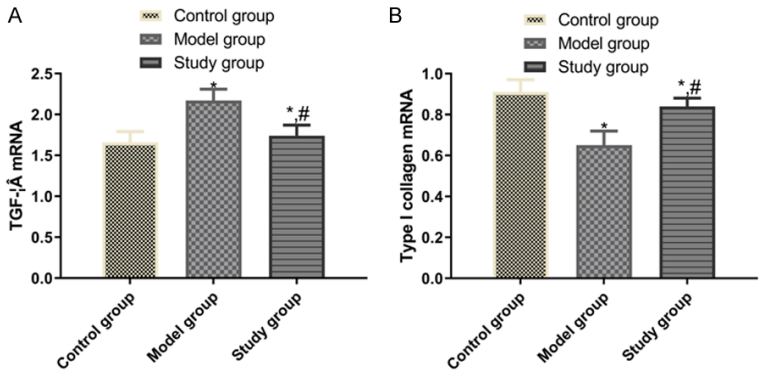

Figure 1. Comparison of the relative expression of TGF-β and type I collagen mRNA. The relative expression of TGF-β mRNA in model group (MG) and study group (SG) was higher than that in control group (CG) ($P < 0.05$). The relative expression of TGF-β mRNA in SG was lower than that in MG ($P < 0.05$) (A). The relative expression of type I collagen mRNA in MG and SG was lower than that in CG ($P < 0.05$). However, the relative expression of type I collagen mRNA in SG was higher than that in MG ($P < 0.05$) (B). Note: Compared with CG, $^{*}P < 0.05$. Compared with MG, $^{\#}P < 0.05$.

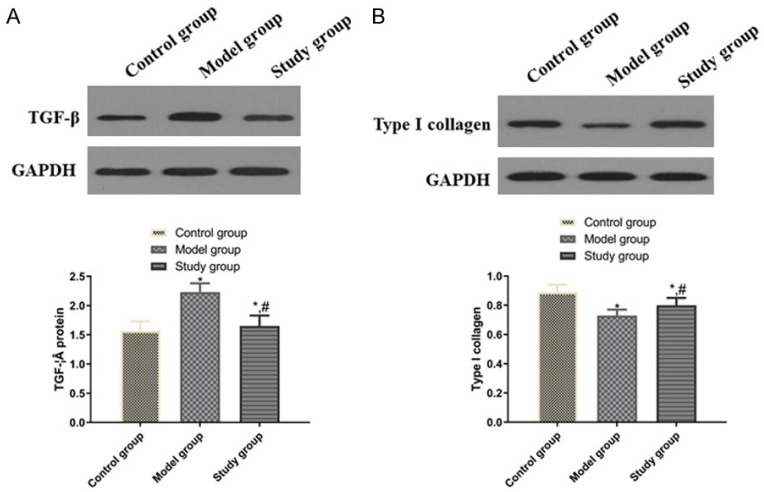

Figure 2. Comparison of the relative expression of TGF-β and type I collagen proteins. The relative expression of TGF-β protein in MG and SG was higher than that in CG ($P < 0.05$). The relative expression of TGF-β protein in in SG was lower than that in MG ($P < 0.05$) (A). The relative expression of type I collagen protein in MG and SG was lower than that in CG ($P < 0.05$). However, the relative expression of type I collagen protein in SG was higher than that in MG ($P < 0.05$) (B). Note: Compared to CG, $^{*}P < 0.05$. Compared to MG, $^{\#}P < 0.05$.

body mass before modeling or body mass after modeling ($P > 0.05$) (**Table 2**).

Comparison of the relative expressions of TGF-β mRNA and type I collagen mRNA

The relative expressions of TGF-β mRNA and type I collagen mRNA were detected among the three groups, and differences were found, which had statistical significance ($P < 0.05$). The relative expression of TGF-β mRNA of MG was higher than that of CG and SG, while CG had lower relative expression of TGF-β mRNA than SG ($P < 0.05$). The relative expression of type I collagen of MG was lower than that of MG and SG, while CG had higher relative expression of type I collagen than SG ($P < 0.05$) (**Figure 1**).

Comparison of the relative expressions of TGF-β protein and type I collagen protein

The relative expressions of TGF-β protein and type I collagen protein were detected among the three groups, and differences were found, which had statistical significance ($P < 0.05$). The relative expression of TGF-β protein of MG and SG was higher than that of CG ($P < 0.05$), and SG had lower relative expression than MG ($P < 0.05$). The relative expression of type I collagen protein of MG and SG was lower than that of CG ($P < 0.05$), and SG had higher relative expression than MG ($P < 0.05$) (**Figure 2**).

Comparison of postoperative injury

In MSG, there was 1 case of eating less (5.00%), 1 case of drinking less (5.00%), 2 cases of poor mental state, 0 case of lusterless fur, and 1 case of movement disorder (5.00%). The incidence of total injury was 25.00%. In MG, there were 2 cases of eating less (10.00%), 1 case of drinking less (5.00%), 3 cases of poor mental state (15.00%), 2 cases of lusterless fur (10.00%) and 2 cases of movement disorder. The incidence of total injury was 60.00%. In SG, there was 1 case of eating less (5.00%), 1 case of drinking less (5.00%), 1 case of poor mental state (5.00%) and 0 case of lusterless fur and

Table 3. Comparison of postoperative injury [n (%)]

Category	Mock Surgical Group (n = 20)	Model Group (n = 20)	Transplant Group (n = 20)	χ^2 value	*P* value
Eating less	1 (5.00)	2 (10.00)	1 (5.00)	-	-
Drinking less	1 (5.00)	1 (5.00)	1 (5.00)	-	-
Poor mental state	2 (10.00)	3 (15.00)	1 (5.00)	-	-
Lusterless fur	0 (0.00)	2 (10.00)	0 (0.00)	-	-
Movement disorder	1 (5.00)	2 (10.00)	0 (0.00)	-	-
Total injury	5 (25.00)	10 (60.00)	3 (15.00)	6.190	0.045

Table 4. Comparison of postoperative ligament stress (Mean ± SD)

Category	Mock surgical group (n = 20)	Model group (n = 20)	Transplant group (n = 20)	F	*P*
Ligament maximum load (N)	301.33±10.56	286.13±10.28	304.71±10.57	17.870	< 0.001
Maximum tensile length (mm)	3.15±0.17	3.02±0.12	3.13±0.14	4.674	0.013
Ligament hardness (N/mm)	165.89±8.57	156.26±8.47	162.76±8.51	6.653	0.003
Maximum load capacity (N·m)	0.43±0.06	0.38±0.06	0.42±0.05	4.330	0.018

movement disorder. The incidence of total injury was 15.00%. The postoperative injury of SG was significantly lower than that of CG and MG ($P < 0.05$) (**Table 3**).

Comparison of postoperative ligament stress

There was significant difference in postoperative ligament stress among the three groups ($P < 0.05$). The ligament maximum load, maximum tensile length, hardness and maximum load capacity of MG were lower than those of CG and SG ($P < 0.05$). No significant difference was observed between CG and SG ($P > 0.05$) (**Table 4**).

Discussion

ACL is one of the most extensively studied structures in human muscular and skeletal system [15]. The ligament is mainly composed of fibroblasts (in ligament extracellular matrix and matrix). Fibroblasts play a major role in ligament repair after ligament injury and in the healing process of ligament injury [16]. ACL injury may be devastating and lead to sequelae of complications. [17]. It causes chronic pain and has adverse effect on long-term health [18]. In this study, adipose stem cells were transplanted into the injured rabbit anterior ligament. The healing condition and mechanism of ligament injury were observed. Thus, it provides a theoretical basis for effective healing of ACL injury in rabbits.

Adipose stem cells promote the healing ability by in situ differentiation and paracrine factor secretion. It has a great prospect in the application of regenerative medicine [4, 19]. A series of paracrine factors secreted by adipose stem cells can also promote the formation of new blood vessels, reduce apoptosis and inhibit fibrosis [20]. The activity of TGF-β can inhibit the degeneration of articular cartilage [21]. Previous studies have shown [22, 23] that a large amount of TGF-β is released at the wound site after ligament injury to inhibit the ligament healing and scar maturation. Type I collagen accounts for 80% in ligament, indicating its importance in ligaments and significance in improving the tension of the ligament tissues [24, 25]. The study of Kew *et al*. has shown [26] that the adipose stem cell group showed less inflammation in the rat model. It may lead to more elastic repair to tendons/ligaments and skin and less scar healing. The analysis results of TGF-β mRNA and TGF-β protein expressions in the three groups of this study showed that the relative expressions of TGF-β mRNA and TGF-β protein in CG were lower than those in MG and SG.

The comparison between MG and SG revealed that SG experienced a remarkable reduction in the relative expressions of TGF-β mRNA and TGF-β protein than the MG, indicating that reduction of expression of TGF-β in tissues can improve the rabbit anterior ligament.

Furthermore, the analysis of type I collagen mRNA and protein indicated that, in CG, the relative expressions were significantly elevated as compared with those in MG and SG while a comparative study between MG and SG found a sharp increase in the relative expression of type I collagen mRNA and protein in SG, indicating that the raised expression of type I collagen in tissues contributes to the repair of rabbit anterior ligament injury. The postoperative injury in SG was remarkably lower than that in CG and MG, which implied that the transplantation of adipose stem cells can better repair the rabbit ACL injury and improve the healing strength.

The healing degrees were different among the three groups. The ligament maximum load, maximum tensile length, the hardness of ligament and maximum load capacity in MG were significantly lower than those in CG and SG. However, no significant difference was observed between CG and SG. The results showed that the strength in MG significantly decreased during the plastic regeneration. However, the healing degree in SG can recover to a similar degree as compared with CG, which indicated that adipose stem cell transplantation had a better effect on repair of the rabbit anterior ligament injury. We know that ADSCs can secrete a certain amount of cytokines. Cytokines with higher expression levels include hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), placental growth factor (PGF), and transforming growth factor-p; Cytokines with moderate expression levels include fibroblast growth factor-2 (FGF-2) and angiopoietin-1 (Ang-1); Cytokines with lower expression levels have angiopoietin-2 (Ang-2). Cytokines maybe conducive to the establishment of a better damage repair microenvironment.

Inclusion, adipose stem cell transplantation can reduce the damage of rabbit anterior ligament, and increase the degree of ligament stress and healing strength by reducing the expression of TGF-β and increasing the expression of type I collagen.

Disclosure of conflict of interest

None.

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