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Original Article

Effect of transplantation of autologous bone marrow mesenchymal stem cells on articular cartilage injuries and its effect on serum u-PA and TGF-p in rabbits

Guangyang Yu[1], Na Luo[2]

[1]*Department of Orthopaedics, The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University, Huaian 223300, Jiangsu Province, China;* [2]*Department of Rheumatology and Immunology, The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University, Huaian 223300, Jiangsu Province, China*

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Abstract: Objective: This study intended to explore the effects and related mechanisms of autologous bone marrow mesenchymal stem cells (BMSC) on the repair and treatment of rabbit knee articular cartilage injuries. Methods: Thirty male New Zealand White Rabbits were chosen to establish the knee joint model of articular cartilage injury, animals were randomly assigned to the BMSC group, the model control group and the blank control group, with 10 rabbits in each group. The BMSC group was treated with BMSCs cell solution. The model control group was injected with 0.6 ml of saline. The blank control group was not intervened with. After 4 weeks of treatment, the status of repair in articular cartilage tissue of each group was evaluated and compared with semi-quantitative scoring. The morphological characteristics of articular cartilage were observed after H&E staining. Immunohistochemistry was used to detect the expression of urokinase-type plasminogen activator (u-PA) and transfer growth factor-p (TGF-p) in cartilage tissue. Results: Compared with the model control group, the BMSC group had better cartilage repair as well as higher scores in cell morphology, matrix staining, surface regularity, and cartilage thickness ($P<0.05$). The expression of u-PA and TGF-p in cartilage tissue of the BMSC group was decreased compared with that of the model control group ($P<0.05$). Conclusion: BMSCs have a positive effect on knee articular cartilage repair, which can significantly improve cell morphology, cartilage thickness and other indicators, and also reduces the levels of serum u-PA and TGF-p in rabbits. The reason may be that BMSC can be induced to differentiate into hyaline cartilage tissue, promoting the recovery of the injured area.

Keywords: Bone marrow mesenchymal stem cells, transplantation, cartilage injury, u-PA, TGF-p

Introduction

In recent years, with the improvement of the health awareness of Chinese residents, fitness activities have gradually become an important part daily life, as well as a nationwide body-building program. While exercise improves the fitness level, the incidence of sports injuries has also been increasing annually. The knee joint is the largest and most complex joint in the human body, and it is also an important weight-bearing joint that we rely on every day. Data shows that knee joint injury is commonly diagnosed in orthopedics, which seriously impacts the normal life of people [1, 2].

Cartilage damage is a common injury type in the knee joint, accounting for about 25% to 50% of knee injuries. Patients with cartilage injury often have clinical manifestations such as decreased knee stability, pain, and joint swelling. The symptoms are usually subtle, leading to misdiagnosis and delayed treatment [3-5]. The joint cavity of the patients is prone to inflammatory lesions, and the blood supply to the injured area is reduced, so the cartilage will be unlikely to heal well without medical intervention. The main treatment option for cartilage injury is surgical reconstruction. However, surgery is invasive, and postoperative complications may occur. On the other hand, due to various surgical factors such as blood supply restoration of the reconstructed tendon and inflammatory responses, *etc*., surgery has a long recovery time. So the current

research focu is on a safer, quicker and more effective intervention [6, 7].

Bone marrow mesenchymal stem cells (BMSCs) are a type of stem cells with high differentiation potential and self-renewal capacity found in mammalian bone marrow stroma, which can be differentiated into bone, cartilage, fat, neural and myoblast cells and other subpopulations [8, 9]. BMSCs are widely present in connective tissues and interstitial tissues, but are mainly distributed in bone marrow tissues. Such cells can not only produce mechanical support for hematopoietic stem cells, but also secrete growth factors to support the hematopoietic function. There have been many studies on BMSCs in recent years. A foreign study has pointed out that after intervention of BMSCs in rats with anterior cruciate ligament injury, the ligament injury of rats was significantly improved, and the biomechanical experiments showed that the load of the ligaments was significantly increased [10]. Another study has found that it is feasible to use autologous bone marrow MSC transplantation to repair the cartilage injury of the knee joint of rabbits, and limb function of experimental rabbits has been significantly improved after intervention [11]. Some studies have pointed out that by adding the osteogenic inducer dexamethasone to the culture medium, the third generation cells will be identified as having osteogenic properties. In addition, studies have found that the traditional Chinese medicine, epimedium stimulates the differentiation of BMSCs into osteoblasts. The highly differentiated characteristics of these cells have been used in the treatment of various clinical diseases [12, 13]. This study aimed to evaluate the effect of autologous BMSC transplantation in the treatment of cartilage injury by establishing a rabbit model of cartilage injury, and analyzed the effect of treatment on serum urokinase-type plasminogen activator (u-PA) and transfer growth factor-p (TGF-p) concentrations.

Materials and methods

Animal sourcing

Thirty male New Zealand white rabbits (Shanghai Jiagan Biotechnology Co., Ltd.) were selected and randomly assigned to the BMSC group, the model control group and the blank control group according to a random number table method, with 10 rabbits in each group. In the BMSC group, the rabbits were aged 11-12 months, with an average age of (11.28±0.34) months, weighed 3.1-3.9 kg, and an average body weight of (3.51±0.14) kg. In the model control group, the rabbits were aged 10-12 months, with an average age of (11.19±0.41) months, weighed 3.2-3.9 kg, and an average weight of (3.52±0.15) kg. In the blank control group, the rabbits were aged 11-12 months, with an average age of (11.21±0.39) months and an average weight of (3.49±0.21) kg.

Reagents and instruments

Rabbit anti-rat u-PA antibody (Thermo Fisher), mouse anti-rat TGF-p antibody (Abcam, UK), ELISA kit (Shanghai Enzyme Biotechnology Co., Ltd.), Biological microscope (Beijing Xiewei Photoelectric Instrument Factory), low-temperature high-speed centrifuge (Beckman Coulter).

Experimental method

Animal feeding: Under a temperature of 22-25°C and humidity of 35%-40%, the rabbits were fed with pelleted animal feed. During the breeding process, the breeding cages were regularly disinfected. The limbs of the white rabbits were examined to ensure that there was no joint swelling and deformity before modeling, followed by a drawer test on knee joints. The operation of experiments preformed on animals conforms to the standards of animal ethics and was approved by the Hospital Ethics Society.

Cartilage injury model establishment: Each rabbit in the three groups of white rabbits were restrained, and general anesthesia was administered by intravenous injection into an ear vein. The right forelimb joints of the white rabbits were depilated, and penicillin was injected intramuscularly to prevent infection. A longitudinal incision was made on the inside edge of the patella of the knee joint. After exposing the articular surface, a 3 mm hole was drilled through the medial surface of the medial condyle to make full-thickness cartilage defects, which was enveloped by the periosteum in the upper extremity of the tibia. After the successful establishment of the model, the wound was sutured and treated with

antibacterial agents. There was no death or infection during modeling.

Preparation of rabbit autologous BMSC: Five ml of rabbit bone marrow was extracted under sterile conditions, centrifuged at 2000 r/min, followed by inoculation at 37°C under 5% CO_2. When the confluence reached 90%, the cells were decomposed with pancreatic proteases, and transferred into bioprotein glue containing fibrin and thrombin. The third-generation BMSC cells were collected and centrifuged to obtain the cell mass, which was mixed with fibrinogen as the intervention drug. In the BMSC group, 1 mL of BMSCs was injected into the tibial and femoral tunnels while 0.6 ml of normal saline was injected in the model control group. The blank control group was not intervened upon. The operation was performed once a week for 4 weeks. Rabbits were fed normally during intervention.

Sample retention and collection: After the successful establishment of the mode and 1 week, 2 weeks and 4 weeks after the last injection, blood samples were taken from the veins of fasting animals and centrifuged at 3000 r/min to obtain the supernatant. The three groups of white rabbits were killed after collection of blood samples, and the right front knee joint was taken, wrapped with normal saline for testing.

Observation indicators

Cartilage repair evaluation: The right knee anterior joint tissues were fixed, trimmed and cleaned, and the cartilage tissue morphology was observed after H&E staining to evaluate the repair of cartilage which was classified as complete healing, incomplete healing and no healing. Complete healing was defined as normal chondrocyte differentiation in the defect area, with smooth tissue surface, tightly coupled with the surrounding tissues. Incomplete healing referred to the poor differentiation of the chondrocytes in the defect area, containing immature cartilage cells which were not arranged neatly and the surface of the new tissue was rough, and their connection with the surrounding tissue was not tight. No healing meant that there was only a small amount of cartilage tissue in the defect area, with sunken defect surface, with poor or no adhesive bonding to surrounding tissue. Healing rate = (complete healing + incomplete healing)/total number of cases × 100%.

Semi-quantitative scoring for cartilage repair: The modified pineda's semi-quantitative scale was used to evaluate the cartilage repair after intervention in the three groups. The scale included cell morphology (0-4 points), matrix staining (0-3 points), surface regularity (0-3 points), cartilage thickness (0-2 points), and degree of integration with surrounding tissues (0-2 points), with a total score of 14 points. The higher score indicates worse cartilage repair [14].

Detection of serum u-PA and TGF-p levels: The serum u-PA concentration was detected with the SABC method, and the TGF-p concentration was detected by enzyme-linked immunosorbent assay (ELISA). Each index was tested three times in succession, and the average value was calculated as the final results.

Statistical analysis

SPSS 220.0 was used for statistical analysis. Measurement data were expressed as ($\bar{x} \pm s$) and were tested by independent sample t. Count data were expressed as [n (%)] and were tested by independent sample chi-square test. F test was used to compare the differences among groups. $P<0.05$ indicated significant differences [15].

Results

Comparison of cartilage repair

In the BMSC group, there were 5 cases of complete healing, 4 cases of incomplete healing, and 1 case of no healing, with a healing rate of 90.00%. In the model control group, there were 0 case of complete healing, 4 cases of incomplete healing, and 6 cases of no healing, with a healing rate of 40.00%. There was no change in the blank control group and the healing rate was 0.00%. There was significant difference in healing rate among the three groups ($P<0.05$) (**Table 1**).

Comparison of semi-quantitative scoring of cartilage repair

The scores of cell morphology, matrix staining, surface regularity, cartilage thickness and degree of integration with surrounding tissues

Table 1. Comparison of cartilage repair [n (%)]

Group	Number of cases	Complete healing	Incomplete healing	Non-healing	Healing rate
BMSC group	10	5 (50.00)	4 (40.00)	1 (10.00)	9 (90.00)
Model control group	10	0 (0.00)	4 (40.00)	6 (60.00)	4 (40.00)
Blank control group	10	10 (100.00)	0 (0.00)	0 (0.00)	10 (100.00)
F	-	-	-	-	5.495
P	-	-	-	-	0.019

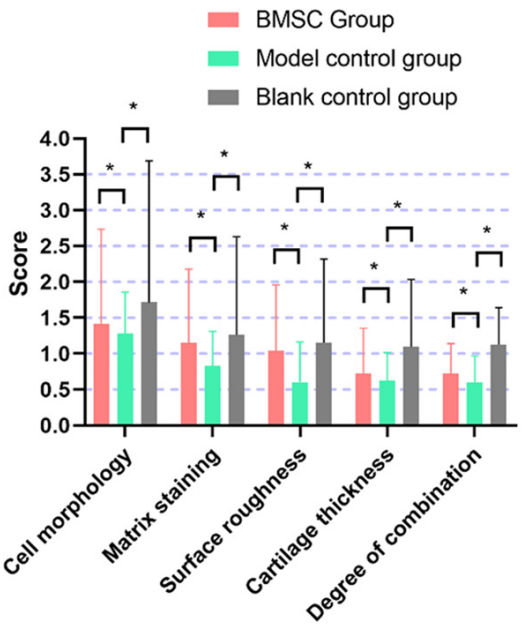

Figure 1. Comparison of the semi-quantitative scoring of cartilage repair among the three groups of white rabbits. The difference was statistically significant ($P<0.05$). *indicates that the difference in the same index between the groups is statistically significant.

in the BMSC group were significantly higher than those in the model control group but lower than those in the blank control group ($P<0.05$) (**Figures 1-3**).

Comparison of u-PA level between the groups

After the successful establishment of the model, the serum u-PA levels of the BMSC group and the model control group were higher than those of the blank control group ($P<0.05$), but no significant difference was found between the model control group and the BMSC group ($P>0.05$). The u-PA level in the BMSC group was lower than that in the model control group after 1, 2 and 4 weeks of intervention ($P<0.05$) (**Figure 4**).

Comparison of TGF-p levels at multiple time points

After the successful establishment of the model, there was no statistically significant difference in serum TGF-p level between the model control and BMSC groups ($P>0.05$). However, after 1, 2 and 4 weeks of intervention, the serum TGF-p level of the BMSC group was gradually decreased after intervention ($P<0.05$) and approached the normal level, while that in the model control group showed little change (**Figure 5**).

Discussion

Articular cartilage is a thin layer of hyaline cartilage on the surface of the connected bones in the joint, generally in the form of hyaline cartilage or fibrous cartilage. Its main function is to alleviate pressure and reduce frictional resistance to joint movement. If the cartilage is damaged, clinical symptoms such as joint pain, swelling, restricted mobility, and tenderness will generally appear, and some patients will also show decreased joint stability [16, 17].

Clinical research shows that knee cartilage injury is a common type of cartilage injury due to long-term compression under high-load exercise. In addition, the high frequency of knee joint movement increases friction, leading to cartilage damage [18-20]. Studies have found that cartilage, which is mainly composed of collagen and proteoglycans, is a connective tissue lacking blood vessels, nerves and a lymphatic system. Once damage occurs, it is difficult to heal. Therefore, if timely intervention is not performed to the defective cartilage, it is likely to cause more serious sequelae [21, 22]. There are many treatments to repair cartilage defects, including microfractures, osteochondral transplantation, chondrocyte transplantation, and cell-free scaffold materials. However, clinical practice shows that the abo-

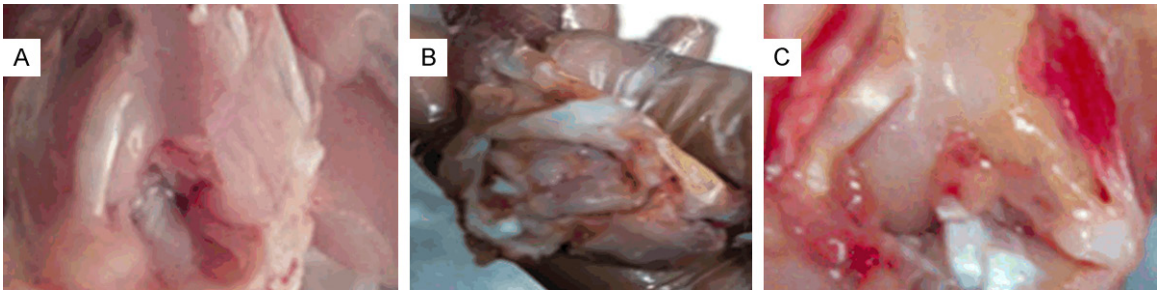

Figure 2. Articular cartilage characters after intervention in the three groups of rabbits. The knee cartilage of rabbits in the blank control group was intact (A); the knee cartilage of rabbits in the model control group was obviously damaged (B); compared with the model control group, the knee cartilage of rabbits in the BMSC group showed significant recovery (C).

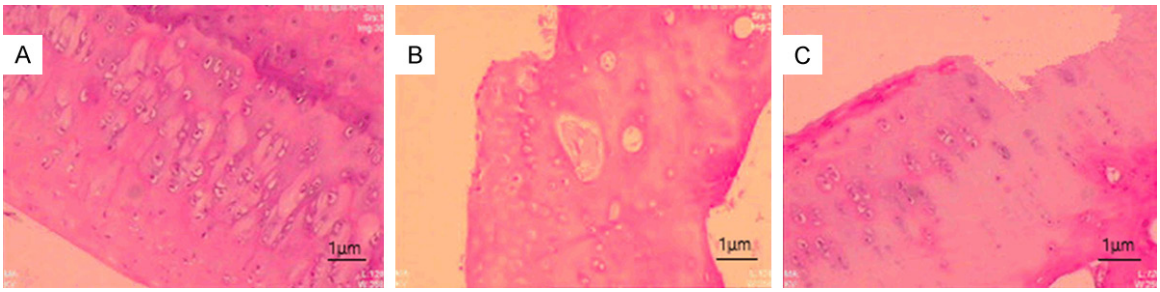

Figure 3. H&E staining after intervention in the three groups of rabbits. In the blank control group, chondrocytes can be seen in regular and dense arrangement (A); in the model control group, chondrocyte defect is severe (B); chondrocytes of the white rabbits in the BMSC group were significantly improved compared with those in the model control group (C).

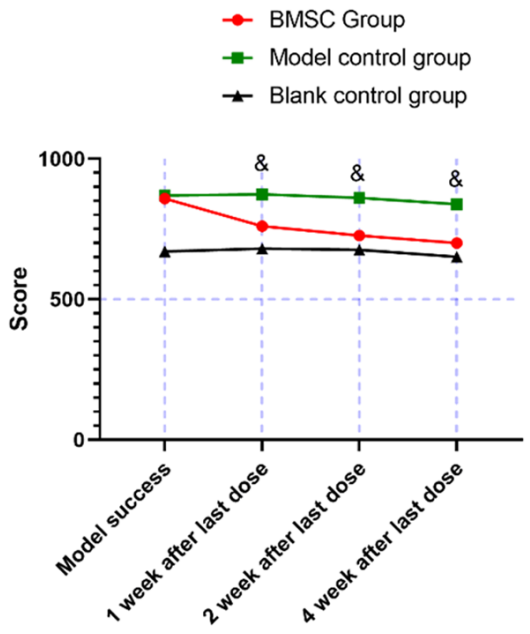

Figure 4. Changes in serum u-PA levels. & represents that the difference in the same index between the groups was statistically significant.

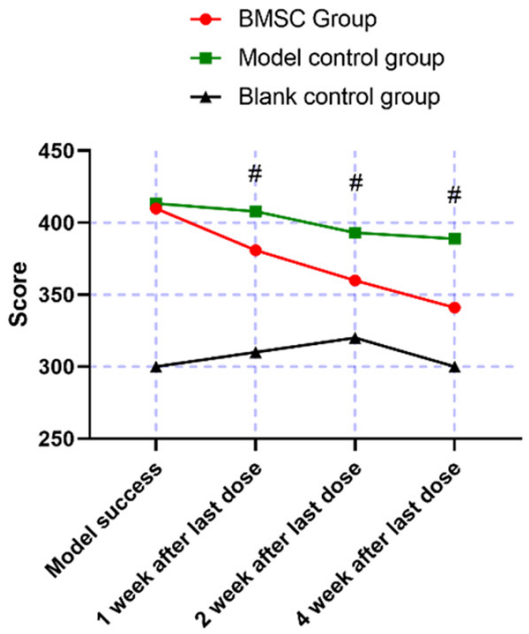

Figure 5. Comparison of serum TGF-p levels. # indicates that the difference in the same index between the groups was statistically significant.

ve treatment options have certain limitations, and the treatment efficacy is limited [23].

In addition to a large volume of hematopoietic stem cells, bone marrow tissue also contains a kind of stem cell that can differentiate into bone marrow interstitial components, namely BMSC cells. BMSCs are undifferentiated primitive pluripotent cells that were retained during embryonic development, and they have the ability of long-term renewal and multidirectional differentiation [24]. In recent years, the differentiation and growth characteristics of BMSCs have been gradually recognized for their importance in clinical practice, and have been used in tissue and functional reconstruction. Studies have pointed out that BMSC transplantation can promote cartilage recovery in the treatment of knee articular cartilage injury, which can promote the regeneration of knee joint cartilage and provide a new way for repair of knee joint injury [25].

In this study, the effect of BMSCs in repairing cartilage injury was explored by setting up controls. The results showed that the healing rate of the BMSC group and the model control group was 90.00% and 40.00% respectively. A study has pointed out that reduced blood supply to the cartilage will result in slower differentiation and growth speed of stem cells. Therefore, the cartilage is difficult to heal after injury. However, injection of BMSCs into the injury area can significantly promote formation of type I and type II collagen, accelerate the healing of the injured area, and increase the ultimate failure load of cartilage [26]. Another study has found that compared with repair using high-density collagen gel, BMSC can better repair cartilage tissue and form a tissue structure similar to the original cartilage, and the newly formed cartilage has better biological characteristics [27]. The results of the above studies are basically similar to the conclusions of this study. In this study, the BSMC group scored higher than the model control group in terms of cell morphology, matrix staining, surface regularity, cartilage thickness, and degree of integration to surrounding tissues. The stained tissues revealed that surface with poor flatness, low volume of fibrous tissue filling defects were observed in the model control group, which was opposite to the condition in the BMSC group, suggesting that BMSC can quickly repair damaged cartilage tissue, and restore joint function as soon as possible.

Finally, this study also analyzed the effect of intervention on the levels of serum u-PA and TGF-p. u-PA belongs to the serine proteases family and promotes the conversion of plasminogen to plasmin. It plays an important role in various processes such as degradation of the extracellular matrix, inflammatory response, cell migration, malignant cell proliferation, and tissue repair. Some studies have found that u-PA is highly expressed in the knee joint fluid of patients with cartilage injury. The reason may be that the microenvironment in the joint cavity has changed following cartilage injury, resulting in hyperplasia of synovial tissue and abnormally increased levels of u-PA, which negatively impacts cartilage repair. Some studies have pointed out that by reducing the level of u-PA in the joint cavity, the inflammatory state of the joint cavity was effectively improved and the repair of cartilage was accelerated. TGF-p could induce cell mitosis and accelerate the differentiation and maturation of cells. At the same time, it is also widely involved in the body's immune response and the repair of damaged tissues. Some studies have confirmed that when individuals have cartilage damage, TGF-p levels in their joint cavity will also present a high expression state. The reason may be that high expression of TGF-p could promote cartilage repair. In this study, the levels of u-PA and TGF-p in the BMSC group were decreased significantly with the extension of the intervention time. The reason may be that the joint is in the inflammatory state upon initial injury, and with the remission of inflammation and the acceleration of tissue repair, u-PA and TGF-p levels will decrease, which also supports the speculation that BMSC could accelerate the repair of cartilage damage.

In summary, BMSC can accelerate the repair of articular cartilage injury, and they have a positive effect in the recovery of joint function. Meanwhile, BMSC also can alleviate the inflammation state of the injury area, which provides a certain theoretical reference for the repair of human knee joint injury. This study has the following shortcomings: (1) The number of animals included in the study is small, and all

of them were adult white rabbits; (2) Only short-term intervention effects were evaluated. A study with a larger sample size and longer follow-up is planned to provide more detailed experimental data.

Disclosure of conflict of interest

None.

Address correspondence to: Na Luo, Department of Rheumatology and Immunology, The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University, No. 1 Huanghe West Road, Huaiyin District, Huaian 223300, Jiangsu Province, China. Tel: +86-14762414787; E-mail: luonnaya@163.com

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