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Effect of mesenchymal stem cells on biomechanics of stress fracture rabbit model after healing

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Abstract: This study aimed to investigate the effects of mesenchymal stem cells (MSCs) on the biomechanics of stress-fractured rabbits after healing. Twenty-Four New Zealand white rabbits were selected to establish a stress fracture model. All rabbits were randomly divided into model group and MSCs group, and another 12 rabbits were chosen as control group. Compared with the control group, the mRNA expression levels of basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF), osteoprotegerin (OPG) and receptor activator of nuclear factor-κ B ligand (RANKL) in the model group decreased significantly, and the OPG/RANKL ratio decreased (p<0.05). Compared with the model group, the mRNA expression levels of b-FGF, VEGF, OPG and RANKL in the MSCs group were significantly increased, and the OPG/RANKL ratio increased (p<0.05). Compared with the model group, the healing and bone mineral density in the MSCs group increased significantly (p<0.05), the biomechanical parameters (maximum load, elastic load, stiffness and maximum deflection) of the rabbits in the MSCs group were significantly improved (p<0.05). In conclusion, MSCs can significantly improve the bone mineral density of stress-fractured white rabbits; promote the release of bone formation factors and fracture healing, which can provide guidance for clinical treatment of stress fractures.

Keywords: Bone marrow mesenchymal stem cells, stress fracture, bone mineral density, biomechanics.

INTRODCTION

Under normal physiological conditions, the osteogenic repair and reconstruction of the human skeleton and the osteogenic absorption are in a dynamic equilibrium. But when the external force below the strength of the bone acts permanently on the bone, it may cause minor damage to the bone or absorptive necrosis. If this force persists for a long time and accumulates to a certain extent, the body would be unable to repair, resulting in incomplete or complete fracture. This type of fracture is called stress fracture, and it is also called a march fracture or fatigue fracture (Tenforde *et al.*, 2010; Moreira and Bilezikian, 2017). This type of fracture is most common in military training or sports training. Stress fractures, if not treated properly or delayed, can lead to chronic pain, decreased mobility and muscle atrophy, so stress fracture treatment has become a hotspot and difficult point for clinical treatment (Matcuk *et al.*, 2016; Welck *et al.*, 2017). Mesenchymal stem cells (MSCs) are pluripotent stem cells, which have the commonalities of stem cells, including self-renewal and multi-directional differentiation (Spees *et al.*, 2016; Fazeli *et al.*, 2018). Current studies have shown that MSCs can migrate to different tissue sites, and can induce differentiation into multi-potential cell subsets in bone, cartilage, fat, nerve, and myoblast cells in a specific environment. It has important clinical significance for body tissue repair (Mao *et al.*, 2017; Moreira *et al.*, 2017). MSCs are clinically applied to solve a variety of hematological diseases, cardiovascular diseases, nervous system diseases, liver cirrhosis, partial meniscus resection injury repair and autoimmune diseases, and have made major breakthroughs to save more patients from sufferings (Wang *et al.*, 2013; Knapik *et al.*, 2017; Bedini *et al.*, 2018; Cao *et al.*, 2020). In this study, a rabbit stress fracture model was used to analyze the therapeutic effect of MSCs on stress fractures, aiming to provide important theoretical basis for further clinical treatment.

MATERIALS AND METHODS

Preparation of MSCs

Thirty six male New Zealand white rabbits weighing (2.01±0.42) kg were purchased from Shanghai Slack Laboratory Animals Co., Ltd., and the white rabbits were kept in SPF animal room at 20-26^{o}C with relative humidity of 40-60%, and 12 hours bright/12 hours dark. The white rabbits were kept in separate cages with standard feed and free access to water. This study was approved by the Experimental Animal Ethics Committee of Xinxiang Medical University, with the approval number: HNXX-2018-002. Animal surgery and post-processing were performed in accordance with the standard procedures. Two rabbits were sacrificed after anesthesia. The tibia and femur of the white rabbits were taken, and the muscles and connective tissues were separated under aseptic conditions to expose the tibia and femur. The femur and the tibia was injected with a syringe filled with phosphate buffered saline (PBS), a cell suspension was prepared by pushing the syringe to blow out the bone marrow cells, and then the cells were separated with lymphocyte separation solution. The white floc cell fraction was washed 3 times with PBS, then

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resuspended in 5 ml of DMEM medium, uniformly blown, and inoculated into a culture flask. When the cells were grown to a density of 90%, the medium was aspirated, washed with PBS twice, then 2 ml of 0.25% trypsin was added and placed in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) for 2 min, then stopped in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA), and passaged three times for subsequent experiments.

Identification of MSCs

The digested MSCs cells were collected and the cell density was adjusted to 1×10^6/ml, 1 ml per tube, divided into 3 groups, each group was washed 3 times with PBS, (1)no antibody was added; (2)5μl CD44-FITC was added; (3)5μl CD45-FITC was added. The three groups of cells were incubated at 4 °C for 1 h, and then washed 3 times with PBS for 5 min. Cells were resuspended in 400 μl of PBS for flow cytometry analysis.

Construction of stress-fracture white rabbit model

36 New Zealand white rabbits were adapted to the environment for 1 week before the experiment. The model group and MSCs group were trained to run and jump in the electric stimulation cage. High-voltage (10 kV), low-current (2 to 3 vA) electric shocks were used to make animals run and jump. The electric shock time was 0.12-0.14 s, 10 times/min. The effective number of running and jumping was about 300 times per day, and the training was 6 days in one week. At other times, the rabbits were kept in the same cage as the control group, and had free access to food and drink. In the MSC group, rabbits in the MSC group were injected with 100 μl (1×10^7/ml) of MSC cells into the bone marrow cavity in the tibia using a needle of 1 ml syringe at the sixth week. The control group and model group were injected with an equal volume of PBS buffer in the same way. The three groups of inoculated white rabbits all had free access to water and food, and tissues from the fracture site were taken 6 weeks after treatment for follow-up experiments.

Treatment of the fracture model

In the MSC group, the rabbits were injected into the medullary cavity with a 1 ml needle syringe with 100 μl (1×10^7/ml) of MSC cells injected into the tibia at the 6^{th} week after modeling. The sham operation group and model group were injected with an equal volume of PBS buffer in the same manner. The three groups of white rabbits inoculated were given free access to water and diet, and the tissues of the fracture site were taken 6 weeks after treatment for subsequent trials.

Detection of cytokine mRNA levels: Fluorescence quantitative PCR was used to analyze the cytokine mRNA levels in the fracture sites of three groups. Three groups of white rabbits were anesthetized, sacrificed, and the tibia and surrounding tissues were taken. Total RNA was extracted and reverse-transcribed into cDNA using reverse transcription kit (Vazyme, Nanjing, China). Rabbit-derived b-FGF, VEGF, OPG and RANKL were provided according to the gene database. β-actin was used as internal control. The fluorescent quantitative PCR primer sequences are shown in table 1. The primer concentration was diluted to 10 pmol/L, cDNA 1; 100 dilution, and the following quantitative PCR reaction system was configured: 20 μl of total reaction system, of which 2×SybGreen mix (Vazyme, Nanjing, China) 10 μl; upstream primer 0.6 μl, downstream primer 0.6 μl, cDNA template (1:100) 8.8 μl. PCR was carried out according to the following reaction conditions: pre-denaturation: 94 °C, 5 min; denaturation: 94 °C, 1 min; annealing: 58 °C, 30 s; extension: 72 °C, 1 min, a total of 39 cycles, and a melting curve was established. The experimental results were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), all data were directly read from a real-time PCR instrument (Applied Biosystems, Foster City, CA, USA).

Fracture healing and callus density analysis

After 8 weeks of treatment, the healing of left tibia fracture was analyzed by X-ray lateral radiograph (Shenghong Medical Instrument Co., Ltd, Tianjin, China). The fracture healing was compared according to X-ray score: no abnormality was 0 point. Blurred edge and reduced density was 1 point; acute periosteal reaction and different density with other cortical bones were 2 points; translucent area appeared in the cortex appears and unidentified lesions in the pain site were 3 points; the occurrences of fracture line were 4 points. The X-ray was judged by 3 professionals and averaged as the fracture healing score. The bone mineral density was measured using a dual-energy X-ray absorptiometry apparatus (Faxitron, Tucson, AZ, USA).

Three-point bending test

The left tibia of three groups of white rabbits was obtained after 6 weeks of treatment. The biomechanical analysis of the tibia was performed. The tibia specimen was fixed with 4% paraformaldehyde, and the tibia was placed at Shimadzu Universal mechanical testing machine after 10 h. A three-point bending test was performed on the test machine. Each sample was placed in the same position and direction during the test, and the sample was protected under the wet conditions, the indicator of the three-point bending test was directly read on the instrument.

STATISTICAL ANALYSIS

All data were analyzed using SPSS17.0 software (SPSS Inc, Chicago, IL, USA). The measurement data were expressed by X±S and compared using *t* test. $P<0.05$ indicates that the difference was statistically significant.

RESULTS

Preparation and identification of MSCs

After 24 hours of inoculation of bone marrow mononuclear cells prepared in this study, some cells were colonized in cell culture flasks, and the cells grew slowly. After 3 passages, as shown in **Figure 1**, it can be seen that a large amount of cells grow in a radial shape, and the cells grow vigorously. Surface marker identification of cells showed that the cell surface marker CD44 positive rate was as high as 100%, while CD45 was negative.

Comparison of cytokine levels in three groups of fracture sites

As shown in **Table 2**, compared with the control group, the mRNA expression levels of b-FGF, VEGF, OPG and RANKL in the rabbits of the model group increased, the OPG/RANKL ratio increased significantly, and the difference was statistically significant ($p<0.05$). Compared with the fracture sites of the model group, the mRNA levels of b-FGF, VEGF, OPG and RANKL in the MSCs group were significantly increased, and the OPG/RANKL ratio was significantly increased, the difference was statistically significant ($p<0.05$).

Fracture healing and bone density in three groups of white rabbits

The results are shown in **Table 3**. Compared with the control group, the bone density of the white rabbits in the model group was significantly decreased ($p<0.05$). Compared with the model group, the bone mineral density of the white rabbits in the MSCs group was significantly increased ($p<0.05$). Compared with the control group, the X-ray scores of the fracture healing of the white rabbits in the model group were significantly increased ($p<0.05$). Compared with the model group, the X-ray scores of fracture healing of MSCs rabbits were significantly decreased ($p<0.05$).

Comparison of biomechanical test results in three groups of white rabbits

The results are shown in **Table 4**. Compared with the control group, the three-point bending index of the left tibia: the maximum load, elastic load, stiffness and maximum deflection of the model group were significantly decreased ($p<0.05$). Compared with the model group, the three-point bending index of the left tibia, the maximum load, elastic load, stiffness and maximum deflection of the MSCs were significantly increased ($p<0.05$).

DISCUSSION

Stress fracture, also known as fatigue fracture or cumulative strain, is a kind of bone damage caused by excessive use. When the muscle is overused, it cannot absorb the vibration caused by repeated collisions and transmit the stress to the bone. Repeated, minor direct or indirect damage can cause small fractures or fractures at specific sites. Stress fractures occur mostly in the weight-bearing parts of the body, such as the tibia and fibra and the foot (calcaneus, scaphoid, and metatarsals) (Knapik *et al.*, 2017). The vulnerable people are soldiers or athletes with more weight on the foot, such as basketball, football, tennis players, as well as track and field, gymnasts and ballet dancers. Once a stress fracture occurs, it will seriously affect the patient's exercise capacity and produce a lot of pain, which will bring a lot of

Table 1: The primer sequences used in this study

Gene	Upstream primer (5'-3')	Downstream primer (5'-3')
b-FGF	TGAATATGAAATCGAACTCT	GTGGGTGCCTTCCAGCTGAC
VEGF	ATCATGCGGATCAAACCTCA	CTCGGCTTGTCACATTTTTC
OPG	CTACTACACA GACACTTGGC	GATCGAGGTAGCGCCCTTCC
RANKL	GCCCAGTCTCATCGTTCTGC	TAGCTGTCAGCGCTTTCCCT
β-actin	CCCATGCCATCCTGCGTCTG	CGTCATACTCCTGCTTGCTG

Table 2: Comparison of cytokine expression levels in the fracture sites of three groups

Group	Cases	b-FGF	VEGF	OPG	RANKL	OPG/RANKL
Control	12	1.02±0.11	0.98±0.13	1.03±0.21	1.18±0.23	0.87±0.18
Model	12	1.26±0.15*	1.31±0.12*	1.76±0.22*	0.89±0.14*	2.21±0.29
MSCs	12	3.32±0.22*#	3.04±0.25*#	3.89±0.24*#	0.65±0.17*#	5.89±0.45#

Table 3: Analysis of fracture healing and bone mineral density after t reatment of three groups

Group	Cases	Bone density	X-ray score
Control	12	0.141±0.003	0
Model	12	0.049±0.004*	4.35±0.37*
MSCs	12	0.120±0.002*#	1.72±0.25*#

Table 4: Comparison of biomechanical indexes of the left tibia of three groups

Group	Cases	Maximum load（N）	Elastic load（N）	Stiffness（N/mm）	Maximum deflection（N/mm）
Control	12	120.11±8.10	89.54±8.41	225.70±12.51	0.80±0.08
Model	12	74.25±6.27*	56.35±5.12*	148.21±8.03*	0.58±0.07*
MSCs	12	120.20±7.55$^{\#}$	79.32±6.25*$^{\#}$	186.39±9.10*$^{\#}$	0.71±0.06*$^{\#}$

Note: *: compared with the sham operation group, $p<0.05$; #: compared with the model group, $p<0.05$.

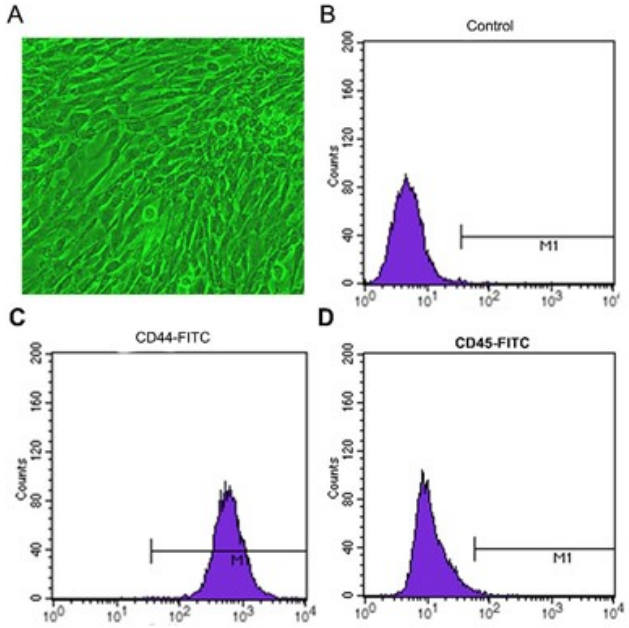

Fig. 1: Culture and identification of bone marrow derived mesenchymal stem cells.
A. The growth state of MSCs under fluorescence microscopy; B. The control of no any antibody detected by flow cytometry; C. The positive expression of CD44 detected by flow cytometry; D. The negative expression of CD45 detected by flow cytometry.

inconvenience to the patient. However, the current treatment of stress fractures is generally conservative, and rest is currently the only method that can completely treat stress fractures. However, the time required for recovery varies greatly. The influencing factors include the location of the injury, severity, strength of the individual's self-repairing function, and individual's nutritional intake. Therefore, choosing a good treatment is of great significance to the patient.

MSCs are a kind of special stem cells derived from mesoderm. They have self-proliferation and multi-directional differentiation potential, and are present in various organs of the human body, among which bone marrow is the most abundant. MSCs in the medullary cavity act as a pluripotent stem cell and an *in vivo* source of osteoblasts. The proliferative capacity of MSCs determines the ability to differentiate into osteoblasts (Kowal *et al.*, 2020). Many studies have shown that MSCs can differentiate into mesoderm cells such as osteoblasts, adipocytes, and chondrocytes, and can differentiate into cells such as nerve cells, hepatocytes, and insulin cells, which have important application value (Lee *et al.*, 2004; Li *et al.*, 2005). Studies have shown that after injection of MSCs in the fracture model, the cells migrate to the fracture site, thereby promoting fracture healing. After entering the bone defect area, MSCs can release cytokines that promote new bone formation, and also indirectly promote cardiovascular formation, which provides the necessary conditions for fracture healing (DeGrendele *et al.*, 1997). In addition, studies have confirmed that in the elderly rat model of osteoporosis, MSCs injected into the medullary cavity of the tibia can significantly increase the density of trabecular bone and cortical bone of the lumbar vertebrae (Wang *et al.*, 2006), indicating that MSCs have a good advantage in the treatment of fractures.

Our study analyzed the therapeutic effects of MSCs on stress fractures, obtained a large number of bone marrow-derived MSCs cells *in vitro*, and transplanted them into a stress fracture rabbit model, and explored its therapeutic effect on stress fractures. The results of the study showed that after treatment, the MSCs group showed that the degree of fracture healing was significantly increased by X-ray analysis, and the bone density of the fracture site

was significantly increased. Biomechanical analysis also found that after treatment with MSCs, the maximum load, elastic load, stiffness and maximum deflection of the tibia at the fracture site of the rabbits were significantly improved compared with those in the model group. The above results indicate that MSCs significantly improve the bone composition of the lesion and have a better therapeutic effect.

This study explored the molecular mechanism of MSCs in the treatment of stress fractures, and analyzed the expression levels of cytokines related to bone formation by real-time PCR. bFGF is a basic fibroblast growth factor, which can promote cartilage repair in the early stage of fracture, on the other hand, promote the formation of bone and cartilage, and shorten the fracture healing time (Huang *et al.*, 2016). Vascular dysfunction occurs after fracture, vascular endothelial growth factor (VEGF) as a highly specific pro-vascular endothelial growth factor, has the effect of inducing angiogenesis, and can provide adequate nutrition for fracture repair and growth (Wagner and Fahrleitner-Pammer, 2010). Osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) are mainly secreted by osteoblasts, which act to inhibit bone and promote bone resorption, respectively. OPG/RANKL/RANK signaling plays an important role in regulating bone formation and bone resorption (Yasuda, 2013; Zhang *et al.*, 2017). Our study found that after treatment with MSCs, the ratio of bFGF, VEGF and OPG/RANKL in the lesions was significantly higher than that in the model group, indicating that the repair ability of the fracture site after MSCs treatment was significantly increased, which was consistent with the post-treatment phenotypic changes. After treatment, X-ray analysis of the white rabbits in the MSCs group showed that the degree of fracture healing increased significantly, and the bone density at the fracture site increased significantly. Biomechanical analysis also found that after treatment with MSCs, the maximum load, elastic load, stiffness and maximum deflection of the tibia at the fracture sites of the white rabbits were significantly improved compared with the white rabbits in the model group, and the difference was statistically significant. The above results are consistent with the results of other experimental animal fracture models using MSCs (Hou *et al.*, 2019; Zhu *et al.*, 2019).

Basic fibroblast growth factor (bFGF) can promote cartilage repair in the early stage of fracture, and can also promote the formation of bone and cartilage, and shorten the healing time of fractures (Huang *et al.*, 2016). Vascular endothelial growth factor (VEGF) has the effect of inducing angiogenesis and can provide sufficient nutrition for fracture repair and growth (Wagner and Fahrleitner-Pammer, 2010). The signal pathway mediated by OPG and RANKL plays an important role in the regulation of bone formation and bone resorption (Zhang *et al.*, 2017). Our research results found for the first time that after MSCs treatment, the bFGF, VEGF and OPG/RANKL ratios of the lesions were significantly increased compared to the model group, indicating that the repair ability of the fracture site was significantly increased after MSCs treatment. This result is consistent with the phenotypic changes after treatment.

In conclusion, this study confirmed that MSCs have a good clinical effect in the treatment of stress fractures, which can significantly improve bone density and tibia biomechanics. Meanwhile, this study provides a certain guiding value for clinical treatment of stress fractures.

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