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Detection on the Promotion Effect on Angiogenic and Osteogenic Capabilities of the Necrosis Parts from Ad-BMP-2-IRES-HIF-1α After Transfecting Ad-BMP-2-IRES-HIF-1αto the Induced Rabbit Bone Marrow Derived Endothelial Progenitor Cells(EPCs) and then Implanting it into the Avascular Necrotic Parts of the Femoral Head

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ABSTRACT

Purpose To discuss the clinical effect of locally treating the parts of necrosis of the femoral head by means of transplanting Ad-BMP-2-IRES-HIF-1αto the endothelial progenitor cells(EPCs) and then implanting it into the necrosis parts of the femoralhead.MethodsTheAd-BMP-2-IRES-HIF-1α was transfected into induced EPCs and then transplanted into avascular necrotic parts of the femoral head(ANFH), and then, the promotion effect on angiogenic and osteogenic capabilities of the necrosis parts from Ad-BMP-2-IRES-HIF-1α was detected. Rabbit bone marrow MNCs were obtained by density gradient centrifugation method, and were induced into EPCs by M199 medium; EPCs were identified in accordance with the cell morphology, specific surface markers and uptake abilities. The Ad-BMP-2-IRES-HIF-1α was transfected to EPCs and then transplanted into parts of ANFH. The models were enthanized2 and 4 weeks after operation, and then the angiogenic and osteogenic indexes of necrotic parts were detected. Results The results showed that more blood vessels generated in group A than that in group B and C (P<0.05), and the statistical differences were found between group B and C(P<0.05); The detection of histology andBMP-2 immuno histochemistry showed that there were statistical differences between group A and B, group A and C (P<0.05). There was no statistical difference between group B and C (P>0.05). Conclusion To sum up, this experiment shows that the EPCs transfected by Ad-BMP-2-IRES-HIF-1α have stronger angiogenic and osteogenic capabilities.

Keywords: *Ad-BMP-2-IRES-HIF-1α; Ad-CMV-IRES-hrGFP-1; EPCs; avascular necrosis of the femoral head; angiogenesis; BMP-2*

INTRODUCTION

The avascular necrosis of the femoral head(ANFH) is usually known as osteonecrosis. The typical feature of ANFH is that when the blood supply of the microcirculation is damaged, the bone cells will generate apoptosis. Once the blood vessels are damaged, the self-recovering abilities of the osteonecrosis will be particularly weak and the prognosis will be inferior as well[1]. The degree of damage and the repair degree of bone tissues mainly depend on the degree of patial damage of blood vessels and if the effective collateral circulation is established[2]. At present, the studies on the repair for the damage of blood vessels and angiogenesis of patients with ANFH are only

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limited to the the changes of mature blood vessel endothelium cells[3]. But more and more researches have indicated that the EPCs which are the sources of the marrow paticipate in the process of vascular repair and angiogenesis but not the repair process that mature blood vessel endothelium cells paticipate[4]. In the meantime, the research group has established Ad-BMP-2-IRES-HIF-1αand verified that Ad-BMP-2-IRES-HIF-1αhas strong angiogenic and osteogenic capabilities[5]. Therefore, this experiment preliminarily discusses the new clinical method of transplanting Ad-BMP-2-IRES-HIF-1αto the endothelial progenitor cells(EPCs) and then implanting it into thenecrosis parts of the femoralhead for the sake of treating osteonecrosis.

MATERIAL AND METHODS

Materials: 36 New Zealand rabbits at the age of 3 to 5 months which were chosen for the experiment. The female and male rabbits were respectively 18 with weight of 2.5 to 3kg, and the rabbits were chosen from Animal Experimental Center of Liaoning Medical University. Ad-BMP-2-IRES-HIF-1αand Ad-CMV-IRES-hrGFP-1 which were supplied by the certral lab of The First Affiliated Hospital of Liaoning Medical University; 10% of fetal calf serum(supplied by Hyclone Company); M199 nutrient solution, EDTA-trypsin(Supplied by Gibco Company); VEGF (Vascular endothelial growth factor), BFGF (Basic fibroblast growth factor) and ECGS (Endothelial cells Growth Supplements)(Supplied by Peprotech Company); Phosphate Buffer Solution(PBS) and Ulex europaeus agglutinin I marked by FITC(FITC-UEA-I)(Supplied by Sigma Company); Acetyl Low-density lipoprotein marked by Dil(DiI-acLDL)(Supplied by Vector Company); Human Fibronectin (Supplied by Chemicon Company); Lymphocyte cell separation media(Supplied by Tianjin Haoyang Biological Co., Ltd.); Anti-CD133/PE and anti-CD34/PE (Supplied by Becton Dickinson Company); SABC Immunohistochemistry kit and BMP-2 monoclonal antibody(Supplied by Wuhan Boshide Biological Engineering Co., Ltd).

Isolation and cultivation of Rabbit bone marrow derived endothelial progenitor cells(EPCs)

50 mg/kg of ketamine was used for anesthesia through the auricular veins of rabbits. Under the aseptic condition, medullo-puncture needles were used to extract the marrow through the bilateral iliac bones of the chosen New Zealand rabbits. Then the density gradient centrifugation method was adopted to acquire mononuclear cells(MNCs). And then the mononuclear cells were placed in M199 culture medium which included 10% of fetal calf serum, 10 μg/L of VEGF, 2 μg/L of bFGF, 150 μg/mL of ECGS, 10 5 U/L of penicillin and 100 mg/L of streptomycin. Next, the cells were inoculated at the density of 1×106 into the culture bottles which were coated by human fibronectin in advance. Then the culture bottles were cultivated into the humidity incubators at 37 °C and with CO2 of 5%.

Ad-BMP-2-IRES-HIF-1α and Ad-CMV-IRES-hrGFP-1 were transfected into EPCs at the best transfection indexes

In accordance with the previous research results, the third generation cells were chosen as the transfected seed cells. The experimental group(group A) transfected the cells with 200 values of MOI, and the blank control group(group C) transfected the cells with 100 values of MOI. Then the cells were put into 2ml of serum-free medium. And next, the mudium was incubated by constant temperature cells incubators at 37 °C and with CO2 of 5% for 3h. After that, every hole was supplemented 2ml of M1999 medium which contained 10% of FBS, and then the medium was continuously cultivated for another 2 to 3d. The inverted fluorescence microscope was adopted to observe the cellular modalities after being transfected.

That no obvious cytopathy occurred meant the transfections succeeded.

The making for animal models of Rabbit ANFH

2ml of equinum serum was injected through the auricular veins of rabbits. Then 4 mg/kg methylprednisolone was injected from the second day and twice a week for total 6 weeks. Then, relevant examinations were arranged to verify if the molding was successful.

The transfected EPCs were implanted into parts of animal models of Rabbit ANFH

Experimental grouping

There were three groups in total, and each group was allocated for 6 female rabbits and 6 male rabbits. In Group A(the experimental group)(12 rabbits in total): EPCs liquid which had been transfected and implanted by Ad-BMP-2-IRES-HIF-1α was added; In Group B(the control group)(12 rabbits in total): EPCs liquid which had been transfected by Ad-CMV-IRES-hrGFP-1(adenovirus vector) was added; In Group C(the blank control group)(12 rabbits in total): only the cell suspension liquid was added.

The preparation for cell suspension liquid and implantation

The quantities of cells in every group were calculated and the quantities of implanted cells in every model were limited in the range of 2×10 4 /g. And every model was configurated into 2ml of cells suspension liquid. The second week(namely the early time of ANFH) after successfully molding the models, in the C-type arm, drill of 3.5mm was used to drill for about 5mm from the underneath of the greater trochanter to the surface of head of femur until the depth extended to the underneath of the surface of catilage in the head of femur. There were three drill ways in total. Then, the rabbits in each group were accordingly handled. 0.5ml cells suspension liquid was injected into the underneath of catilage in the head of femur along with the drill way by means of the injection syringe of 1ml, and then the openings of the drill ways were sealed by bone wax in case the suspension liquid outflowed. And the other groups did the same operations.

Detection of indexes

Identification for the induced and cultured rabbit bone marrow derived endothelial progenitor cells(EPCs)

(1)The cellular morphologies were observed after the liquid was quietly incubated for 5d in the incubators. After the incubation of 14d, the cell morphologies was observed for one more time. (2)The third generation cells which had been induced and incubated were made into cells suspension liquid at the concentration of 1×10 6 /mL. Next, CD34 antibody marked by 10 μL of FITC and VEGFR2 antibody marked by R-PE were respectively added into the cells suspension liquid. Then the liquid was incubated for 25min away from the light. Then the flow cytometry was used to analyze the specific mark on the face of EPCs, and if the homotypic R-PE or IgG1-FITC was dyed they would be treated as the negative control groups. (3)The induced and incubated cells were inoculated into six pores plate with prepared cells grow on cover slip at the density of 5×10 4 /cm 2. After being incubated by5 μg/mL of DiI-ac-LDL at 37 °C for 3h, the cells were rinsed by PBS and fixed by paraformaldehyde of 4% for 20min. Then 10 μg/mL of FITC-UEA-1 was added for the continous incubation of 1h at 37 °C. Then the fluorescence microscope was used to observe thus detect the ingestion capacity of EPCs.

Detection for the animal models

2 weeks and 4 weeks later, X-ray normotopia radiography(the conditions for radiography were same) and histological observations were arranged for the sake of detecting if the animal model-making in each group succeeded.

Detection for the angiopoiesis in necrosis zones

2 weeks and 4 weeks after the operations, CD34 dyeing was arranged immunohistochemistry detection. The status of newly born capillaries was observed by optical microscope in the perspective of 200 times and then the quantities of CD34 and blood capillary were calculated. With regard to each slice, the zones that had most qunantities of CD34 and blood capillary were chosen for the calculation and three zones were chosen in each slice. And each specimen was calculated in six pespectives and then the average value was selected.

Detection for the dyeing of immunohisto-chemistry

The expression of BMP-2: The partial tissues that had been transfected for 4 weeks in the necrosis zones of Group A, Group B and Group C were chosen. After they were digested, they were inoculated at the density of 3×104 /hole into the cover glasses with six pores plate. When the cells basically covered the glasses, the supemate was sucked up and then the sediment was fixed by paraformaldehyde of 4% for 30min. Next, the sediment was dyed in accordance with the specifications of SABC immunohistochemistry kit. When the sediment was observed by the inverted phase contrast microscope, if there were claybank particles found in the cytoplasm, then it meant that the expression of BMP-2 was positive. 5 perspectives were randomly selected from each group, then the artificial counting method was used to calculate the quantities of positive cells. Finally, the average value was adopted.

Histological detection for the stat used of the necrosis of femoral head after being transplanted

The experimental animals of each group were respectively proceeded histological detection 2 weeks, 4 weeks and 8 weeks after the transplantation for the sake of comparing the differences between the groups.

Statistical analysis

All the above-mentioned experimental data were counted and analyzed by SPSS17.0 software. The data were expressed by the mean plus/minus standard deviation. $P<0.05$ was regarded that the differences had statistical significance.

RESULTS

Authenticate for EPCs

The cellular modality

The mononuclear cells in rabbit bone marrow occurred the phenomenon of growing adhering to the wall 48h after being induced and cultivated. On the fourth to sixth day, the modality of cells which grew adhering to the wall changed and turned into the shape of shuttle; On the seventh to nineth day, the modality of cells turned into polygon and gradually conjugated into sheet pattern; On the 15 day, the modality of cells presented typical appearance of paving stone pattern (See in Figure 1).

Detection for the peculiar marks on the surface of EPCs

Flow cytometry was used to detect the peculiar marks on the surface of EPCs. The results showed that the positive rates of peculiar marks CD34, CD133 and VEGFR-2 were repectively 56.61%±6.24%, 49.36%±5.16% and 54.70%±4.28%(See in Figure 2).

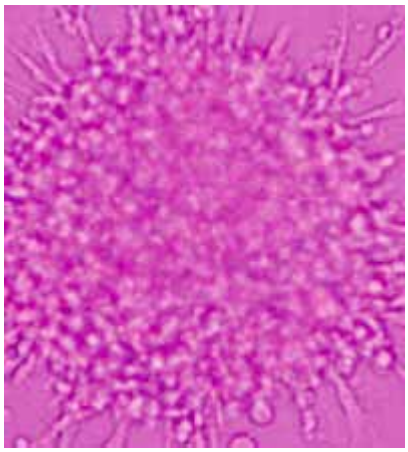

Fig. 1: The modalities of EPCs

Under the observation of inverted microscope, the induced and cultivated cells that grew adhering to the wall presented typical appearance of paving stone pattern(400×) on the fifth day.

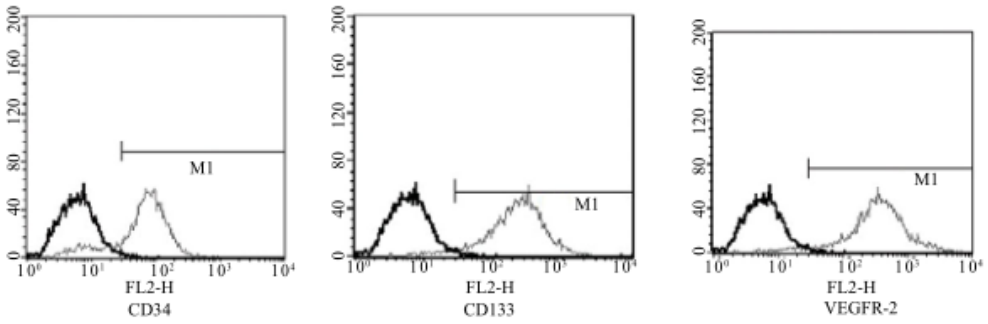

Fig. 2: Detection for the peculiar marks on the surface of EPCs by flow cytometry

The detection results of flow cytometry showed that the positive rates of peculiar marks CD34, CD133 and VEGFR-2 were respectively 56.61%±6.24%, 49.36%±5.16% and 54.70%±4.28%

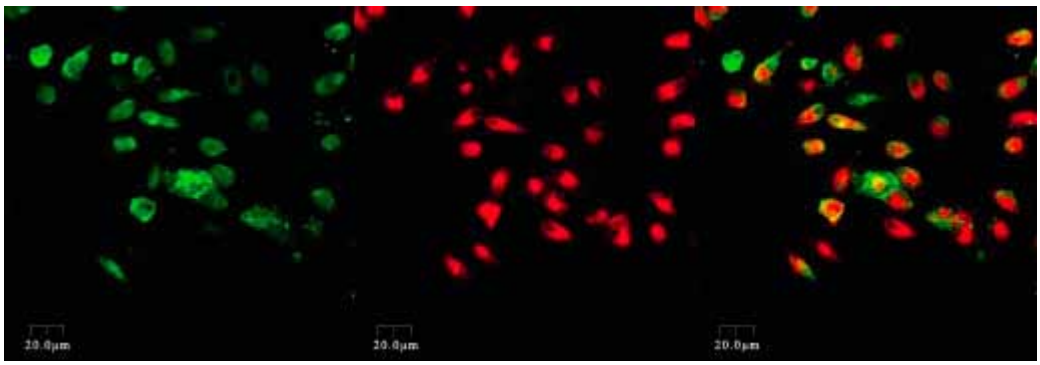

Fig. 3: The capacity that endothelial progenitor cells conjugated with FITC-UEA-1and DiI-ac-LDL(200×)

Under the fluorescence microscope, the cells with dual-fluorescence were endothelial progenitor cells whose positive rate was about 50%.

(A) (B)

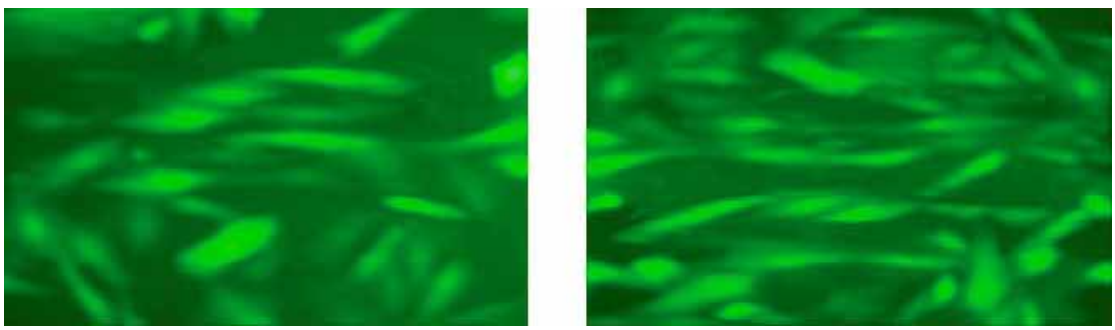

Fig. 4: The immunofluorescence observation results under the fluorescence microscope after the transfection

The immunofluorescence observation results under the fluorescence microscope 3d after the cells were transfected (200×). A: The MOI value was 200; B: The MOI value was 100

(A) (B)

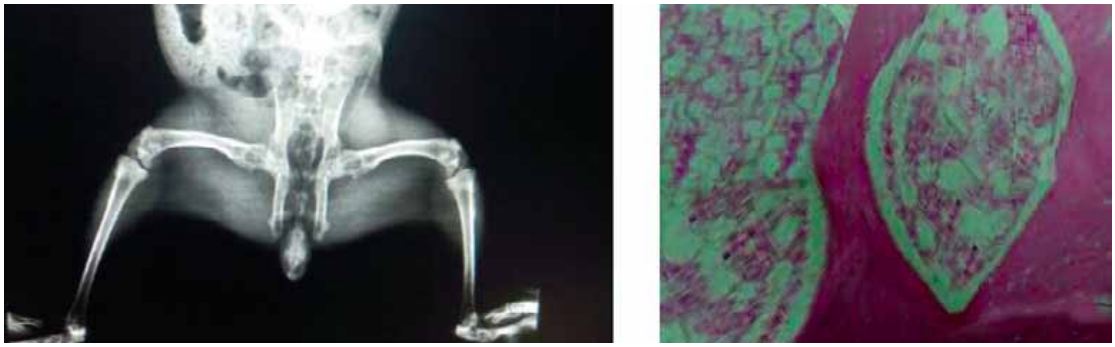

Fig. 5. Detection results of the indexes of animals models

A: The X-ray detection results showed that the head of femur occurred necrotic changes 4 weeks after the model-making; B: The histological examination showed that the quantities of osteoblasts and other histocytes reduced, the bone trabecula became thinner and the fossa of bones was empty 2 weeks after the model-making(40×).

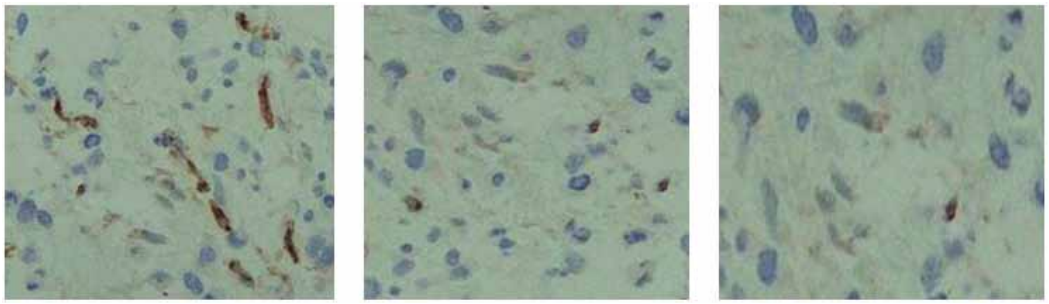

Fig. 6. Immunohistochemical results of CD34 in group A, B and C(200×)

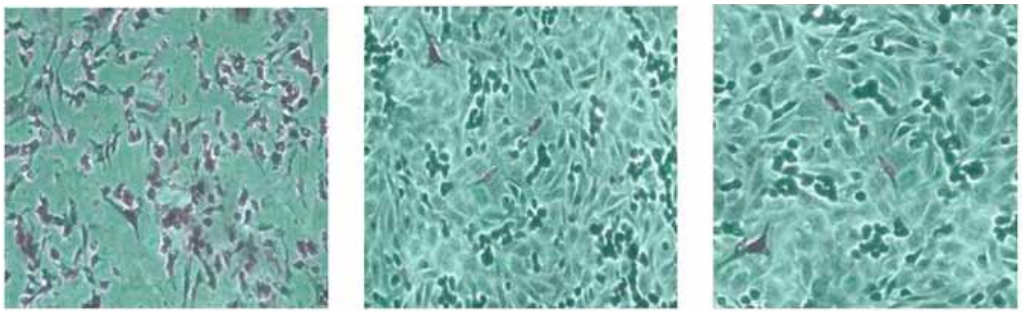

Fig. 7. Immunohistochemical results of MBP-2 in group A, B and C

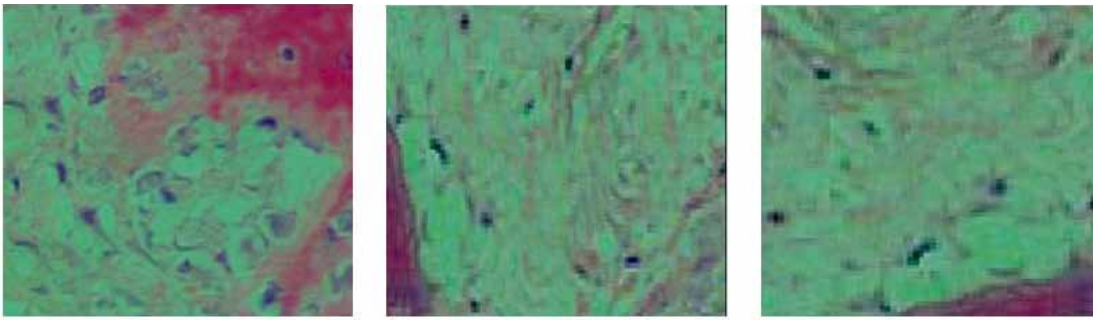

Fig. 8. Histological detection for the repairing statuses of necrotic zones in groups A, B and C

Detection for the combining capacity of EPCs

The quantities of positive cells that presented dual-fluorescence under the fluorescence microscope accounted the quantities of cells that grew adhering to the wall for about 50%(See in Figure 3).

Observation for the transfected effect of venom

The observation results under the inverted fluorescence microscope showed the transfected effects when group A was with MOI value of 200 and group B was with MOI value of 100. And the results showed that there was no obvious cytopathic effect(See in Figure 4).

Detection for the indexes of animal models

The X-ray detection results showed that the head of femur occurred necrotic changes 4 weeks after the model-making(See in Figure5 A). The histological examination showed that the quantities of osteoblasts and other histocytes reduced, the bone trabecula became thinner and the fossa of bones was empty 2 weeks after the model-making(See in Figure 5 B).

Detection results for the new vessels

CD34 immunohistochemistry results showed that within the scope of 200 times, the mean values of new blood vessels 2 weeks after the transplantation in Group A, Group B and Group C respectively were 10.96±1.34, 6.42±0.84 and 2.72±0.78. 4 weeks after the transplantation, the mean values respectively were 16.84±1.76, 9.28±1.14 and 3.72±1.32(See in Figure 6).

Immunohistochemical dye detection for the expression of BMP-2

The observation under the inverted phase contrast microscope showed that the among the transfected cells in Group A, the positive cells expressed BMP-2 and the quantities of positive cells were 15.40±1.85; the transfected cells in Group B and C mildly expressed BMP-2 and the positive cells of Group B and C respectively were 6.40±1.36 and 5.80±1.44(See in Figure 7).

Histological detection for the repairing statuses of necrotic zones in femoral head

4 weeks and 8 weeks after the transplantation, the histological detection was arranged. The results showed that there were new bone trabecula, blood capillary and osteoblast and other cellular constituents in Group A. In Group B, there were new blood vessels, the quantities of osteoblast and other histocytes were less, the bone trabecula became thinner and the fossa of bones was empty. And there was no obvious change found in Group C(See in Figure 8).

DISCUSSION

With larger quantities of patients, the avascular necrosis of femoral head was one of the most common diseases in department of orthopaedics. In western developed country, more than 50 million patients took the replacements of the artificial hip joints prosthesis and artificial knee joints prosthesis due to ANFH[6]. When the avascular necrosis occurred in femoral head, not only were the structures of bones damaged but also the bone density reduced. Therefore, the disability rate was pretty high if the osteonecrosis happened. In this article, the way of equinum serum-hormone was adopted to make the models. It had been proved that this method was easy to operate and with significant effects[7]. And the results of X-ray detection and histological examination proved that the model-making was successful. Considering that some animals may die and other reasons during the process of model-making, this experiment chose 50 animals in total. Among the 50 animals, 5 died during the process of model-making, 4 died after the model-making, and then 36 animals in the rest of the living animals were randomly chosen for the experimental sectionalization. At present, the adoption of stem cell transplantation for the sake of treating the avascular necrosis of soft tissues had obtained obvious curative effect. But the researches on the treatment for the avascular necrosis

were less and needed further exploration[8]. Recent researches showed that in blood vessels, endothelial progenitor cells played the key roles in aspects of treating the oxteonecrosis and angiogensis and had superiority than other stem cells[9]. EPCs was the important primitive cells in the field of medicine. EPCs were Initially extracted by Asahara in 1997. And it had been proved that EPCs had angiogenic abilities and were the original endothelial cells[10]. Among the bone growth factors, BMP-2 was the most outstanding in terms of accelerating the bone defect repairing and fracture healing. So far, most researches adopted the way of adenovirus carrying BMP-2 gene to transfect the seed cells for the sake of promoting the seed cells to differentiate into osteoblasts thus this method was used for the bone defect repairing[11]. As the DNA binding protein, HIF-1 could accelerate the endogenous expression of VEGF and produce endogenous VEGF and the receptors thus accelerate the generation of blood capillary. And the induced new blood vessels were anti-leakage, with less inflammatory reaction, without tissue edema, without bending of blood vessels and without generation of cystic vessels[12]. Therefore, this experiment transfected EPCs by means of utilizing the established Ad-BMP-2-IRES-HIF-1αand under the immunofluorescence observation of the inverted microscope, the transfectiong was successful.

In recent years, with the constant and in-depth study on the biological characteristics of EPCs and the update of separation and depuration methods, it was deemed that the special marks on the surface of EPCs were CD34, CD133 and VEGRR-2[13]. Therefore, this experiment authenticated those marks for the sake of detecting if the induced and cultured cells were EPCs. And the results showed that the positive rate was higher. The induced cells which grew adhering to the wall could specifically combine with DiI-ac-LDLand FITC-UEA-1. The fluorescence dyeing results showed that positive cells were more, which indicated that the inducing of rabbit bone marrow could culture MNC into EPCs.

In this experiment, the transfected EPCs were made for cell suspension fluid and then the cell suspension fluid was implanted into the necrotic parts. CD34 immunohistochemistry detection results showed that 2 weeks after the transplantation, in every scope of 200 times, the quantities of new blood vessels were 10.96±1.34 in Group A, 6.42±0.84 in Group B, 2.72±0.78 in Group C; 4 weeks after the transplantation, the quantities of new blood vessels were 16.84±1.76 in Group A, 9.28±1.14 in Group B, 3.72±1.32 in Group C. In every period, the comparisons of the status of new blood vessels between Group A and Group B, Group A and Group C had statistical significance($P<0.05$), and the comparisons between Group B and Group C had statistical differences($P<0.05$). The BMP-2 dye showed that among the transfected cells in Group A, the positive cells expressed BMP-2 and the quantities of positive cells were 15.40±1.85; the transfected cells in Group B and C mildly expressed BMP-2 and the positive cells of Group B and C respectively were 6.40±1.36 and 5.80±1.44. The quantities of positive cells in Group A were obviously higher than those in Goup B and Group C and the differences had statistical significance($P<0.05$), the differences between Group B and Group C had no statistical significance($P>0.05$). 4 weeks and 8 weeks after the transplantation, the histological detection was arranged. The results showed that there were new bone trabecula, blood capillary and osteoblast and other cellular constituents in Group A. In Group B, there were new blood vessels, the quantities of osteoblast and other histocytes were less, the bone trabecula became thinner and the fossa of bones was empty. And there was no obvious change found in Group C.

The above-mentioned results indicated that in the aspect of angiopoiesis, the comparisons

between the experimental group and blank control group, the control group and blank control group had statistical significance, and the comparisons between the experimental group and control group had statistical significance, which indicated that EPCs had angiogenic functions but the functions of EPCs were stronger after being transfected by Ad-BMP-2-IRES-HIF-1α. The results of osteogenesis detection for necrotic zones showed that the comparisons between the experimental group and control group, the experimental group and blank control group had obvious differences, but the comparisons between the control group and blank control group had no statistical differences. It was initially proved that the transplanted EPCs transfected by Ad-BMP-2-IRES-HIF-1α had stronger angiogenic abilities and osteogenic functions and could better improve the generation of new blood vessels in necrotic parts, increase the density of blood capillary and promote the generation of new bones, which could help suspend the necrosis of femoral head and even was beneficial to the repairing of necrosis of femoral head.

CONCLUSION

To sum up, this experiment testifies that the transplanted EPCs transfected by Ad-BMP-2-IRES-HIF-1αhave stronger angiogenicand osteogenic capabilities in the necrotic zones, which provides new ideas in terms of clinical treatments for the avascular necrosis of femoral head.

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