Int J Clin Exp Pathol 2016;9(2):888-898
www.ijcep.com /ISSN:1936-2625/IJCEP0018998

Original Article

VEGF gene modified allogenic bone marrow-derived mesenchymal stem cells therapy in diabetic hindlimb ischemia rats

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Received November 2, 2015; Accepted January 5, 2016; Epub February 1, 2016; Published February 15, 2016

Abstract: Diabetes mellitus (DM), a worldwide difficult problem, is still lack of effective treatment so far. Bone marrow-derived mesenchymal stem cells (BMMSCs), with a low immunogenicity, is a promising source which can be used for revascularization and repair of hindlimb ischemia of DM. Vascular endothelial growth factor (VEGF), a strongest cytokine to promote the growth of blood vessel, could do function on revascularization or not is unknown yet. In this study, rat type 2 diabetes mellitus (T2DM) hindlimb ischemia model was established by intraperitoneal injection of streptozotocin (STZ) and thereafter ligating of the bilateral femoral arteries and their branches around. BrdU-labelled passage 3 BMMSCs were randomly divided into three groups including BMMSCs group, empty vector of green fluorescent protein labelled adenovirus (Ad-GFP) transfected BMMSCs (Ad-GFP-BMMSCs) group and VEGF gene-expressing Ad-GFP modified BMMSCs (Ad-VEGF-GFP-BMMSCs) group. Then those cells in each group were respectively injected into the left rectus femoris and gastrocnemius of the corresponding T2DM models. Digital subtraction angiography (DSA) of hindlimbs arteries were performed after transplantation and H&E staining and it showed there were more abundant blood vessels in the Ad-VEGF-GFP-BMMSCs group than the other two groups. The capillary density of bilateral rectus femoris and gastrocnemius in Ad-VEGF-BMMSCs group was superior significantly to that in the other two groups at the corresponding time point. Combining the result of immunofluorescence staining, we could confirm VEGF gene modified BMMSCs could survive in situ and contribute to neovascularization in T2DM hindlimb ischemia rats.

Keywords: Bone marrow derived-mesenchymal stem cell, transplantation, vascular endothelial growth factor, diabetic angiopathies, disease models

Introduction

Diabetes mellitus (DM) especially the type 2 (T2DM) has always been a global and social problem [1-3], one main factor of which is that ischemia can result in diabetic foot (DF). DF, with an extensive lesion, has always been an important influence on the bilateral hindlimb arteriolae, especially the arteriolae below the popliteal artery. Once the abscission of atherosclerotic plaque arises, ischemia and putrescence of local tissues follow it subsequently, which finally results in the amputation. This serious complication has been the first cause of non-traumatic amputation [4, 5]. Although the traditional methods such as surgery and interventional therapy can alleviate the symptoms of hindlimb ischemia to some degree, it is difficult for DF patients, a majority of whom are the old, to bear the blow caused by surgery. The interventional therapy is suitable only for the patients with a minor pathological change in the distal arteries of lower limb. Finally, restenosis is hard to be avoided [6-8]. Therefore, more effective methods need to be further explored.

Early in 2001, autologous bone marrow cells were performed for the therapy of hindlimb ischemia patients by intramuscular injection [9], which tentatively confirmed the safety and validity of these cells therapy. It indicated that BMMSCs might be hopefully the resource of

seed cells for DF. Thus it's necessary to study intensively the mechanism of autologous BMMSCs transplantation treatment on DF. And whether it's an effective method for DF or not mainly depends on whether it can promote neovascularization in diseased region. BMMSCs have the abilities to differentiate towards almost all types of the cells from endoderm, mesoderm and ectoderm at optimum conditions such as osteocytes, chondrocytes, adipocytes, cardiomyocytes, tenocytes, epicytes and endotheliocytes and so on [10-15]. It has been reported that BMMSCs possess the differential ability towards vascular endothelial cells (VECs) which can stimulate neovascularization at suitable condition [16]. In addition, vascular endothelial growth factor (VEGF) plays a most important role in neovascularization. Although VEGF gene modified BMMSCs therapy on myocardial ischemia has been reported [17, 18], whether it can improve the neovascularization and blood supply or not has not been definitely confirmed. In this view, human VEGF gene-expressing green fluorescent protein labelled adenovirus vector (Ad-VEGF-GFP) transfected rat allogenic BMMSCs were transplanted to treat T2DM hindlimb ischemia rats, and through this research we tried to provide the valuable experimental evidence for VEGF gene modified BMMSCs therapy on DF.

Materials and methods

Animals, adenovirus vectors

Sprague Dawley (SD) rats (N=72) in a specific pathogen free (SPF) grade (production licence: SCXK Yu 2012-0005) and the high-fat feet containing 65% basal feed, 20% lard, 10% white sugar and 5% yolk were purchased from the Animal Center of Third Military Medical University. Animals and the operational program of this experiment were approved by the ethics committee of the animal experiments of Zunyi Medical College. Ad-VEGF-GFP and empty vector of green fluorescent protein labelled adenovirus (Ad-GFP) were a gift from Professor Jicheng Yang of Soochow University.

Isolation, culture and immunophenotypic identification of rat BMMSCs

The isolation/culture of rat BMMSCs referred to the previous relevant report [19]. Then, as for phenotypic identification of rPBMSCs by flow cytometry analysis, the rPBMSCs of second passage (P2) were collected and treated with 0.125% trypsin (Gibco, New York, USA), after which the cells were suspended by 0.1% BSA and the cell concentration was adjusted to 2×10^6/ml. Afterwards, the liquid was placed into flow tubes, 100 μl per tube, Alexa Fluor® 488 anti-mouse/rat CD29, Alexa Fluor® 488 anti-rat CD90/mouse CD90.1, Alexa Fluor® 488 anti-rat CD45, Alexa Fluor® 488 anti-rat CD44 and Alexa Fluor® 488 anti-rat CD34 (BioLegend, San Diego, USA) were added into tubes respectively for labeling the cells. The results were detected and analyzed using the FACS Calibur (BD, Franklin L, USA) and the analytic software was Cell Quest.

Ad-VEGF-GFP modified BMMSCs

The P3 BMMSCs were seeded at 5×10^5/ml into T25 disposable plastic flask at 37°C, 5% CO_2 in the CO_2 incubator (3141, I/R, Thermo Forma, USA). When cell growth reached 50%-80% confluence approximately, the supernatant was poured. Then, 50 μl Ad-VEGF-GFP, the virus titer being 8×10^8 pfu/ml, and 5 ml LG-DMEM (Gibco, New York, USA) including 2% FBS and 10 ng/ml bFGF (Sigma, Saint Louis, USA) were added for the subsequent culture. The cultured cells were shaken slightly every 12 h and the cells were collected for transplantation 48 h later. The method of Ad-GFP transfected BMMSCs was same as the above.

Label of transfected BMMSCs with BrdU in vitro

The P3 BM-MSCs were seeded at 1×10^4/ml per well in 24-well plate with cover glass and incubated with 1 ml LG-DMEM containing 10% FBS and 10 ng/ml bFGF at 37°C, 5% CO_2 atmosphere. Then the wells were randomly divided into the BMMSCs transplantation group, Ad-GFP transfected BMMSCs (Ad-GFP-BMMSCs) group and Ad-VEGF-GFP modified BMMSCs (Ad-VEGF-GFP-BMMSCs) group. After 6 h, the media in the BMMSCs transplantation group were replaced with 1 ml LG-DMEM including 10% FBS, 10 ng/ml bFGF and BrdU (10 μmol/L, Sigma, Saint Louis, USA), away from light. BrdU labeling rate of BM-MSCs was detected by immunofluorescence according to the previous report [20]. In the Ad-GFP transfected BMMSCs (Ad-GFP-BMMSCs) group and Ad-VEGF-GFP modified BMMSCs (Ad-VEGF-GFP-BMM-

SCs) group, when the cell growth reached 50%-80% confluence, the media were respectively replaced with Ad-VEGF-GFP solution and Ad-GFP one, whose multiplicity of infection (MOI) were both 50, and 1 ml LG-DMEM containing 2% FBS, 10 ng/ml bFGF and 10 μmol/L BrdU away from light. Then the plate was shaken slightly every 12 h and 48 h later the BrdU labeling rates were detected.

Establishment of rat T2DM and hindlimb ischemia model

Male SD rats (N=22) weighting 160-180 g were selected for the establishment of T2DM model. The total rats were divided into 2 groups randomly, the experimental group used for the establishment of model which were fed with the high-fat diet and the control group which were fed with normal diet for one month. Subsequently the animals above were weighed and the serum lipid level from rat tail vein blood was measured. After 15-hour abrosia the experimental rats were performed single intraperitoneal injection of 30 mg/kg streptozotocin (STZ, Sigma, Saint Louis, USA) to establish the T2DM model. The blood glucose level was measured at 1st, 7th, and 14th day respectively after STZ injection. Diabetes was finally confirmed when the blood glucose concentration was higher than 16.7 mmol/L. Afterwards, the experimental rats were fed with the same diet continually until the finish of the whole experiment. Then, the T2DM model, after an intraperitoneal injection of 10% chloral hydrate at 0.33 ml/100 g, was performed ligation of bilateral femoral artery below the inguinal ligaments and its branches around were stripped to prepare for hindlimb ischemia models.

Transplantation of different BMMSCs into T2DM model

The left hind of T2DM model (N=36) was respectively injected with different BMMSCs, the right one with equivalent normal saline for own control. Briefly, the P3 BMMSCs, Ad-GFP-BMMSCs, Ad-VEGF-GFP-BMMSCs, all labelled with BrdU, were prepared to be single cell suspensions (the cell number being 2×10^6) in 400 μl normal saline respectively and then injected separately into the rectus femoris and gastrocnemius of the left hindlimb at ten different injection points from the ligation site of the left femoral arteries. After the finish of the injection, the syringe needle was retained for 30 s.

Digital subtraction angiography (DSA) after different BMMSCs transplantation

Three rats in different BMMSCs transplantation group (N=27) were selected randomly for DSA by DSA machine (Lntegris CV, Philips, Netherlands) at 2nd, 6th, 10th week respectively. Briefly, the abdominal wall was opened longitudinally along the ventral midline to manifest the abdominal aorta. After the occlusion of the proximal part of the abdominal aorta by vessel clamp, anterograde abdominal aorta catheterization was operated between the abdominal aorta branch and vessel clamp. Thereafter, the contrast medium containing 30% iohexol was injected at 1 ml/s, 111 KPa to show the perfusion of the bilateral hindlimb arteries by DSA.

Detection of hindlimb capillary density after different BMMSCs transplantation

The bilateral fresh rectus femoris and gastrocnemius tissues of T2DM rats after different BMMSCs transplantation in every group were collected respectively at 2nd, 6th, 10th week. Then, those tissues, after 24-hour fixation by 4% paraformaldehyde (PFA), were sliced into 5 μm by paraffin slicing machine (RM 2235, Leica, Germany). After H&E staining, the sections were observed under the light microscope (YS100, Nikon, Japan). Ten fields were selected randomly at high magnification and the number of blood capillary and muscle fibers of rectus femoris and gastrocnemius were counted and calculated the blood capillary density as the formula: blood capillary density=blood capillary number/muscle fiber number.

Tracing the survival of transplanted BMMSCs

After obtainment of rectus femoris and gastrocnemius tissues (N=9), they were fixed in 4% PFA for 2 h and then were performed dehydration in 20% sucrose solution for 6 h until the tissues stayed sink. Then the tissues were taken out for the preparation of frozen sections by freezing microtome (CM 1850, Leica, Germany). The transplanted BMMSCs, Ad-GFP-BMMSCs, Ad-VEGF-GFP-BMMSCs labelled with BrdU in vivo were detected after the counterstain by DAPI fluorescent cell linker (Sigma, Saint Louis, USA) combining the immunofluorescence by rat PE-labelled antibody (BioLegend, San Diego, USA) for BrdU and observed under the inverted fluorescence microscope (Leica Mirb, Nikon, Germany) respectively.

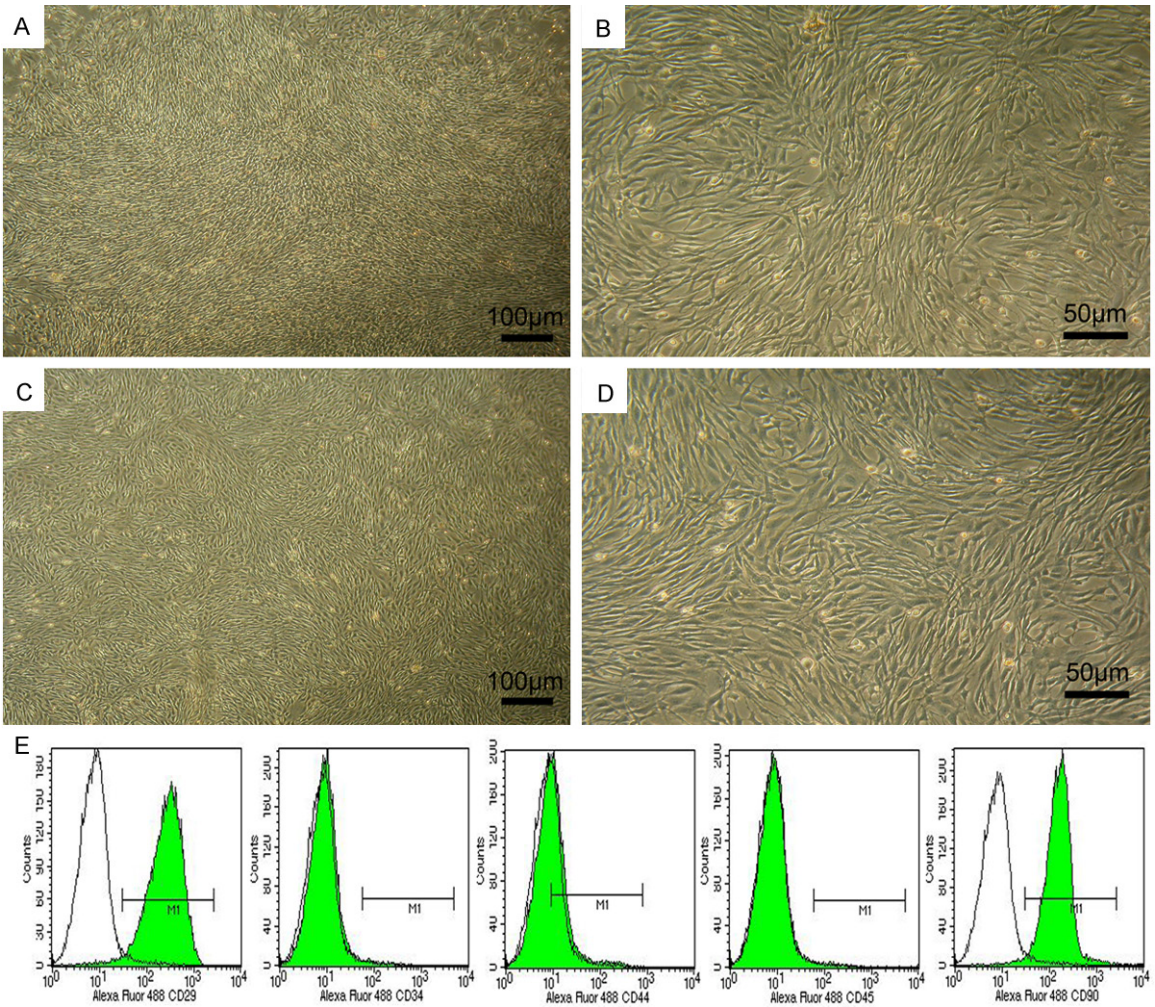

Figure 1. Morphology and Immunophenotypes of culturing BMMSCs. Morphology of primary culturing BMMSCs at 4th day under light microscope at 40 times (A) and 100 times (B); Morphology of BMMSCs after subculture at 2nd day at 40 times (C) and at 100 times (D); (E) Immunophenotypes of BMMSCs after subculture.

Statistical analysis

Experiment was operated in triplicate and the data were presented as mean ± standard error (SEM). Statistical analysis was performed using the SPSS 13.0 software package and one-way ANOVA was conducted for comparison between groups. Statistical significance was set at $P < 0.05$.

Result

Growth and immunophenotypic characteristics of rat BM-MSCs

There were spindle adherent cells observed after a 48-hour primary culture and colonies of adherent cells formed obviously after a 72-hour primary culture, and those cells grew to 50-80% approximately confluence after a 4-day primary culture (**Figure 1A**, **1B**). The cells after a 48-hour subculture possessed classic long-shuttle-like morphology showing a slabstone-like arrangement (**Figure 1C**, **1D**). The phenotypic identification results by flow cytometry and immunocytochemical stain suggested cultured BMMSC overexpress CD90, CD29 andCD44, but expressed negatively CD45 and CD34 (**Figure 1E**).

Detection of BrdU labeling rate of different BMMSCs by immunofluorescence

Under the fluorescence microscope, ten fields were randomly selected to calculate the BrdU labeling rate according the specific value of the fluorescyte number labelled with BrdU and DAPI. The rates were 86±2% in the BMMSCs

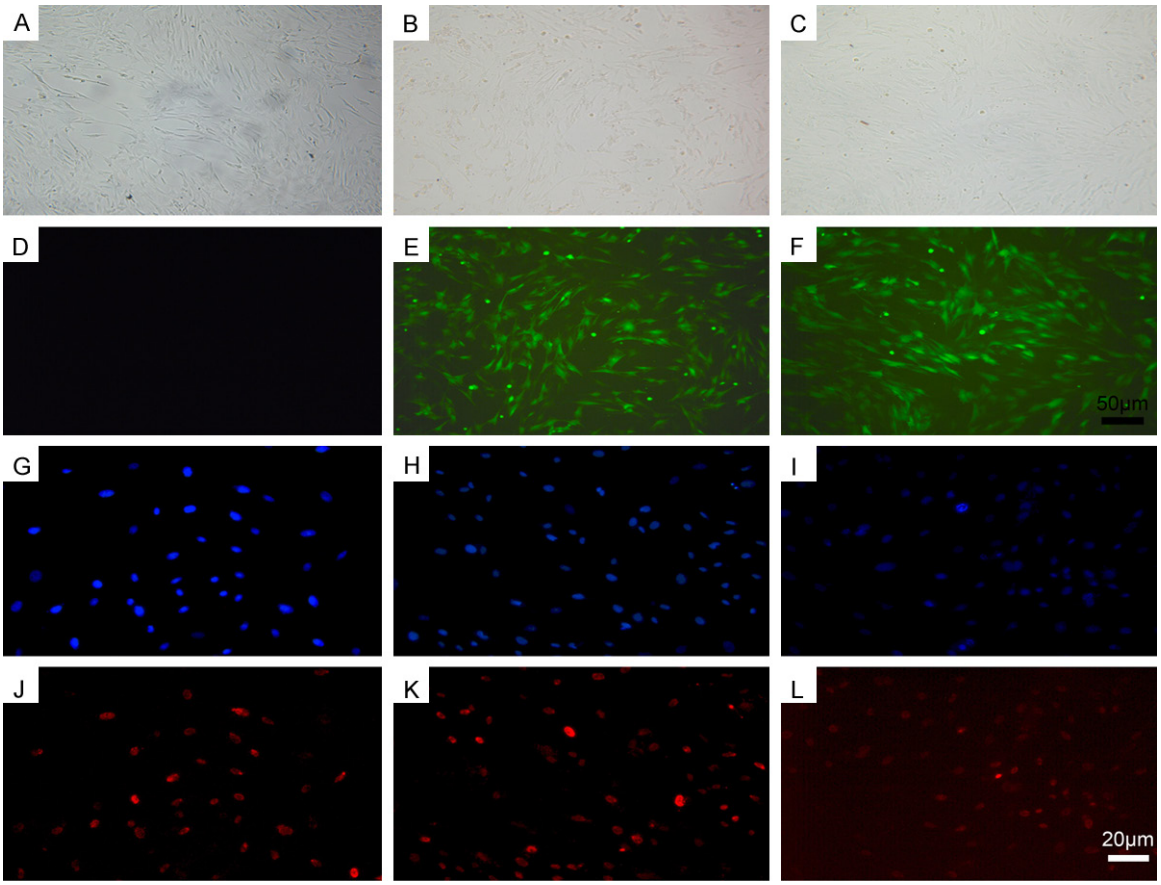

Figure 2. Transfection of recombinant adenovirus vectors into BMMSCs and Immunofluorescence detection of BrdU-labelled BMMSCs. (A, D) PBS group; (B, E) Ad-GFP group; (C, F) Ad-VEGF-GFP group; (A-C) Views under phase contrast microscope; (D-F) Corresponding views of (A-C) after 48 h under fluorescent microscope (Original magnification: 100 ×); (G, J) BMMSCs group after BrdU labelling *in vitro*; (H, K) Ad-GFP group after BrdU labelling *in vitro*; (I, L) Ad-VEGF-GFP group after BrdU labelling *in vitro*; (G-I) Views under phase contrast microscope; (J-L) Corresponding views of (G-I) after 48 h under fluorescent microscope (Original magnification: 200 ×).

Table 1. Wight, TG, TC and BSL change between control and high-fat diet group

Group	Wight (g)	TG (mmol/L)	TC (mmol/L)	BSL (mmol/L)
Control	276.5±13.19	0.62±0.24	1.61±0.12	5.21±0.85
High-fat diet	404.4±18.41**	1.46±0.29**	2.39±0.39**	24.12±3.35**

Compared with control group, **$P < 0.01$; BSL was measured 14 days after establishment of T2DM model.

group, 84±2% in the Ad-GFP-BMMSCs group and 84±3% in the Ad-VEGF-BMMSCs group respectively (**Figure 2A-F**).

Estimate of rat T2DM and hindlimb ischemia model

The results showed definitely the rats after high-fat feeding had hyperlipidemia, glucose metabolic disorder, overweight and obesity to a certain extent, which are the classical symptoms of human T2DM. It indicated the T2DM model was established successfully (**Table 1**). After the vascular ligation, hindlimb muscles presented an acute ischemia sign with a dark violet. Meanwhile, bilateral hindlimbs was ice-cold and couldn't move. In addition, there was obvious swelling of the ankle joint existed. The symptoms were relieved after 3-4 w and there were a less flexible movement.

DSA after different BMMSCs transplantation

After angiography through abdominal aorta (**Figure 3A**), the bilateral femoral arteries of the T2DM model without femoral ligation had clear branches and less collateral circulations (**Figure 3B**). As for the bilateral femoral arteries after femoral ligation, by contrast, had vascular disruptions at the inguinal ligaments, and there

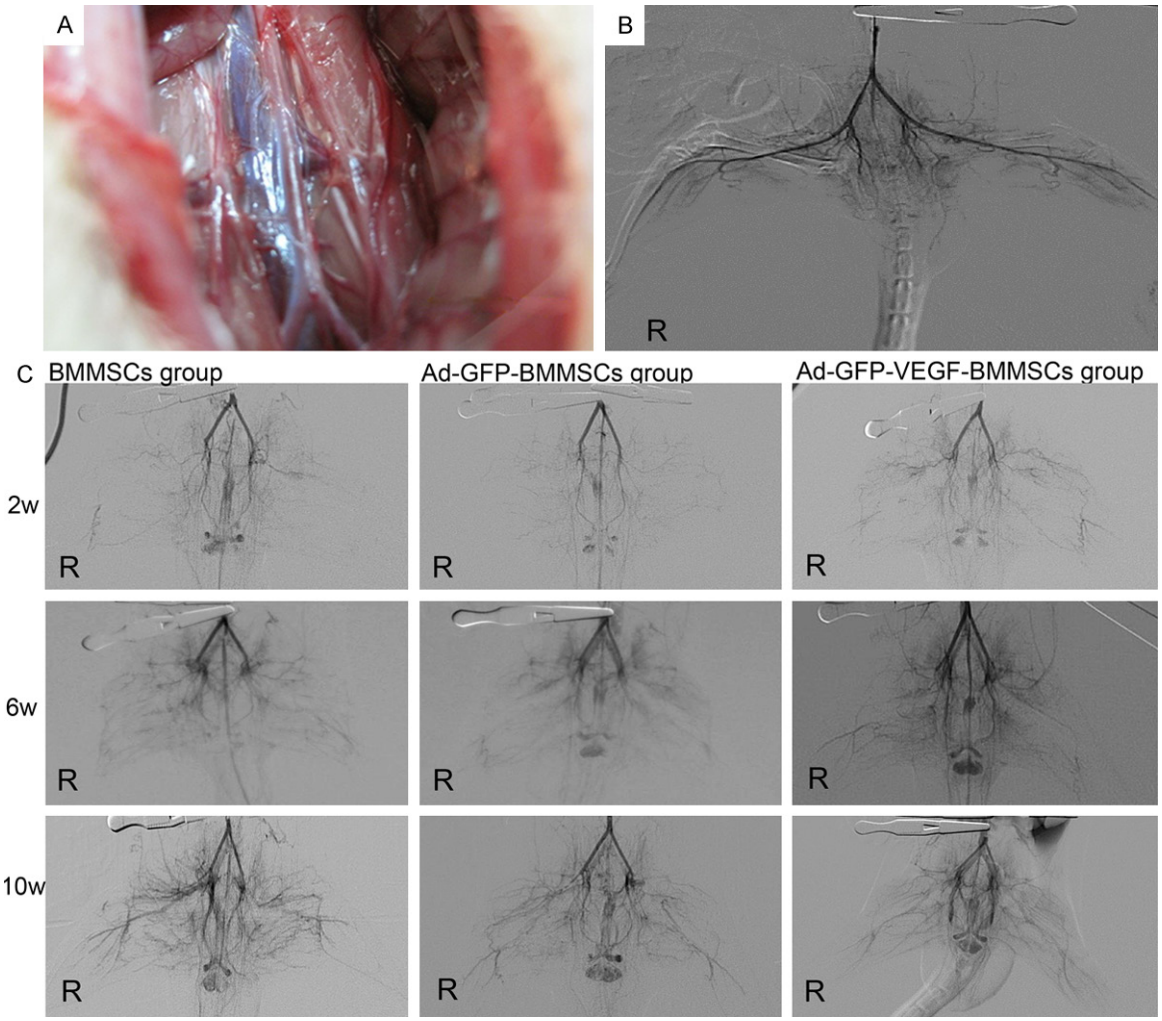

Figure 3. Surgical procedure and DSA of lowerlimb ischemia in T2DM rats. A. View after ligation of the left lowerlimb femoral artery; B. View of DSA before ligation of bilateral femoral arteries; C. View of DSA after ligation of bilateral femoral arteries at 2 w, 6 w and 10 w respectively in BMMSCs group, Ad-GFP-BMMSCs group and Ad-VEGF-GFP-BMMSCs group after cell transplantation.

was no angiography occurred in the branches of femoral arteries. After transplantation, angiography of bilateral hindlimbs could be observed at 2 w, 6 w and 10 w in those three groups respectively, among which there were more abundant blood vessels in the Ad-VEGF-BMMSCs group. However, comparing with the left hindlimb, there wasn't significant difference observed at the right hindlimb in all these three groups (**Figure 3C**).

Analysis of blood capillary density

After transplantation, the blood capillary densities of bilateral rectus femoris and gastrocnemius had a more obvious increase in the Ad-VEGF-BMMSCs group than that in the other two corresponding groups ($P < 0.05$) at 2 w, 6 w, and 10 w respectively. While, the difference between the BMMSCs and Ad-GFP-BMMSCs wasn't significant ($P > 0.05$) (**Figure 4**).

Tracing the survival of transplanted BMMSCs

Result by Immunofluorescence showed that BMMSCs labelled by BrdU existed in the muscle fibers of the left rectus femoris while there wasn't any fluorescyte observed at the right (**Figure 5**).

Discussion

Until now, there are more and more gene modified stem cells therapies reported [9, 17, 18], which preliminarily confirms its feasibility and safety. BMMSCs, with a low immunogenicity,

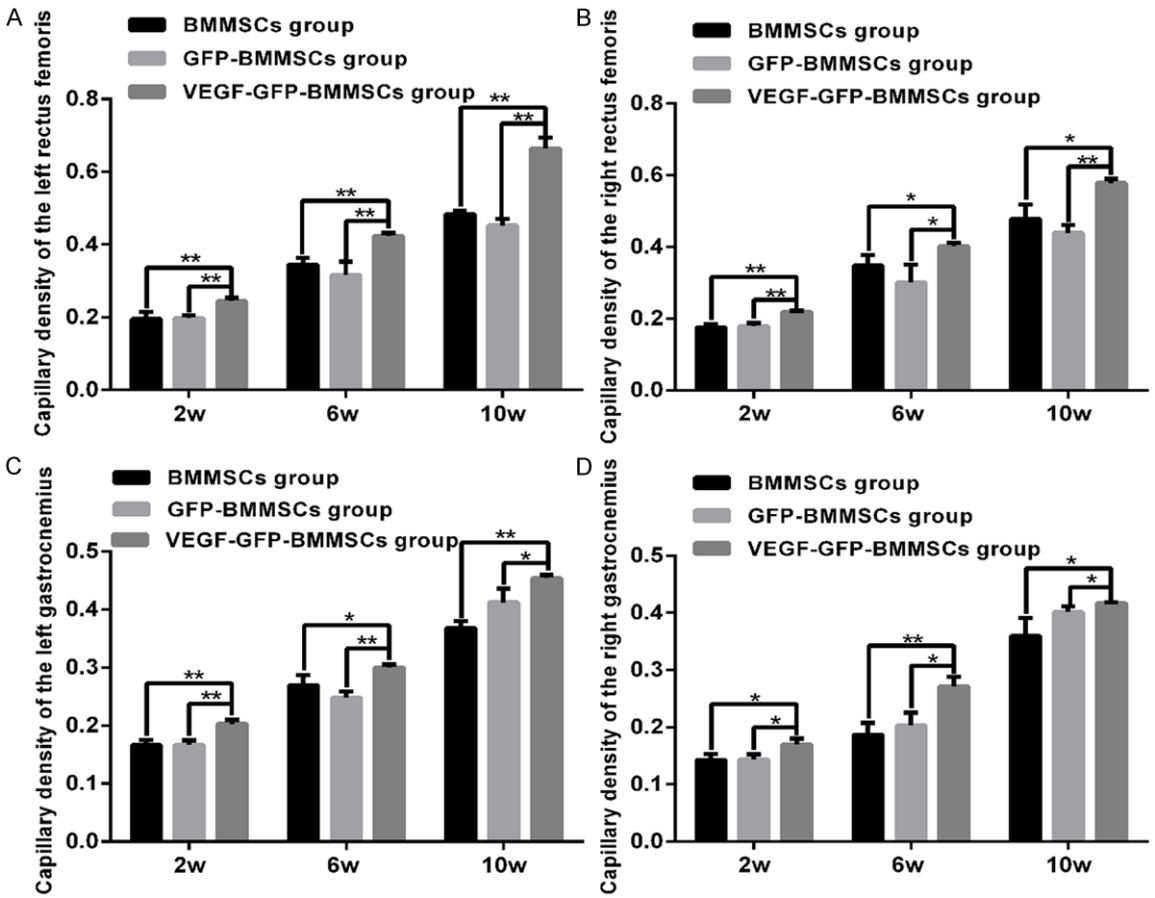

Figure 4. Capillary density of hindlimbs in diabetic hindlimbs ischemia rats after cell transplantation. $*P < 0.05$, $**P < 0.01$ *vs.* Ad-VEGF-GFP-BMMSCs group.

not only can help the recombinant viruses avoid the rejection, alleviate the inflammatory and extend the time of gene expression, but also has the advantages of convenient collection, self-renewal ability and multiple differentiation potential, thus it has been the main stem cell carrier of foreign genes [21, 22]. Because of the low ratio in the bone marrow karyocytes ($1/10^6$-$1/10^5$) [23], BMMSCs needs isolation, purification and culture in vitro when transplanting for treatment. In this paper, phenotypic analysis of P2 BMMSC showed overexpression of CD90, CD29 and CD44, but negative expression of CD45 and CD34, which definitely confirmed the obtained cells were highly purified BMMSCs. After the transfection of recombinant viruses, plenty of VEGF gene modified allogenic BMMSCs (the number being $4\text{-}8\times10^6$) could be obtained, enough for the transplantation of two models. The high labeling rate of Ad-VEGF-BMMSCs by BrdU could reach up to 75%, which laid a sound foundation for the trace of the survival of transplanted BMMSCs.

It is always difficult to recombine the BMMSCs with the virus carriers which carry target genes for cytotherapy owing to the cytotoxicity. Although the recombinant adenoviruses can stably and efficiently transfect stem cells in vitro, the transfection efficiency will obviously decrease when the virus titer is lower than 1×10^8 pfu/ml and the transfection efficiency will not be increased even though the volume of virus liquid is increased, resulting in the aggravation of cytotoxicity oppositely [24, 25]. In this study, after amplification the virus titer of Ad-VEGF was 8×10^8 pfu/ml, which indicated that we successfully obtained the virus liquid for the experiment subsequently. Ad-VEGF-GFP-BMMSCs used for the therapy of diabetic hindlimb ischemia could not only reduce the therapeutic dose of adenovirus into organism, but also avoid direct contact with endothelial layer, which was beneficial to the alleviation of cytotoxicity. Meanwhile, the expression of VEGF gene increased, as a consequence, promoting the revascularization.

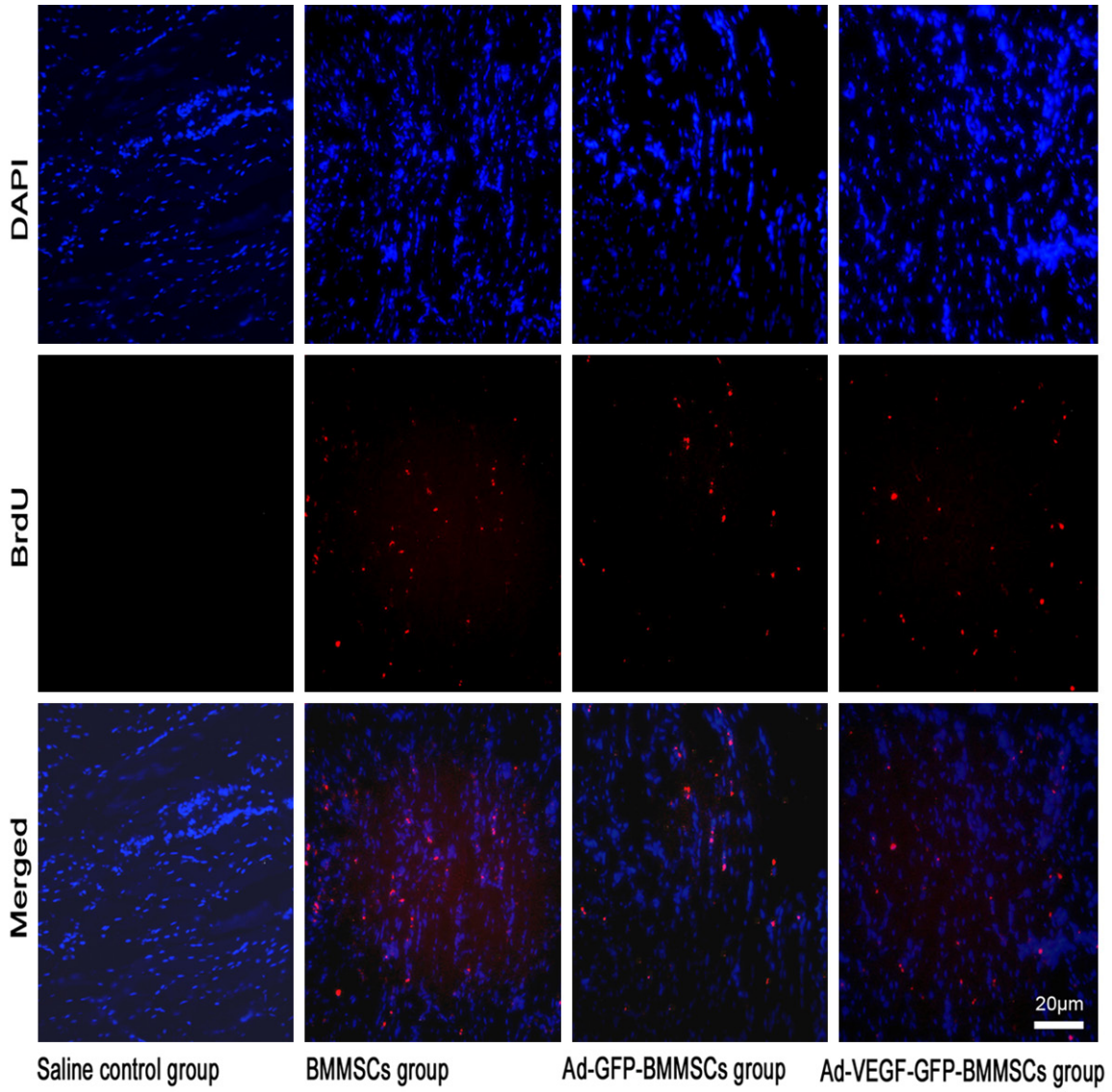

Figure 5. *In vivo* distribution of BrdU-labeled BMMSCs 2 weeks after transplantation by immunofluorescence. All pictures showing dual immunofluorescence stains by DAPI (blue fluorescence, displaying nucleus) and phycoerythrobilin (PE, red fluorescence, displaying BrdU-labeled implanting cells, original magnification: 200 ×).

So far, the method to establish the DF of T2DM model, which is similar to that of human, has been always limited. In this research, rats were fed with high-fat diet to induce the disorder of lipid metabolism and the intraperitoneal injection of STZ for the destruction of islet cells to induce the disorder of blood glucose regulation. In this way could we similarly get the T2DM model, and the blood glucose levels were all higher than 16.7 mmol/L [26, 27]. After the ligation of bilateral femoral arteries and its branches around, the symptoms, including acute ischemia sign with dark violet and ice-cold bilateral hindlimbs, were in accordance with the previous report [28]. However, there was no skin ulcer or gangrene occurred all the time which might be related to the increase of compensatory circulation in rat.

VEGF has always been known as the strongest cytokine to promote the growth of blood vessel, and the main research on VEGF is the revascularization function, which is crucial to the tissue repair and organ development [29]. Recently, it has been reported that the VEGF gene knockout in the skeletal muscles has induced the significant decrease of capillary density, finally resulting in the insulin resistance in mice [30]. Whether VEGF gene modified allogenic BMMSCs can promote the revascularization of the

blood supply of DF or not, to our knowledge, has not been reported so far.

After the respective intramuscular injection of BMMSCs, Ad-GFP-BMMSCs and Ad-VEGF-GFP-BMMSCs at situ, the results by DSA showed there were plenty of new vessels occurred around the femoral artery, although there was no vascular recanalization after ligation. The phenomenon turned more significant as the transplanted time extended. In addition, the blood vessel density of the left rectus femoris in the Ad-VEGF-GFP-BMMSCs group were superior than that in the BM-MSCs group and Ad-GFP-BMMSCs one, which strongly indicated an obvious increasing expression of VEGF gene due to the modification of BMMSCs by foreign VEGF. The results by H&E staining after a 2-week transplantation affirmed the capillary density of the left rectus femoris and gastrocnemius exceeded that of the right, which demonstrated definitely the revascularization function of BMMSCs, Ad-GFP-BMMSCs and Ad-VEGF-GFP-BMMSCs to an extent. Meanwhile, Immunofluorescence staining showed the transplanted BrdU-labelled cells still existed in the transplantation sites in all three groups after 2 weeks. It proved the transplanted cells can survive for at least 2 weeks in vivo and take effect in the tissue repair. However, there was no significant difference observed in the capillary densities between the bilateral hindlimbs and it was difficult to find the survival of BrdU-labelled cells. It might be related to the gradual loss of BMMSCs after transplantation, which needs a further exploration on the expression change of the related cytokines in the local lesion tissues. It may lay a new theoretical foundation for the therapy of limb ischemic diseases by VEGF gene modified BMMSCs.

In summary

Human VEGF gene-expressing adenovirus vector can be successfully introduced into rat BMMSCs which could be obtained successfully by adherent culture. After the intramuscular injection of VEGE gene modified BMMSCs into the T2DM hindlimb ischemia model, the transplanted cells can survive in the transplantation site and improve the function of revascularization. It preliminarily validates the feasibility of VEGF gene modified BMMSCs transplantation in hindlimb ischemia of diabetic model.

Acknowledgements

This work was supported by grants from the Nomarch Foundation of Guizhou Province for Excellent Scholars of Technology and Education, No. 2008-104; The Doctors Priming Foundation of Zunyi Medical College, No. F-267.

Disclosure of conflict of interest

None.

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